JCCLS MM6-A1
(2017.10. 1)

An Approved Guideline for the Quality Management of Specimens for Molecular Methods (Part 2) New Technologies and Sample Quality Control

Japanese Committee for Clinical Laboratory Standards (JCCLS).
2-7-13 Uchi-Kanda, Chiyoda-ku, Tokyo 101-0047 Japan

CONTENTS

(1) Introduction

In molecular methods, to ensure accurate measurements, it is necessary to standardize workflow processes in the pre-examination (pre-analysis) stage that have a potential to greatly influence the results. There are many points at which various properties of specimens can affect nucleic acid extraction and the measurement process. However, methods for evaluating and avoiding the influences of sample preparation on results have not yet been adequately established. Against this background, the WG-2 of Technical Committee on Standardization of the Gene based Tests in the NPO Japanese Committee for Clinical Laboratory Standards proceeded with the development of a quality evaluation system for specimens. Based on survey results regarding the actual situation in the field and obtained evidence, a draft of the "Specimen Quality Control Manual for Molecular Methods" was issued in the year 2009, and thereafter, an approved version of the document was released in 2011.

Analytical validity is greatly affected by the quality of the specimen. In securing analytical validity, this manual plays an important role in the quality assurance of molecular methods in the care of individual patients and in promoting the development and application of new therapeutic reagents into the clinical practice. This manual lists the representative types of the specimens used in three categories of molecular methods (nucleic acid test for pathogens, somatic gene test, genetic tests of the germ-line). Preparation of these specimens, requirements at the time of storage, transportation, processing, and collection of various categories of specimens also have been clearly described.

This manual is a comprehensive and practical document that fills a void in the Japanese setting regarding the quality of specimens for the purpose of quality assurance of molecular methods. It is widely used at the national level at the Advanced Medicine Experts Conference of the MHLW, and seeks to be adopted as a set of standards for a facility acting in advanced medical practice and receiving consignments from external medical institutions. Meanwhile, even in fields not described in the manual, progress in gene-based technologies have been accelerated in the development from research to clinical application, and the use of molecular methods for services is expanding (Table 1). Chromosomal analyses are not included in the framework of the molecular methods, but chromosomal analyses are handled in the same way as genetic testing in the diagnosis of congenital disease. In addition, testing for prenatal diagnosis based on molecular methods has become the subject of laboratory services in recent years, and the number of testing requests is increasing. For hematopoietic tumors, chromosomal and genetic analyses are the basis of the disease type diagnosis according to the WHO classification. Depending on the results of the chromosomal analysis, FISH or DNA

analysis can be later requested using specimens that were stored in Carnoy's fixative (Acetic acid: methanol = 1: 3). Appropriate management of samples is also important for quality assurance of examination even in those fields not included in this manual.

As new areas for technical implementation and application, there are circulating tumor cells, circulating cell-free nucleic acids in blood, fetal DNA in blood, microRNA, microarray methods, gene expression profiling analysis, array CGH, whole genome sequencing and more. Even when these methodologies are conducted for the purpose of research, the results could be returned to patients for medical care. In the fields of gene-related testing technology, development and clinical applications for laboratory use are being actively promoted. As a result, in creating data on clinical validity and clinical utility based on analytical validity, methods for quality control of specimens to ensure measurement accuracy are important.

In view of this background, the WG-2 Committee selected items to be added to the "Specimens Quality Control Manual for Molecular Analytic Methods." With regard to the selected items, based on the recognition that securing the quality of the specimen is important for securing the quality of the measurement results, the "recommended operation method" for the preparation of specimens is presented, followed by (1) Inappropriate conditions unsuitable for testing, (2) Cause, (3) Troubleshooting, and (4) Measures to avoid inappropriate situations. This manual also has clarified the requirements at the time of collection of various specimens subject to molecular methods. The WG-2 has decided to proceed with the document development and to publish it as “Specimen Quality Control Manual of Molecular Methods (Part 2).”

Table 1 Items Requiring Additional Description in "Sample Quality Control Manual for Molecular Methods"

Purpose	Specimen types	Target Sample of Interest	Examination method
1. Cancer diagnosis	Peripheral blood	Trace cells DNA RNA	Blood circulating tumor cells (CTC) measurement Blood circulating cell-free nucleic acid Targeted sequencing
	Tumor tissue Tumor cell	Viable cells Mitosis image	Chromosomal analysis Targeted sequencing
		Fixed tissue / cell (Cell block)	FISH Targeted sequencing
2. Prenatal diagnosis	Peripheral blood	Trace DNA	DNA analysis of fetal origin in maternal blood
	Amniotic fluid Villus Umbilical cord blood	Viable cells Mitosis image	Chromosomal analysis
		Fixed cell Viable cells	FISH
3. Diagnosis of mitochondrial disease	White blood cell Muscle tissue	Mitochondria DNA	Mitochondrial DNA analysis
4. Whole genome analysis	White blood cell Tumor tissue	Viable cells Mitosis image	Chromosomal analysis
		Viable cells / Fixed cell / DNA	Array CGH
		DNA	Whole genome sequencing Microarray
		RNA	RNA sequencing Microarray

(2) Quality Assurance and Standardization Activities for Molecular Methods

1. Promoting Practical Applications of Genomic Medicine and Specimen Quality Management Manual

Molecular Methods have been developed for clinical applications based on advancements in analytical techniques as well as elucidation of molecular pathological conditions of diseases such as infectious diseases and cancer. In recent years, analysis of human genes has progressed, and not only single gene diseases but also molecular pathologies with a genetic component such as common diseases and drug response, in which genetic factors and environmental factors are intertwined, are being elucidated. The application of diagnosis, treatment, and prevention of diseases by development and clinical application of Molecular Methods is in progress. Personalized medicine is being promoted in the development of molecular targeted drugs and companion diagnostic drugs based on elucidation of individual differences in drug reactivity and molecular pathological conditions of diseases. In addition, with the enforcement of the Act on Medical Care for Patients with Intractable/Rare Diseases, diseases that are difficult to diagnose, and specific chronic pediatric diseases have greatly expanded in number. As a result, in FY2008 medical fee reimbursement, D006-4 genetic test insurance listings increased from 36 diseases to 72 diseases.

Next-generation analysis systems based on simultaneous multi-item analysis (multiplex analysis) and comprehensive analysis, including Next Generation Sequencing (NGS), have been developed and are being used for clinical trials and patient diagnosis as a development and deployment of new technologies. As the number of Laboratory Developed Tests (LDT) using new analysis systems increase, there are various problems such as practical application, system implementation, appropriate use, and arrangements for social infrastructure. In the clinical use of these new analytical techniques, it is necessary to ensure the quality of molecular methods in response to patient needs and advancement of technology.

In recent years, domestic and overseas activities have been focused on standardization of molecular methods as a way of ensuring accuracy. Based on the interim report (July 2015) of the Genome Medical Realization Promotion Council (established under the Health and Medical Strategy Promotion Headquarters, Health and Medical Strategy Promotion Council) promoting the realization of genome medical practice, in November 2015, a task force to promote the practical application of medical care using genome information (Genome Medical TF) was set up. Discussions at the national level were held regarding the establishment of systems such as quality assurance and accreditation of laboratory performing molecular methods, as the basis of genomic medicine. The final report was released in October 2016. A policy proposal stated, "To ensure the quality and accuracy of molecular methods, it is

considered that the required level of Japanese version best practices / guidelines specialized in molecular methods is necessary, and based on the discussion at the task force, we should consider and formulate concrete measures etc. from now on."

The quality of molecular methods largely depends on the quality of the pre-examination processes, and in particular, the analyte to be measured. The use of this manual is expected to contribute to the improvement of the quality of molecular methods in Japan and the provision of high-quality medical care / health care based upon it.

2. International Standardization of Molecular Methods

In contrast to the first-generation molecular methods aimed at the detection and quantification of a single target of interest, in recent years, simultaneous multi-item analysis / multivariate analysis or multiplex analysis and more comprehensive genome wide analysis have been developed (Figure 1). Detection methods including multiplex PCR method, DNA microarray methods, differentiation of gene mutations using melting curve analysis of amplified products after nucleic acid amplification, and the like are being used.

As a genome-wide analysis technology, NGS technology has begun to be used not only for research but also for clinical sequencing with limited detection targets. With the elucidation and clinical significance of biomarkers in cancer and the development of molecular-targeted therapeutics, the need for simultaneous multi-item detection or multiplex analysis is increasing. Based on the biological elucidation of complicated disease pathology and diagnostic algorithms, simultaneous biomarker measurements of multiple items may be required for individual assessments. Gene mutations as specific biomarkers are mutually exclusive. As an example, there are fusion genes of *ALK, ROS1, RET* in non-small cell lung cancer, and fusion (chimeric) genes associated with typical chromosomal reciprocal translocation in hematopoietic tumors. Also, there are various mutations (e.g. *ABL1*) in one molecule or various molecular mechanisms of resistance (e.g. *BRAF*) that are used as indices for predicting the resistance to treatment of a molecular targeted therapeutics. A multi-molecular-targeted drug (e.g. multi-kinase inhibitor, MKI) has also been developed and clinically used for the purpose of improving therapeutic effect and overcoming resistance. In actual clinical examination, the use of multiplex analysis is expected, given the small amount of specimen available, and to achieve reductions in time and cost. As a result, paradigm shifts have occurred in FDA approval, and the platform of multi-gene diagnostics and many drugs is now being adopted over conventional one-drug, one-gene diagnostics (*EGFR, KRAS, BRAF*).

In multiplex molecular testing, quality control of nucleic acids to be used is important in securing high performance measurement. Based on that recognition, the JCCLS Technical Committee on Standardization of Gene-Related Testing, in cooperation with the Japan bio Measurement & Analysis Consortium (JMAC), made a proposal on "International standardization related to sample quality for multi-item gene analysis technology - *In vitro* diagnostic medical devices - General requirements and terminology of quality evaluation of nucleic acid for multiplex molecular testing -" at the Singapore plenary meeting (November 2013) of the ISO / TC 212 Technical Committee (Clinical laboratory testing and *in vitro* diagnostic test systems). The proposal was approved as a preliminary work item (PWI). This

proposed standard was approved as a proposal for a new work item at the plenary meeting (Toronto) in November 2014 (ISO NP 21474) and approved by voting. At the plenary meeting of November 2016 Kobe), it has been renamed to ISO 21474 "*In vitro* diagnostic medical devices - Multiplex molecular testing for nucleic acids - Part 1 - Terminology and general requirements for nucleic acid quality evaluation" and is under discussion.

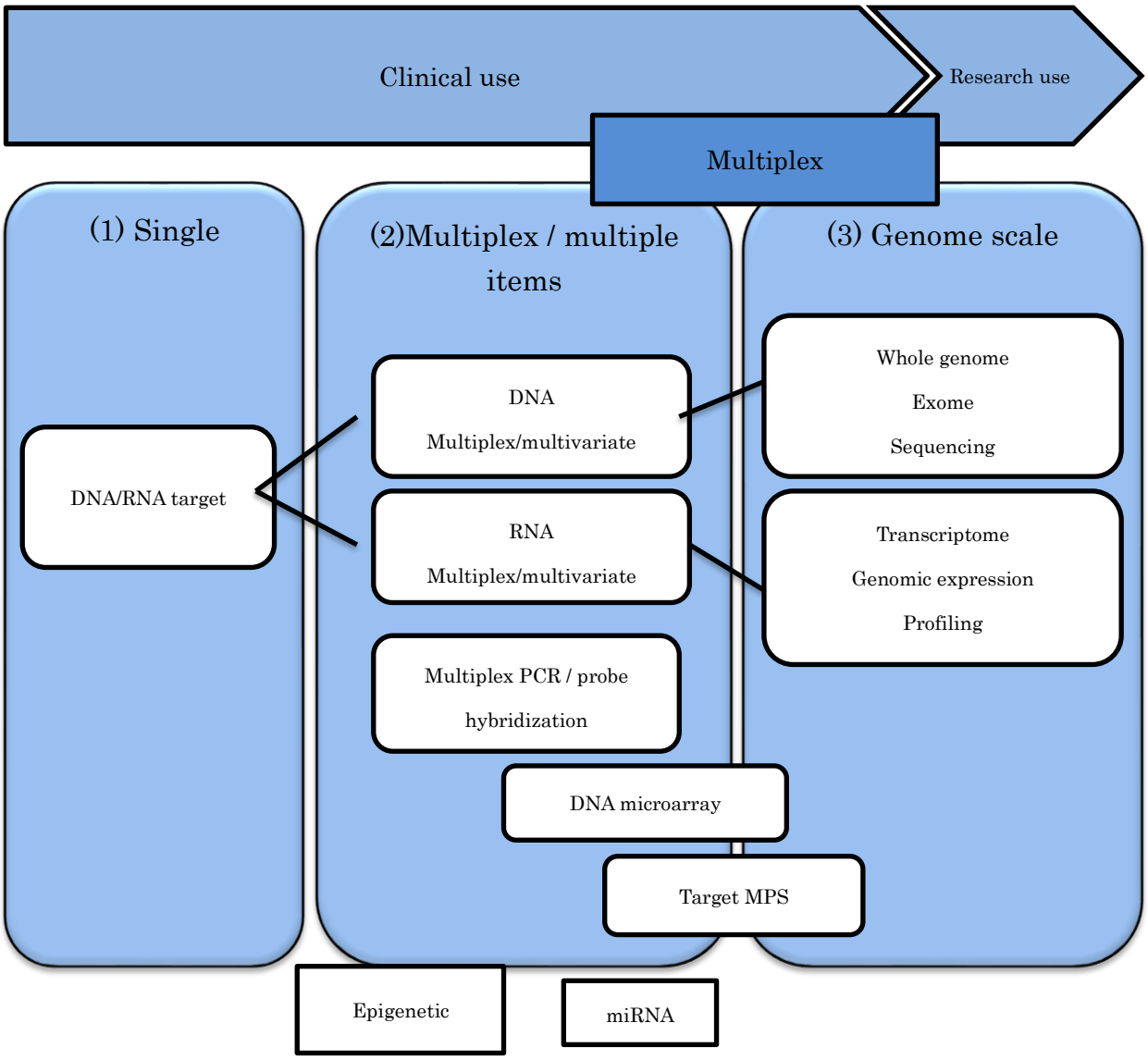

Fig. 1 Development of utilization of genome information

2.1 International Standard ISO 15189 "Medical Laboratories - Requirements for Quality and Competence"

For standardization of Molecular Methods, it is necessary to standardize the technology to be used, and to perform testing in clinical laboratories standardized in terms of quality, competency and the way they provide the results of the test. ISO / TC 212 (Clinical laboratory testing and *in vitro* diagnostic test systems) of the International Organization for Standardization (ISO) 212, which has contributed to the quality improvement of clinical laboratory testing and *in-vitro* diagnostic test systems, has emphasized the importance of pre-analytic process in ensuring measurement accuracy. In this technical committee, discussions and standard document development are now active, and international standards for definition and procedures are being developed to ensure sample quality.

The international standard ISO 15189 : 2012 “Medical laboratories - Requirements for quality and competence” was formulated as a standard for the evaluation of reliability and objectivity in the clinical laboratory in ISO / TC 212. In its introduction, the significance of the primary sample (specimen) preparation as an object of standardization in a series of processes of clinical laboratory services is clearly stated as follows. “Medical laboratory services include arrangements for examination requests, patient preparation, patient identification, collection of samples, transportation, storage, processing and examination of clinical samples, together with subsequent interpretation, reporting and advice, in addition to the considerations of safety and ethics in medical laboratory work.”. In the technical requirement of the standard document, regarding preparation of specimens in the pre-examination process, items of 5.4.4 "Primary sample (specimen) collection and preparation ", 5.4.5 “Sample (specimen) transport", 5.4.6 "sample (specimen) reception" and 5.4.7 "Pre-examination- handling, preparation and storage" are developed in 5.4 "Pre-examination process". While the international standard ISO 15189: 2012 provides chapter 5.4 "pre-examination processes", the procedure description on patient safety and specimen quality currently is inadequate.

2.2 International Technical Specifications Regarding Pre-examination Process

ISO / TS 20658 "Medical laboratories examinations - Requirements for collection, transport, receipt and handling of samples" as a technical specification complementing the requirements of the pre-examination process of ISO 15189 has been proposed and relevant documentation has been developed.

The chapter is divided into "Integration, Stability of the primary sample (specimen)", "Transport of the primary sample (specimen)", "Receipt and evaluation of the primary sample

(specimen)", and " Storage of primary sample (specimen) until testing" for the purpose of securing the quality of specimens. It is stated that proper temperature, storage conditions and time are important for ensuring the integrity of the primary specimen in the preservation of the sample and it is necessary to confirm the validity of these steps and periodical audit.

2.3 Various Standards for Pre-examination Process of Molecular Tests

Recently, remarkable progress has been made in molecular diagnosis and clinical applications using nucleic acids, proteins and metabolites in human tissues and body fluids. The profile and integrity of these molecules will change in the pre-examination process, lowering the reliability of subsequent measurements. In Europe, the SPIDIA (Standardisation and improvement of generic Pre-analytical tools and procedures for *In-vitro* DIAgnostics) project formed by a consortium composed of 13 countries was established in 2009 to standardize the work procedures of pre-examination process. Based on the outcomes, standard documents were described and submitted to the CEN/TC 140 committee to develop the European standards. In ISO/TC212, the series of CEN documents are currently under development as an international standard aiming at standardization and systematization of pre-examination processes of molecular diagnostic testing (Fig. 2). The object of the standard documents is three specimen types of formalin fixed paraffin embedded tissue, frozen tissue and blood. That is, ISO 20166 (Molecular *in vitro* diagnostic examinations - Specifications for pre-examination processes for formalin-fixed and paraffin-embedded (FFPE) tissue - Part 1: Isolated RNA, Part 2: Isolated proteins, Part 3: Isolated DNA) respectively for RNA, protein and DNA in formalin-fixed paraffin-embedded tissues, ISO 20184 (Molecular *in vitro* diagnostic examinations - Specifications for pre-examination processes for frozen tissue - Part 1: Isolated RNA, Part 2 : Isolated proteins) respectively for RNA / protein in frozen tissues, and ISO 20186 (Molecular *in vitro* diagnostic examinations - Specifications for pre - examination processes for venous whole blood - Part 1: Isolated cellular RNA, Part 2: Isolated genomic DNA, Part 3: Isolated circulating cell free DNA from plasma) respectively for peripheral blood cell RNA, peripheral blood genome and blood circulating free DNA in blood.

In addition to those developing tests in clinical laboratories and molecular pathology laboratories, the intended users of these standards are supposed to be *in-vitro* diagnostic device manufacturers, development and research institutions, and biobanks. (Figure 2).

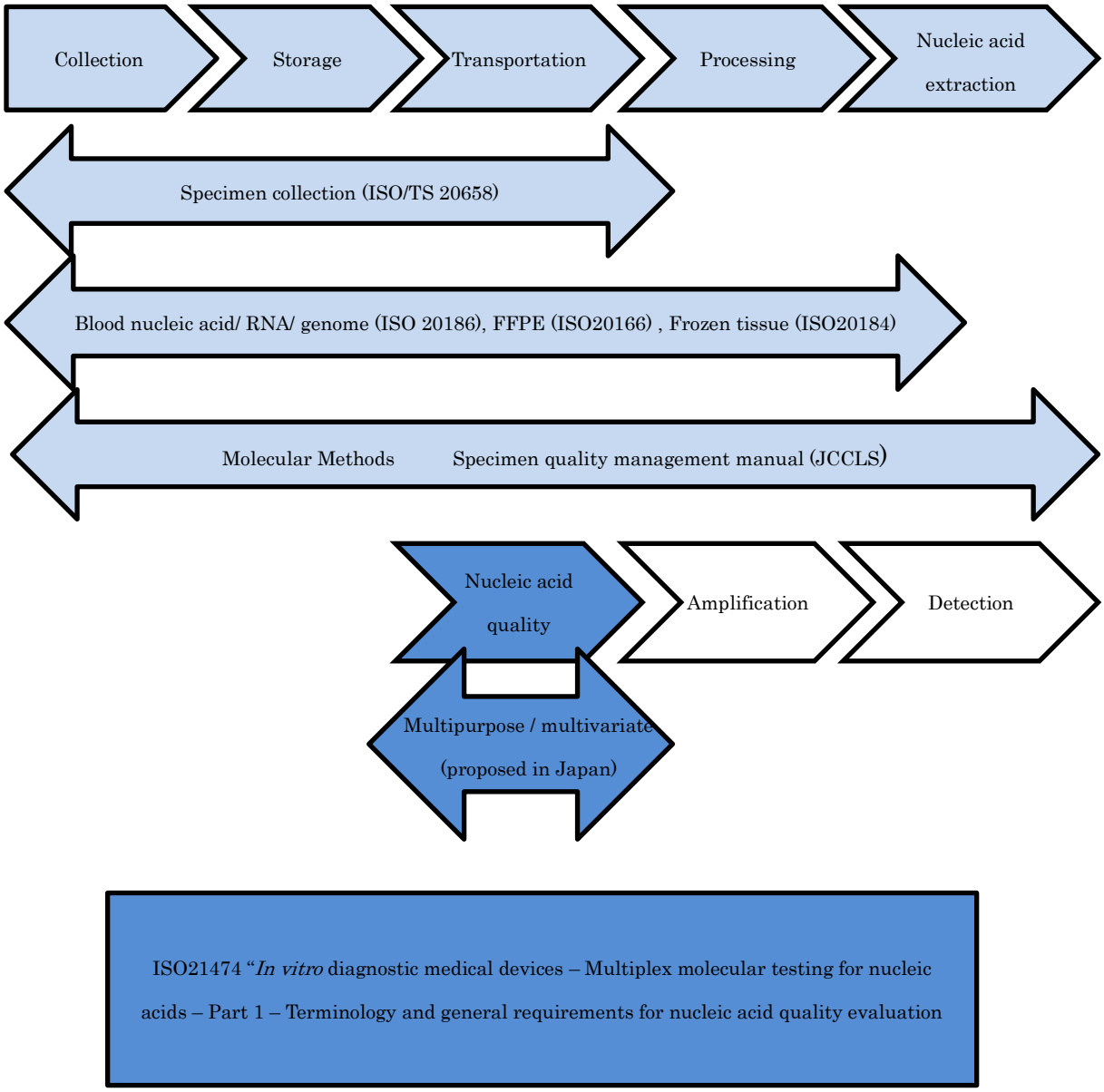

Fig. 2 Standardization activity of pre-examination process

(3) Various Examination Technologies / Analytical Sample Quality Control

1. Chromosomal Analysis and FISH

1.1 Chromosomal Analysis of Tumor Cells (cancer diagnosis / tumor cells / living cells, mitotic figures)

When venous blood is used as a sample, collect it with a heparin-containing blood collection tube, thoroughly invert and mix the tube, and store it in a refrigerator. In the case of bone marrow fluid, collect it in a dedicated preservation solution (RPMI 1640 culture medium or the like) and store it in a refrigerator. Both cultures should be started within one day of sample collection. Depending on the condition of the patient, adequate numbers of white blood cells may not be obtained, so specimens should be collected at an appropriate time.

(1) Inappropriate conditions for testing

1) Relevant cells undergoing mitosis may not be observed and it may be impossible to judge an appropriate karyotype due to the disease or treatment (nucleated cells can be extremely low in number after administration of anti-cancer agents), or due to the method of preparing or preserving the sample.
2) There is a possibility that specimens will have a large proportion of cell components other than the tumor cells as the target of interest, such as normal cells and non-tumor cells, among the total cells in the specimen. This will result in difficulty in interpreting the karyotype of target cells.
3) If a specimen is coagulated, it can interfere with establishing a proper culture.
4) Depending on the tumor cells of interest, their interphase mitosis images are not obtained at the time of chromosomal analysis and only normal karyotypes of mixed normal cells can be observed.

(2) Cause

1) Sample collection was performed after administration of anti-cancer agent. Sample preservation method was inappropriate, such as use of a frozen specimen.
2) Samples with low content of tumor cells were collected.
3) Anti-coagulant other than heparin was used. Alternatively, the admixture of anticoagulant (heparin) was insufficient.
4) The division cycle of the target cancer cell is slow.

(3) Troubleshooting

1) In some cases, cell proliferation can occur by culturing, but there is no countermeasure when cells are poorly proliferating because nucleated cells die or are scarce.

2) There are no measures to avoid inappropriate situations.
3) Culture the cells after eliminating coagulation.
4) There are no measures to avoid inappropriate situations.

(4) Measures to avoid inappropriate situations

1) Collect specimens before anticancer agent administration. Comply with appropriate storage conditions of specimens.
2) Collect sample areas with a high proportion of the tumor cells.
3) Collect with a heparin containing blood collection tube, and thoroughly invert and mix the tube after taking blood.
4) Perform FISH using interphase nuclei.

1.2 FISH (paraffin section) of Tumor Cells (cancer diagnosis / tumor cells / fixed cells)

In the diagnosis of some solid tumors, the FISH method using formalin-fixed paraffin-embedded section (FFPE section) has been performed, meanwhile it is important to prepare a specimen appropriate for the test, minimizing possible risk of influence by examination procedure or operation.

As a recommended condition for fixation, 10% neutral buffered formalin is used and the fixing time is set to 6 to 48 hours. However, the penetration of formalin to tissue varies depending on the type (such as how much fat is present), size, and shape, of the tissue to be used, and thus the observation of the fluorescence signal is affected. Therefore, depending on the specimen, it would be necessary to adjust the fixation time.

A thickness of 4-6μm is recommended for slicing of the tissue (the optimum thickness varies depending on the cell size).

If the cancer cell to be observed has a large nucleus, and the sliced section is thin, the nucleus will be also sliced and thus will be only partially visible. If the section is thick, in the case of a small nucleus, the nucleus becomes multilayered, making it difficult to observe and interpret the patterns. Accordingly, it would be necessary to adjust thickness in consideration of the characteristics of the cancer cell to be observed and the probe to be used.

(1) Inappropriate conditions unsuitable for testing

1) No fluorescent signal is observed, and the judgment can not be made appropriately.
2) The number of cells for counting is less than that required.
3) A tissue is disrupted and thus can not be judged properly.
4) A tissue is necrotic, and thus the fluorescence signal is not observed.
5) A slice of tissue has peeled off from the surface of slide glass during heat treatment.

6) The background is too high.

7) There are many fragments of nuclei, or there are many overlapping nuclei, and thus proper observation is difficult.

(2) Cause

1) Possible causes include autolysis of tissue due to long time from collection to decalcification treatment and formalin fixation, improper fixation (excessive or insufficient fixation) during formalin fixation, fragmentation of DNA due to not using neutral buffered formalin (acid decalcification treatment), and degradation due to leaving sample for a long time after slicing.

2) Biopsy material with few tumor cells was collected.

3) Material was disrupted at the time of tissue collection.

4) A necrotic portion is selected for the test material.

5) An uncoated slide glass was used.

6) A tissue is colored with a fluorescent dye, or a slide used has been insufficiently washed or dried.

7) The tissue section is not sliced at the appropriate thickness.

(3) Troubleshooting

1) Overfixation may be improved by strengthening the digestion treatment of tissue to the extent that the cell morphology does not collapse. In addition, when there are many overlaps in the nucleus to be observed, reducing the thickness of the thin section can strengthen the digestion of the tissue and improve it. There is no troubleshooting for tissue autolysis, insufficient fixation, fragmentation of DNA and degradation of sample.

2) There is no troubleshooting for biopsy materials with very few tumor cells.

3) If there is a part that is not disrupted, use that part for analysis.

4) If there is a part that is not necrotic, use that part for analysis.

5) Coated slides should be used instead of uncoated ones.

6) Observe the degree of fluorescence after the deparaffinization treatment, and if there are background issues, adopt a decolorization procedure.

7) Slice the tissue at a suitable thickness and re-stain.

(4) Measures to avoid inappropriate situations

1) In preparing formalin-fixed paraffin-embedded (FFPE) samples, comply with the precautions described in the HER2 examination guide (Breast Cancer HER2 Examination Pathology Working Group or Gastric Cancer HER2 Guidelines Committee in the Japanese Society of Pathology), Guidance on ALK Fusion Gene

Testing in Lung Cancer Patients（developed by the Japan Society of Lung Cancer), etc., and consider the preservability of the nucleic acid.

2) Use a tissue section with a sufficient number of tumor cells as a sample.
3) Avoid squashing tissues during collection.
4) Do not subject a necrotic part of a sample to a test.
5) Prepare a sample slide using a coated slide glass. If peeling is still observed, use an appropriate heating procedure (60-65 °C., about 30 minutes).
6) Use non-fluorescent dyes and reagents that do not affect observation of fluorescence of FISH. Clean the slide firmly in each step and pay attention to drying.
7) Slice tissue at a proper thickness. Highly trained and skilled pathologists should do the slicing.

2. Liquid based Cytology sample: Focusing on Cervical Cytology Examination

Liquid-based cytology (LBC) samples can be classified into gynecological ones and others by the type of LBC vials. The uterine cervical cells are obtained with a brush and collected in a preservative solution prior to a smear sample being prepared.

Before and after the cytological examination, a nucleic acid amplification test (NAAT) for detection of pathogens such as human papilloma virus, *Chlamydia trachomatis*, *Neisseria gonorrhea* and *Trichomonas vaginalis* can be performed. NAAT should be performed on pre-processed aliquots of the specimen.

In addition, LBC are also utilized for fine needle aspiration biopsy specimens such as thyroid, mammary gland, pancreas, body fluids specimens such as urine, pancreatic juice and bile specimens, and pancreatic and biliary duct brush wash specimens, and others.

Depending on the manufacturer / provider, the collection method after obtaining the cervix cells using a brush differs, for instance with or without the brush head in the solution of a vial. Although it is less invasive, collection using cotton swabs in pregnant women is acceptable only if doctors understand the risk of having far fewer cells in the specimen.

Immunohistochemical tests may be performed, especially in cases where it is difficult to make a diagnosis by cytological examination.

When performing NAAT after the cytological test, handle the sample carefully to avoid cross-contamination from others.

In addition, when an aggregate of cells is found, vortex or mix, as necessary, to disperse it.

As the preservative solution of the LBC sample is part of a medical device for preparing a cytology sample slide, the appropriate storage temperature and period for the cytological examination is described. Furthermore, NAAT should be performed within evaluated test

temperature and period for the stability of the nucleic acid.

Descriptions here are focused on the cervical cytology examination.

(1) Inappropriate conditions unsuitable for testing

1) The target sequence (pathogen) is not retained.
2) The proportion of cancer cells is too low.
3) Excessive peripheral blood is obtained.
4) Excessive normal cells (mucus) are obtained.
5) Presence of substances that interfere with NAAT (contraceptive jellies and anti-fungal medication).

(2) Cause

1) A sample is not stored at the appropriate temperature and period of the preservative solution for NAAT (nucleic acid of pathogen).
2) The presence and proportion of cancer cells can't be confirmed immediately.
3) Brushing or fine needle aspiration cytology may obtain excess peripheral blood. In the cervical cytology test, blood may be present in the sample due to bleeding from a lesion, menstruation and strong scraping.
4) Sampling without removing mucus, cervical cells are not scraped adequately, and the number of cells is reduced.
5) Obtain contraceptive jellies and anti-fungal medication.

(3) Troubleshooting

1) Although the amount of nucleic acid at each stage of extraction, storage and transport can be confirmed by an internal control derived from the human genome, this control does not guarantee preservation of a nucleic acid sequence for detection of a target of interest (pathogen).
2) The presence / proportion of cancer cells is confirmed using methods such as a cytological examination on the same specimen, specific gene expression (of epithelial cells), FISH and the like.
3) Perform a hemolysis treatment and prepare the sample again. Check the extent of blood volume and its effects. In the case of excessive blood affecting the test results, re-collection should be also considered.
4) Use a nucleic acid extraction reagent and amplification / detection system which is not affected by mucus contamination. Depending on the reagent to be used, re-sampling may need to be considered. Regarding the extracted nucleic acid, the proper ratio of extracted amount to the cell number is confirmed by an appropriate internal

standard.

5) Estimate an inhibitory effect on NAAT by an internal standard, using contraceptive jellies and antifungal creams.

(4) Measures to avoid inappropriate situations

1) Perform the test within validated conditions (time and temperature) of the preservative solution for NAAT (nucleic acid of pathogen).
2) Train a personnel for proper sampling methods to collect a sufficient number of cells.
3) Specimen collection at menstruation should be avoided. Collect specimens from suspected lesion.
4) Upon removal of mucus, cervical cells are taken with a brush. Train a personnel for proper sampling methods.
5) Make sample collectors aware of the interfering effects of contraceptive jellies and anti-fungal medication in NAAT using LBC specimens.

3. Array CGH

Based on the quality control of pathological samples used for measurement by array CGH (comparative genomic hybridization), specimen collection, preservation, transportation and handling should all be performed appropriately to obtain genomic DNA of recommended quality.

The containers for transport of genomic DNA should be made of a material such as Styrofoam, capable of maintaining a stable temperature inside and of preventing the collapse of tubes. The sample can be transported in refrigerated devices.

Samples may be stored in a container filled with dry ice for keeping at temperatures -70°C or lower, when the examination is not scheduled for a certain time. Avoid repeated cycles of freeze and thaw. Compact tubes of DNase-free and low temperature resistant are used for DNA samples.

3.1 Blood

Blood is collected with a blood collection tube containing an anticoagulant (e.g. EDTA), then immediately mixed thoroughly by inverting. The tubes are stored refrigerated, if nucleic acid extraction is not performed on the day of blood collection.

Blood collected in EDTA-tubes can be stored at refrigerated for 3 days. Storage temperature and transportation methods are selected based on the length of the period until DNA extraction.

(1) Inappropriate conditions unsuitable for testing

The quality of the extracted DNA is outside the criteria of the purity index. Severe degradation in the extracted DNA will affect the results. Intact (high molecular weight) DNA is desirable, whenever possible.

(2) Cause

The quality of the extracted DNA is influenced by the storage conditions of the sample, DNA extraction method, DNA storage condition and handling.

■Assays using degraded DNA can result in a large error in results.

■Contamination with protein, sugar, lipid components, EDTA, and other impurities in the DNA solution may affect the final result, for example, by reducing the efficiency of fluorescence labeling.

(3) Troubleshooting

When the quality of the recovered DNA is evaluated and judged as unsuitable for CGH analysis, perform re-examination from the DNA extraction step. If the sample itself is of poor quality, do the re-examination from the step of re-sampling.

(4) Measures to avoid inappropriate situations

In order to obtain genomic DNA suitable for array CGH, store the specimen/DNA sample, process and extract the DNA using appropriate conditions and methods.

3.2 Tissues / Cells

The samples confirmed to substantially contain tumor cells are used, after performing cytology and pathological examination on a split part of the samples, for the purpose of ascertaining the abundance ratio of tumor cells. Normal cells (non-tumor cells) are removed from the tissues by microdissection as needed. The samples are handled appropriately, depending on the needs of the target of sample, using suitable conditions of collection, storage, preparation, for obtaining sufficiently high-quality genomic DNA for array CGH. DNA is extracted, stored and-handled using appropriate conditions and methods.

(1) Inappropriate conditions unsuitable for testing

Detection sensitivity is remarkably diminished when a tumor tissue or cells are contaminated with a substantial proportion of normal tissues or composed with a heterogeneous cell population. A part of normal tissues or cells need to be removed as much as possible.

When the quality of the recovered DNA is low, the error of the examination results can be significant.

(2) Cause

The low ratio of tumor cells in tissue / cell samples is caused by insufficient removal of the normal cells from the whole sample used for DNA extraction. The quality of the recovered DNA is influenced by the target of the sample, conditions of storage, sample preparation, recovery and storage of extracted DNA.

(3) Troubleshooting

Remove normal cells by an appropriate procedure such as micro/macro dissection.

The abundance ratio of tumor cells can be confirmed in the samples by analysis including cytology and pathological examination on a split part of the samples.

If the quality of the recovered DNA is judged to be inappropriate for array CGH analysis, repeat the DNA extraction and re-examine. If the specimen itself is of poor quality, re-sample the specimen and re-examine.

(4) Measures to avoid inappropriate situations

A portion of the collected sample should be stored for reexamination. If the specimen itself is of poor quality, perform reexamination from the re-sampling.

4. Next Generation Sequencing (NGS)

Quality of samples used for DNA or RNA analysis by next generation sequencing (hereinafter referred to as NGS) is assured by conforming to specimen type-specific collection, storage, transportation and treatment.

When performing sequencing using NGS, appropriate procedures and quality assurance in all the steps are important, regarding (1) proper collection and storage of specimens, (2) quality control of DNA or RNA, and (3) processing for sequencing reaction (library preparation). These qualities affect the final sequencing efficiency and accuracy.

Although general quality management in NGS analysis will be described below, the quality control method can be different depending on the components of NGS system used for analysis.

4.1 Sample Quality Control for NGS Analysis

4.1.1 Blood

When DNA or RNA derived from nucleated cells is used for NGS analysis, a specified amount is collected using a blood collection tube containing a nucleic acid stabilizer or a blood collection tube containing an anti-coagulant (EDTA or the like). Immediately thereafter, the tube should be inverted and mixed, and DNA or RNA is prepared from the blood specimen according to the recommended method of a reagent kit that can extract DNA or RNA.

When circulating free DNA (ccfDNA) is used for NGS analysis, blood samples are collected into a blood collection tube containing an anti-coagulant (EDTA or the like) or a blood collection tube containing a reagent with cytoprotective action. DNA is prepared according to the recommended method of a reagent kit which can separate plasma (or serum) under appropriate conditions and extract ccfDNA.

(1) Inappropriate conditions unsuitable for testing

Coagulated blood or blood after freezing and thawing is unsuitable. When ccfDNA is used for NGS analysis, specimens in which nucleated blood cells are lysed are also inappropriate. Specimens that should not be used for DNA or RNA extraction can not be visually distinguishable in some cases. For this reason, record the history of collection, storage, and transportation specimens so that they can be tracked.

(2) Cause

Inadequate properties such as coagulation and lysis of nucleated blood cells are caused by insufficient mixing after blood collection. In addition, it is considered that the quality of DNA or RNA to be extracted depends on long-term exposure to high temperatures, repeated cycles of freezing and thawing, and storage or transportation out of the appropriate conditions (temperature and period). The quality of DNA or RNA deteriorates when the reagent kit for DNA or RNA extraction and the blood collection tube are stored in an environment deviating from the recommended conditions, and when used in a method other than the recommended method.

(3) Troubleshooting

If it is judged that the quality of the recovered DNA or RNA is not suitable for NGS analysis, re-testing is performed from DNA or RNA extraction. If the specimen itself is of poor quality, re-collect the specimen.

(4) Measures to avoid inappropriate situations

Immediately invert and mix tubes after taking blood. Samples should be stored under the conditions suitable for DNA extraction shown below. Generally, when DNA derived from nucleated cells is extracted, it is desirable to perform DNA extraction within 24 hours after blood collection, and it is possible to store up to 3 days under refrigeration conditions. If DNA cannot be immediately extracted, it can be stored frozen, but should not undergo repeated cycles of freezing and thawing. When ccfDNA is extracted, it is recommended to use a blood collection tube containing a ccfDNA profile stabilizer (for details see section 7.1 Collection of blood circulation free nucleic acid (1)). The preservation method of the blood specimen after the blood collection should comply with the conditions described in the package insert of the blood collection tube. If a dedicated blood collection tube is not used,

immediate refrigeration or plasma separation is necessary. On the other hand, refrigerated specimens for RNA extraction can be stored up to 24 hours, but it is desirable to treat with appropriate RNase inhibitors as soon as possible after collection. The blood collection tube and the reagent kit for DNA or RNA extraction should be selected to conform to the type of specimen and the purpose of analysis, and appropriately stored and managed as described in the accompanying instruction manual. Operate whole processes according to the methods prescribed.

4.1.2 Tissues / Cells

The RNA profile can change prior to stabilization by freezing of tissues by a variety of factors, such as induction of gene expression/suppression of RNA degradation. The degree of influence depends on temperature, length of time exposed to ischemia, and storage time in ambient temperature. Warm ischemia time during surgery has a greater effect than cold ischemia time after organ tissue removal. The degree of influence due to the length of the storage period depends on the amplicon size and the position of the primer or probe. When using fresh frozen tissue specimens for NGS analysis, cryopreservation in a condition as close as possible to their *in vivo* status is ideal. To do this, it is important to recognize lesions correctly in their fresh state before the surgical specimen is fixed with formalin or the like and to collect the viable cell population of the lesion as quickly as possible. However, when collecting tissue specimens for research, do not prevent pathological diagnosis, such as mistakenly collecting the parts necessary for pathological diagnosis. Tissue samples for research should be collected from residual samples that are not necessary for pathological diagnosis.

From the surgical specimen, collect tissue specimens for NGS analysis from suitable sampling sites and avoid compromising pathological diagnosis (samples shall be taken without having patients at a disadvantage) by avoiding sampling from areas where nucleic acid and proteins have been denatured such as bleeding lesions and areas of necrosis. For example, in case of a cancer, specimens are taken from both the cancerous and the non-cancerous part (appropriate control part). A minimum description about the collection site shall be recorded. From the surgical specimen, it is desirable to collect tissue specimens for NGS analysis promptly. If this is not possible, temporarily store the specimens in a refrigerator (4 °C) or the like and collect the tissue specimens stored at 4 °C within 3 hours alternatively. When extracting DNA from tissues or cells for NGS analysis, freeze the tissues (pellets in the case of cells) or store them in a refrigerator or freezer after immersing in the designated preservation solution.

When the specimen is to be cryopreserved, the amount to collect depends on the judgment of the pathologist or clinician who is familiar with the requirements of pathology diagnosis.

However, when there is no particular obstacle to pathological diagnosis and an adequate collecting site can be secured, it is appropriate to collect tissue about the size of the tip of the small finger (about 1 × 0.5 × 0.3 cm, about 50 to 100 mg). Cut the tissue into cubes with 2-3 mm sides. Place a piece of tissue into a screw-capped tube that is resistant to low temperatures, and immerse the tissue in a nucleic acid protective agent or place the tube directly in liquid nitrogen. Quick freezing should be performed, within 30 minutes of removal of the surgical specimen. When storing for a long term, keep the samples in a liquid nitrogen storage container (about -180 °C.) until they are used. It is also possible to preserve for a long term in a deep freezer (-80 °C.).

In NGS analysis using FFPE specimens, the existing methods used in pathological diagnosis work at each facility should be respected. On the other hand, care should be taken in obtaining DNA or RNA suitable for NGS analysis as much as possible. When handling tissue specimens for treatment purposes, follow the methods recommended or required in the "Guidelines on the handling of pathological tissue samples for genomic research and treatment: Standard operating procedures based on empirical analyses" issued by the Japanese Society of Pathology.

After removing the specimen, immerse it in a fixative as soon as possible and fix it. Even if fixation can not be performed immediately, it is desirable that the specimen be stored in a refrigerator (4 °C.) or the like and fixed within about 3 hours. Transport the specimen promptly at 2-8 °C. It is preferable to use a neutral buffered formalin solution for fixation instead of a non-buffered (acidic formalin) solution. Also, when mainly considering extraction of DNA and genetic mutation analysis, it is desirable to fix with 10% formalin (3.5% formaldehyde) rather than 20% formalin (7% formaldehyde). Some commercially available tissue fixing solutions that do not contain formalin can be used to enable histological observation and have been confirmed to be superior in the preservation of nucleic acids, proteins and the like. It is most desirable to avoid over-fixing and to process within 24 hours of surgery. However, if processing takes place within 48 hours after surgery, a reasonably good retention of nucleic acids and the like can be expected.

In general, it is recommended that nucleic acid extraction from unstained specimens be carried out promptly after sectioning and if unstained specimens need to be preserved for an indefinite period of time for research, they should be stored at 4 °C. However, if it is not extremely long term, storage at room temperature would not generally affect the quality of the nucleic acid. In the case of analysis from sliced sections of cancer tissue, it is necessary to recover as much of the tumorous part as possible, in order to reduce the influence of background noise caused by a large amount of normal cell tissue. There are no strict

restrictions on paraffin coating of unstained specimens. A paraffin coating on the surface of unstained specimens can reduce dust and physical damage and does not generally affect the quality of nucleic acid. Exposure to extreme conditions such as direct sunlight should be avoided when preserving unstained specimens for long periods of time. Where there is a possibility of subjecting a specimen containing hard tissue to genomic research, avoid rapid decalcification using acid such as by the Plank-Rychlo method which has a strong effect on the quality of nucleic acid. Instead, perform slow decalcification using EDTA.

When DNA is extracted from tissues and cells and NGS analysis is performed, only tissues (pellets in the case of cells) should be frozen. Alternatively, store tissue in a special preservative solution and maintain in a refrigerated or frozen state.

The success of mutation analysis by NGS depends on DNA amount, dsDNA (double-stranded DNA) concentration, type of specimen, and cellularity of specimen. The amount of DNA required for the analysis depends on the measurement method to be used, the number of target sequences of interest to be detected, the method of separating or concentrating the target cells, and the method of preparing the library. In the case of puncture aspiration or needle biopsies, the amount of DNA obtained tends to be small. In NGS analysis targeting cancer, panel tests using DNA or RNA have become widespread. For small tissue specimens and cell specimens, a method of simultaneously extracting DNA and RNA is used as needed. The proportion of tumor needs to be 10-20% or more (due to the possible influence of inflammatory cells, blood, mucin, necrosis). The main contributors to poor quality such as DNA degradation are the use of formalin-fixed specimens or decalcification of metastatic bone cancer specimens using strong acid.

(1) Inappropriate conditions unsuitable for testing

In the case of tumor tissues or cells which are highly contaminated with normal tissues, the detection sensitivity decreases remarkably and false-negative results are common. If the quality of the recovered DNA or RNA is low, errors in the measurement results will increase.

(2) Cause

In a specimen used for DNA or RNA extraction, the proportion of the tumor cells in the tissue specimen decreases when the normal tissue is not adequately removed. The quality of the recovered extracted DNA or RNA is influenced by the storage condition of the specimen, the DNA recovery method, the extracted DNA storage conditions, and its handling.

(3) Troubleshooting

Confirm the ratio of tumor cells by cytology, and pathological examination. If necessary,

remove normal cells by micro/macrodissection or the like.

If it is judged that the quality of the recovered DNA or RNA is not suitable for NGS analysis, perform the re-examination by repeating the DNA or RNA extraction process. If the specimen itself is of poor quality, collect a fresh specimen.

Efficiencies of adapter ligation and DNA amplification for sequencing could decrease when using DNA derived from FFPE due to factors such as fragmentation, crosslinking with protein, and a low proportion of single-stranded DNA. This may make it difficult to prepare libraries. There are reagents available to check the quality of DNA for NGS analysis.

NGS FFPE QC kit (Agilent Technologies)

TruSeq FFPE DNA Library Prep QC Kit (Illumina)

KAPA hgDNA Quantification and QC Kit (Kapa Biosystems)

Fragmentation can also be high in RNA derived from FFPE. Because of this, efficiencies in adapter ligation and PCR amplification can decrease, making it difficult to prepare libraries. Since the quality [RIN (RNA Integrity Number) value] that can be handled for each library preparation reagent is different, it is desirable to consider whether the acquired extracted RNA is suitable for analysis based on the information from the manufacturer and distributor.

If the quality of RNA does not satisfy the recommendations of the reagents being used to prepare the library, defects such as misjudgment of the gene expression level and inaccuracy of the detection of fused genes can occur in some cases.

(4) Measures to avoid inappropriate situations

Divide the collected sample, and subject a part of it to cytology, pathological examination and the like to confirm the relative ratio of tumor cells, and submit a sample that has been confirmed to have tumor cells present. The normal cells should be removed from tissue by microdissection or the like as necessary. If it is judged that the quality of the extracted DNA or RNA is not suitable for NGS analysis, perform re-examination by repeating DNA or RNA extraction. Extraction, storage and handling should be carried out under appropriate conditions and methods. Keep a portion of the collected sample for re-examination. In cases where there is only a small amount of specimen, such as aspiration cytology, pay attention to the low amount and diminished quality of extracted nucleic acid. Properly stored FFPE specimens that are up to 5 years old are considered suitable for analysis. Avoid using FFPE specimens that have been stored for more than 10 years. If the specimen itself is of poor quality, collect a fresh specimen.

4.2 DNA and RNA Quality Control for NGS Analysis

4.2.1 General Precautions on Preparation of DNA and RNA

In preparing DNA or RNA for NGS analysis, the following general points should be noted.

・When performing the procedure, to avoid contamination of nuclease into reagents, always wear powder-free laboratory gloves, and use pipettes fitted with nuclease-free pipette tips and aerosol prevention filters.

・The experimental area shall always be kept clean, and experiments are carried out under conditions that satisfy Biosafety Level 1 (BSL 1).

4.2.2 DNA Quality Control

Length, amount, chemical purity and structural integrity can be all used as indicators of the quality of DNA.

(1) Length

Generally, electrophoresis is used to confirm the length (degree of degradation) of DNA. For genomic DNA with a good quality and a low degree of degradation, high molecular weight DNA can clearly be confirmed, whereas low molecular smear bands and peaks are not observed.

In the case of DNA extracted from a FFPE sample, it may be suspected that the nucleic acid is low molecular weight (fragmented) due to formalin fixation. In evaluating the quality of such DNA, it is effective to adopt a method of estimating the degree of DNA degradation by real-time PCR using a couple of primer sets designed to have PCR product of different lengths at the same site.

In the case of ccfDNA extracted from plasma or serum, the majority of fragments range from 140 to 200 bp in length. When high molecular weight DNA is observed, it is suspected to be due to contamination by genomic DNA from nucleated cells, so it is not suitable for analysis of ccfDNA.

Since high molecular weight DNA can be fragmented by physical stress, attention is required such as gently tapping with a finger when mixing solutions. Avoid repeated cycles of freezing and thawing as much as possible.

(2) Amount

Since the concentration of DNA affects the efficiency of library preparation, it is necessary to accurately quantify only the appropriately long dsDNA. Incorporation of single-stranded DNA, RNA, or nucleotides can compromise accurate quantification.

In the ultraviolet-visible spectrophotometer, substances having absorption near 260 nm are quantitatively measured together, so there is a tendency to overestimate the amount

of DNA. Therefore, it is desirable to quantify a sample using a fluorescence assay method or the like showing selectivity for dsDNA. In this case, it is necessary to prepare a calibration curve at an appropriate frequency and measure the DNA of known concentration every time to obtain the measurement value as accurate as possible.

When extracting ccfDNA from plasma or serum, generally 1 to 50 ng of ccfDNA can be recovered from 1 mL of plasma. Although there is a range depending on the state of the pathological condition or other factors, in the case of an excessive yield, it is suspected to be due to contamination with genomic DNA from nucleated cells, and thus not suitable for analysis of ccfDNA.

When frozen DNA stock solution is used for preparation of a library for NGS, it is desirable to quantify immediately before use.

(3) Chemical purity

A good index of chemical purity of DNA is that the value of A_{260}/A_{280} is 1.8 to 2.0 and the value of A_{260}/A_{230} is greater than 1.0. In addition, when EDTA, ethanol, phenol or the like is contained in a solvent dissolving the DNA, it can inhibit the reaction of the library preparation reagent.

Refer to the instruction manual provided by the manufacturer and distributor and check that the buffer does not include substances that would contaminate your sample.

(4) Structural integrity

There is a method of calculating the proportion of dsDNA as an index of structural integrity. DsDNA is quantitated using a fluorescence spectrophotometer and compared with the DNA concentration measured with a spectrophotometer.

Percentage of dsDNA (%) = dsDNA concentration / DNA concentration × 100

When DNA with a high degree of degradation such as derived from FFPE is used, it may be effective to prepare a library by calculating the necessary amount of DNA based on the dsDNA concentration.

4.2.3 RNA Quality Control

The quality of RNA is defined by indexes of length, amount, chemical purity and structural integrity.

(1) Length

Generally, electrophoresis is used to confirm the length (size) of RNA. It is desirable to use an electrophoresis system that can analyze a trace amount for quality of RNA for NGS analysis. For RNA with a good quality and a low degree of degradation, 28S and 18S bands can be clearly confirmed, and low molecular smear bands and peaks are not observed.

(2) Amount

If the concentration of RNA is not accurately quantified due to DNA or nucleotide contamination, library preparation efficiency can be reduced.

(3) Chemical purity

A good chemical purity of RNA is indicated by an A_{260}/A_{280} value of 1.8 to 2.0 and an A_{260}/A_{230} value greater than 1.0. In addition, when EDTA, ethanol, phenol and the like are contained in a solvent dissolving RNA, or DNA is mixed in a sample, the reaction of the library preparation reagents can be inhibited in some cases. Refer to the instruction manual provided by the manufacturer and distributor, and check that the buffer does not include substances which listed as contaminants in the manual.

(4) Structural integrity

Measure the RIN (RNA Integrity Number) value using electrophoretic method (Bioanalyzer, etc.) as an index of structural integrity. The closer the RIN value is to 10, the higher the quality of the RNA.

4.3 Library Quality Control for NGS Analysis

For NGS analysis for research, whole genome sequencing may be performed in some cases. However, when conducting NGS analysis from the aspect of genetic testing, in most cases only specific genes and genomic regions are used as targets for sequencing. In order to efficiently extract the maximum sequence results from valuable specimens and accurately evaluate genetic mutations, it is extremely important to properly perform the quality checks at each step described below. A library preparation reagent for NGS analysis is commercially available from various manufacturers and distributors, and the quality check methods at each step are described in detail in each protocol, which are to be referred to.

4.3.1 General Precautions on Library Preparation

In the preparation of DNA or RNA for NGS analysis, the following general points should be noted:

・When performing the procedure, to avoid mixing with nuclease into reagents, always wear powder-free laboratory gloves, and use pipettes fitted with nuclease-free pipette tips and aerosol prevention filters to avoid mixing with nuclease into reagents.

.

・Keep the measurement area shall be always clean, and measurements are carried out under conditions of Biosafety Level 1 (BSL 1).

・In order to avoid mutual contamination between DNA libraries for NGS, it is preferable to

perform the procedure before and after PCR in different laboratories, and also to use dedicated equipment and instruments. Also, after completing library preparation work, instruments, laboratory benches and other work surfaces should be cleaned with bleaching agent (sodium hypochlorite).

4.3.2 Quality Check of Fragmented DNA

In order to analyze the target region, when enriching the region by applying hybridization between the probe and the target region, genomic DNA is fragmented first.

In order to decrease the loss of genomic DNA as much as possible, it is desirable to use equipment that intensively sonicates the sample with a high powered and stable ultrasonic frequency. Alternatively, there are reagents that combine multiple restriction enzymes or use DNA fragmentation enzymes that cut at random sites.

Fragmented DNA is confirmed by an electrophoretic histogram of a microelectrophoresis apparatus to have a single peak, and also checked for a peak shape, a size of the peak top, and a size of the peak tail, according to the instructions of the manufacturer or distributor. The optimum fragment length differs depending on the platform and reagent of NGS used for the analyses, and the purpose of them.

Agilent Technologies' HaloPlex provides dedicated Enrichment Control DNA (ECD) along with reagents to check if restriction enzyme treatment is proceeding as expected.

For the experiment, it is recommended that a quality check is performed by including this ECD for one specimen. ECD contains genomic DNA and 800bp PCR product with cleavage sites for all restriction enzymes used. When there is no problem with restriction enzyme digestion, smear bands derived from genomic DNA are observed between 100 and 2500bp. Three major bands around 125, 225 and 450bp corresponding to restriction enzyme digestion fragments derived from the 800bp PCR product should be also observed. If these three bands are seen, restriction enzyme digestion is regarded as successful.

4.3.3 Confirmation of Success or Failure of Adapter ligation and Quality Check

In order to analyze the target region, when enriching the region by applying hybridization between the probe and the target region, an adapter for the NGS reaction is added to both ends of the fragmented DNA.

If the amplification product is obtained as expected by PCR amplification using the adapter sequence, it can be considered that the adapter addition reaction is successful.

In order to obtain a high capture efficiency in hybridization, it is important to accurately use the specified amount of the DNA library to which the adapter is added.

The adapter-attached DNA library is analyzed by an electrophoretic histogram of a micro electrophoresis apparatus, confirmed to have a single peak, and checked for peak shape, size length of peak top, size of peak tail and the like. The optimum fragment length differs depending on the platform and reagent of NGS used for the analyses, and purpose of the analyses. In addition, quantitation of the library concentration by quantitative PCR is recommended. Since quantitative PCR can selectively quantify only the library to which the adapter is added at both ends, the accuracy of the quantitative value is high. Quantitation can be also performed using a fluorescence assay (or the like) showing specificity for dsDNA. In such quantification, one should obtain as accurate measurement values as possible by creating standard curves at an appropriate frequency and measuring DNA of known concentration each time.

4.3.4 Quality Check of DNA library for NGS

The quality of the DNA library for NGS is confirmed by electrophoretic histogram diagram of microelectrophoresis apparatus.

When preparing a sample by target enrichment using the hybridization principle, confirm that it has a single peak, and check for the peak shape, a size of the peak top, a size of the peak tail and the like. When preparing a library based on the amplicon principle, multiple peaks can be detected in some cases. The optimum peak shape and fragment length will differ depending on the platform and reagent of NGS used for analysis, purpose of analysis and so on. In addition, quantitation of the library concentration by quantitative PCR is recommended. Since quantitative PCR can selectively quantify only the library to which the adapter is added at both ends, the accuracy of the quantitative value is high. Quantitation can be performed using a fluorescence assay or the like, specific to dsDNA. In such quantification, one should obtain as accurate measurement values as possible by creating standard curves at an appropriate frequency and measuring known concentration each time.

5. Circulating Tumor Cell (CTC) Measurement (cancer diagnosis / peripheral blood / trace amount cells)

CTC is a beneficial tool for obtaining information on cancer metastasis. Since the number of CTCs flowing in the blood is extremely small, it is necessary to be able to capture the CTC existing as low as only one cell per 100,000 leukocytes. Extreme caution is required for the specimen preparation. A variety of measurement methods are commercially available, including the only equipment approved by the FDA, namely the Cell Search system. It is important to capture a very small number of CTC without damages, measure the number of captured cells, and if possible, analyze the captured cells for subsequent genetic testing.

A general sample processing method can be described as follows,

1) The necessary amount of blood (for example 5 to 20 mL) is collected from the patient and stored in an appropriate tube (e.g. Cell Save) for stable storage. The possible storage period varies depending on the type of tube and the measuring method. The analyzing process should be performed within the period instructed by the manufacturer. When the collected blood sample is transported during this period, storage conditions (temperature, humidity) should be controlled in accordance with the conditions instructed by the manufacturer.
2) Buffer solution provided in a kit and a density gradient separating reagent are added to part or all the blood collected in process 1) and mixed. The sample is centrifuged to obtain blood cell components including CTC. Red blood cells can be removed by the centrifugation or hemolysis treatment.

CTCs are concentrated using various methods from the blood cell components after the process 2). It is important to consider that CTCs need to be recovered with a high yield through the concentration process, and damage of CTC should be minimized. Processes should comply with protocols provided by manufacturer and distributor of the CTC measurement system.

(1) Inappropriate conditions unsuitable for testing

Samples with a low CTC recovery rate or with damaged CTC due to inappropriate conditions during collection, transportation, storage, or concentration are unsuitable for testing, as CTC-derived DNA will not be obtained or detected sufficiently.

(2) Cause

The number of CTCs in the specimen is extremely small, and they can be lost or damaged during the collection process.

(3) Troubleshooting

Review the processes as instructed by the manufacturer and select appropriate procedure

in those processes. Concentrate and recover cells with less stressful conditions.

(4) Measures to avoid inappropriate situations

Avoid any collection methods that stress the cells and adopt a measurement method that allows observation of CTCs.

CTC ; Circulating Tumor Cell

"Circulating tumor cell" or "peripheral blood circulating tumor cell" is defined as cells released from a primary tumor tissue or metastatic tumor tissue and leaked into blood.

6. miRNA · Exosome

6.1 microRNA (miRNA)

miRNA is a very small, highly conserved RNA molecule that has a crucial role in biological processes. Most RNA extraction methods are specifically provided to obtain only mRNA. It is difficult to efficiently prepare short RNA molecules. For this reason, extraction reagents or kits intended to prepare short RNA molecules are desirable.

Unless analysis occurs immediately after extracting miRNA from the collected specimen, the specimen should be kept under appropriate conditions. The extracted miRNA should be stored in RNase-free conditions at -80 °C in a storage container preventing RNA adsorption.

If body fluids such as serum and plasma are used as a specimen and miRNAs secreted from cells are targeted, cell contamination and hemolysis shall be avoided. When plasma is used, an anticoagulant such as EDTA or citric acid should be used. Anticoagulants of biological origin such as heparin should be avoided.

In the case of using frozen sections, tissue pieces should be frozen in liquid nitrogen and stored in an airtight container at -70 °C or below.

The extraction method set for each test or sample type should be followed. The storage conditions for the extracted RNA should be controlled as recommended.

(1) Inappropriate conditions unsuitable for testing

1) Degradation of RNA and contamination with impurities other than RNA.
2) When the amount of RNA is extremely small, it may not be possible to measure accurately.

(2) Cause

1) RNA is degraded by being unnecessarily exposed to ambient or high temperature.
2) RNA is degraded by an RNA degrading enzyme.
3) RNA is adsorbed by contamination with impurities, containers and instruments.
4) In transportation, storage and measurement, RNA is adsorbed during one of the procedure steps.

(3) Troubleshooting

1) Storage conditions of RNA should be -70 °C or lower.
2) For RNA storage containers, use containers designed to prevent adsorption of nucleic acids.
3) When transporting RNA, keep it cool below -70 °C or use dry ice packaging.
4) The containers for transport shall be of a shape that is expected to maintain temperature such as Styrofoam and that do not collapse RNA-containing tubes.
5) When RNA is used, confirm the presence or absence of contamination by impurities

as much as possible by electrophoresis or absorbance measurement, and purify if impurities other than RNA are observed.

(4) Measures to avoid inappropriate situations

1) When RNA is to be extracted from tissues or cells, use RNA protectant until extraction or keep it at -70 °C or lower. When storing at -70 °C or lower, RNA degradation can still occur depending on the tissue or cell type, so it is preferable to validate that a specimen is fit for purpose and not excessively decomposed beforehand.
2) If the sample type from which RNA is extracted is blood-derived, blood that has chyle or has undergone hemolysis should not be used.
3) When RNA is being extracted from serum, the storage temperature of the specimen should be -70 °C or lower, and RNA extraction should be completed within 7 days from the start of storage.
4) When RNA is being extracted from serum, the cycle number of freezing and thawing of the serum should be less than 3 times.
5) At the time of extracting RNA, ensure operating procedures are being followed, such as procedures and conditions specified by each extraction reagent.
6) When storing RNA, in order to avoid repeated cycles of freezing and thawing, use freezer without a defrost function.
7) The personnel handling RNA shall wear masks and gloves after removing RNase in the handling environment, in order to prevent RNA degradation by RNA degrading enzymes.

6.2 Exosome

Methods for separating exosomes include ultracentrifugation, precipitation, filtration and other methods. Comply with each isolation method or recommended isolation method for each sample type. Avoid adding anti-coagulants of biological origin such as heparin in blood analysis. Comply with the recommended storage conditions for isolated exosomes. When extracting nucleic acid or protein from the isolated exosome, comply with the established method.

(1) Inappropriate conditions unsuitable for testing

1) Influence of impurities other than exosome affects the results.
2) When the amount of isolated exosome is extremely small, it can not be measured accurately.

(2) Cause

1) Contamination of impurities in transportation, storage and isolation operation steps.

2) An isolation method inappropriate for the measurement method is selected.
3) Exosome is adsorbed to storage container.
4) Sample is damaged by repeated cycles of freezing and thawing.

(3) Troubleshooting

1) Remove cells and debris before exosome isolation.
2) After isolation of exosomes, resuspend the sample in phosphate buffer saline (PBS) or the like.
3) Store exosomes at 4 °C or less.
4) Use a container with low adsorption for storage of exosomes.
5) When transporting exosomes, use a refrigerator below 4 °C or pack in dry ice.
6) Transport container is expected to maintain temperature such as styrofoam, and it should be of a shape that does not collapse tubes containing exosomes.

(4) Measures to avoid inappropriate situations

1) When extracting nucleic acid or protein from exosomes, extract immediately after exosome isolation.
2) At the time of the extraction procedure, comply with the operation procedure, such as procedures and conditions specified by each extraction reagent.
3) Avoid repeated cycles of freezing and thawing when storing exosomes.

7. Blood Circulating Cell Free Nucleic Acid

7.1 Blood Circulating Cell Free Nucleic Acid: Tumor

The general procedure from blood collection to plasma separation and storage is shown. (See 4.1.1 Blood section for inappropriate conditions, cause, troubleshooting, and avoidance methods)

(1) Collection

· Collect blood using a blood collection tube according to the package insert of the reagent or the device to be used or the instructions of the product/service provider.
· The use of a blood collection tube containing ccfDNA profile stabilizer is recommended. These types are currently marketed in Japan:
  PAXgene ccfDNA blood collection tube (Japan Becton · Dickinson)
  Blood collection tube for cell free DNA extraction (Roche・Diagnostics)
· When a blood collection tube with stabilizer can not be used, it is expected that inactivation of endogenous DNase will be required, therefore a blood collection tube with EDTA should be used than other anticoagulants.

· Immediately invert and mix the tube after collecting blood. Mix slowly to avoid DNA release due to destruction of cells. Invert and mix in accordance with the description of the reagent to be used or the package insert of the device or the instructions of the service provider.

(2) Transportation

· Be careful not to expose the sample to ambient temperature for a long period before plasma separation, in order to avoid blood cell destruction.

· Plasma separation is performed according to the instructions of diagnostic reagent manufacturer, the equipment manufacturer or laboratory service provider. Also note the recommendations for storage conditions.

· For blood collection tubes with stabilizer, maintain the stability of the ccfDNA profile by following the package insert or information provided. If there are instructions from the service provider, follow these.

· When using a blood collection tube with EDTA, detrimental effects can be avoided if processing is within 6 hours at 2-6 °C, depending on the measurement application. If there is a package insert or instruction sheet, follow the instructions.

· Validate the time and temperature conditions as necessary.

· As the ccfDNA profile changes due to freezing, the primary sample (whole blood sample) should not be frozen.

(3) Pretreatment of ccfDNA before specimen treatment

· Perform processing according to the package insert of the reagent or the equipment, or the instructions of the service provider. The following is a general procedure to use a blood collection tube with EDTA, if one is not described in the package insert or instructions.

· Centrifuge at 1,600-2,500 × g, 2-8 °C, for 10 minutes. In order to avoid contamination of nucleic acids from leukocytes, pay attention not to disturb the interface between plasma and cell layer, and then transfer it to another tube.

---

ccfDNA; circulating cell-free DNA

Extracellular free DNA fragments called circulating cell-free DNA exists in the liquid layer of human blood. It is also called cell-free DNA (cfDNA), or blood circulating free DNA. DNA derived from a cancer, and that has been released into the blood is called circulating tumor DNA (ctDNA).

· Perform the second centrifugation. This should be carried out under the conditions of 14,000 -16,000 × g, 2-8 °C for 10 minutes.

・In the absence of a high speed centrifuge, the second centrifugation can take place at a lower relative centrifugal acceleration, for example 3,000 - 5,000 × g, 2-8 °C, for 20 minutes.

(4) Storage

· The preservation of plasma should be in accordance with the package insert of the reagent, or the instructions from the service provider. If there are no instructions, it can be stored for up to 24 hours at 2-8 ℃. Longer term storage should be at or below -20 ℃. Validate the temperature and duration of storage.

7.2 Noninvasive Prenatal Genetic Testing

A prenatal genetic test using pregnant women's peripheral blood (maternal blood) has been developed and started to be used in Japan, following overseas usage. For this test, peripheral blood is collected as a specimen and used for measurement. Because it minimizes physical burden on the patient, it is called non-invasive prenatal genetic testing (NIPT).

Fetal-derived DNA fragments account for 3-10% of the total maternal blood ccf DNA. Its length tends to be short (less than 300 bp), suggesting it arise from apoptosis rather than cell necrosis. There are various ways to detect fetal numerical chromosome abnormalities by analyzing fetal-derived DNA present in maternal plasma. The shotgun method of sequencing all DNA in plasma and the method of detecting specific sequence markers are analytically validated in clinical trials.

NIPT measures the relative number of specific autosomes (13, 18, 21) by quantifying both fetal-derived free DNA and mother-derived DNA fragment present in maternal plasma, and thus determines the presence or absence of a numerical abnormality of a fetal chromosome. NIPT is a screening test and non-definitive test. Besides the specific autosomal chromosome change, now detection methods of the numerical abnormalities of sex chromosomes have been developed and applied to a practical use in oversea countries.

The emergence of fetal-derived DNA fragments in maternal blood is observed from as early as week 5 of pregnancy, or after 9 weeks as the latest. Therefore, the examination should be done after 10 weeks gestation.

For sample collection, it is recommended to use a blood collection tube containing ccfDNA profile stabilizer (for details see section 7.1 Collection of blood circulation free nucleic acid (1)). Note the gauge size of the blood collection needle to avoid the effects of cell destruction due to excessive pressure. Procedures such as inverting and mixing immediately after collection should follow the method described in the package insert of the blood collection

tube.

NIPT in Japan is conducted in clinical laboratories which have been certified by the Japan Medical Association as a clinical research facility. Since plasma separation and analysis procedures are strictly defined and implemented according to the standard work protocol in each clinical laboratory, from the viewpoint of quality control of NIPT, it is necessary to consider collection and transport conditions of blood specimens. For this reason, concrete methods for risk management such as transport means of specimens and prevention of mistakes should be presented. In addition, this examination should be requested in a limited facility capable of providing adequate genetic counseling.

(1) Inappropriate conditions unsuitable for testing

Coagulated blood. Samples that include lysed nucleated blood cells. Samples stored for inappropriate times and temperature after collection and transportation can deteriorate in quality and reduce the reliability of measurement results.

(2) Cause

Mixing after blood collection is insufficient. Plasma separation is insufficient. Influence of freezing of specimen and pressure. Inadequate use of blood collection tube, long-term storage of sample after collection, transport time and conditions and storage temperature are not appropriate.

(3) Troubleshooting

If measurement is difficult due to inappropriate nature, blood should be taken again.

Relative centrifugal acceleration RCF (Relative Centrifugal Force): The so-called "centrifugal force" used for centrifugal separation is referred to as relative gravitational acceleration because it is expressed as a ratio with the gravitational acceleration of the earth.

(4) Measures to avoid inappropriate situations

Choose an appropriate blood collection tube, collect blood, immediately invert thoroughly and mix, and store and transport appropriately as described in the attached instruction manual.

In general, blood collection tubes with EDTA are designed to be kept refrigerated after blood collection, but some blood collection tubes dedicated to ccfDNA extraction are designed to be stored at ambient temperature without refrigerating or freezing of blood specimens. Set the storage temperature appropriately. It is recommended to transport in dedicated shipping box where specimen temperature control is secure from collection until delivery. When transportation exceeds a predetermined time due to holidays or delays, plasma separation and freezing (-80 °C) is performed at the registered commercial laboratories in accordance with the standard operating procedure document prescribed by the NIPT clinical laboratory.

8. Mitochondrial DNA

Genetic analysis targeting mitochondrial DNA (mtDNA) is performed by several methods including using a PCR-RFLP method for detecting specific point mutations, Southern blotting, and whole genome sequencing with next-generation sequencers.

Blood is collected using a blood collection tube containing an anticoagulant (e.g. EDTA), then immediately mixed by inversion. When nucleic acid extraction is not performed on the day of blood collection, store the sample refrigerated.

Cryopreservation is not contraindicated but better to avoid in some cases. It depends on the downstream processing method. For example, although platelets do not contain nuclear DNA, they do contain mtDNA. Thus, platelets may be used as a source for mitochondrial DNA isolation. Freezing the blood will make centrifugation more difficult, due to hemolysis or destruction of white blood cells.

Tissue samples are stored frozen (-70 ℃ or below) in a sterilized container. The maximum storage period for frozen tissues is recommended to be less than 1 month at -80 °C or less than 1 week at -20 °C.

(1) Inappropriate conditions unsuitable for testing

1) Blood is contaminated with impurities or clotted (platelets are absorbed in blood clots, and mtDNA content in the supernatant is thought to be reduced).
2) Accurate measurements can not be possible with extremely small amounts of isolated mtDNA. It is difficult to judge if the measurement result has been affected by contamination.

mtDNA has a high copy number per cell and the risk of contamination is high.

(2) Cause

1) Impurities contaminate the sample during transportation, storage and isolation processes.
2) Genomic DNA is contaminated in the isolation procedure. It is known that the sequences homologous to mtDNA called NUMT (nuclear mitochondrial DNA segment) are interspersed in genomic DNA. Nuclear DNA contamination should be controlled when analyzing mtDNA regions containing sequences homologous to NUMT.

(3) Troubleshooting

1) The containers used for collection, and prevention of acquiring the operator's mtDNA should be checked. An isolation procedure with a high recovery rate of mtDNA should be selected. The contamination of genomic DNA should be avoided as much as possible. Pay attention to the PCR primer design for analyzing the samples prepared from tissues or whole blood, where the genomic DNA contamination cannot be completely avoided, and take into account the existence of NUMT.

(4) Measures to avoid inappropriate situations

1) Use a sterile, specialized container is used for storing the sample.
2) Use samples suitable for mtDNA extraction including platelets.
3) Use a reagent specifically designed for mtDNA extraction is used for the isolation operation.
4) Select an extraction method that takes into consideration that mtDNA is circular.

(4)Appendix

3-1: Quality Control of DNA for Array CGH

In the case of detecting changes in genome copy number using CGH microarray, quality control of DNA is important because the quality of DNA used will affect the noise level of the data finally obtained and the accuracy of the analysis result.

In the quality control of DNA, length, amount, chemical purity and structural integrity can be used as indices. For confirming the length (degree of degradation) of DNA, electrophoresis is used. For good DNA with a low degree of degradation, high molecular weight DNA can be clearly confirmed, and smear bands and peaks of low molecular weight are not observed. It is preferable that the DNA is free of carbohydrates, organic solvents, proteins, and RNAs. Further, the index of chemical purity of good DNA is that the value of A_{260} / A_{280} is 1.8 to 2.0 and the value of A_{260} / A_{230} is higher than 1.0. There is a method of calculating the ratio of double stranded DNA (dsDNA) as an index of structural integrity. dsDNA is quantitated using a fluorescence spectrophotometer and compared with the DNA concentration measured with an absorptiometer.

Percentage of dsDNA (%) = dsDNA concentration / DNA concentration × 100

The quality of DNA is very important as it affects the final analysis result. It is important to prepare DNA that meets the quality recommended by each manufacturer.

【index】

Evaluate with purified DNA quality.

1) A_{260}/A_{280} ≧1.5
2) A_{260}/A_{230} ≧1.0
2) Confirm that there is a band of 20kbp or longer by electrophoresis and that fragmentation is not evident.
3) For example, the amount of double-stranded DNA is specifically measured using a fluorescent reagent or the like.

* The amount of DNA required depends on the array platform to be used.

3-2: Quality Check of CGH Microarray DNA Specimen: Agilent CGH / CGH + SNP Microarray

When detecting changes in the genome copy number using the CGH microarray, the quality of the DNA to be used affects the noise level of the data to be finally obtained, that is, the accuracy of the analysis result. Therefore, before and after labeling DNA quality control is important. Here, a direct labeling method by Agilent Technology's CGH microarray and DNA fluorescent label (hereinafter referred to as labeled) reagent for CGH + SNP microarray, SureTag DNA Labeling Kit, SureTag Complete DNA Labeling Kit is cited as an example.

With reference to these kits, we describe the method of DNA quality check for CGH microarray, relevant for both the control DNA and the DNA to be analyzed.

Quality check of genomic DNA and labeled DNA for CGH and CGH + SNP microarrays

(1) Notes on operation

- To avoid nuclease contamination into reagents, always wear powder-free laboratory gloves and use pipet tips with an appropriate solution, and pipet tips with nuclease-free aerosol prevention filters.
- Clean the measurement (or work) space at all times.
- Water used for dilution / buffer solution shall be free of nucleases.
- Do not mix solutions containing genomic DNA using a Vortex Mixer. Mix the liquid by gently tapping the tube with your finger.
- Solutions containing genomic DNA should not be subjected to repeated cycles of freezing and thawing.
- When using a frozen stock solution, follow these directions.
  ① Melt the dispensed solution as quickly as possible so as not to heat at ambient temperature or higher.
  ② Lightly mix with a Vortex Mixer for a few seconds, centrifuge for 5 to 10 seconds in a centrifuge, and spin down the liquid adhering to the tube wall and lid.
  ③ If DNA is not completely dissolved, dissolve by heating at 37 ℃ for 30 minutes.
  ④ Store on ice or in a cooling block until use.
- Perform measurement under conditions of Biosafety Level 1 (BSL 1).

(2) Quality check of genomic DNA before labeling operation

- Confirm the values of A_{260}/A_{280} and A_{260}/A_{230} using ultraviolet visible spectrophotometer such as NanoDrop (Thermo Fisher Scientific) to confirm that high concentrations of salt, protein, organic solvents are not mixed in with the DNA. It is desirable that the value of A_{260}/A_{280} is between 1.8 and 2.0, the value of A_{260}/A_{230} is higher than 1.0. It is also desirable to confirm that there is no contaminant affecting absorbance at 260 nm by continuously scanning the absorbance at 220-320 nm.
- To confirm that there is no contamination by a large amount of RNA in the DNA, quantification is carried out by both a fluorescence analysis method specific to double-stranded DNA and an ultraviolet-visible spectrophotometer. Then, confirm that there is no significant difference between the two values. Since quantitative values obtained by ultraviolet and visible spectrophotometer include those attributable to substances having absorption in the vicinity of 260 nm such as RNA, dissociation of the quantitative values

of both methods suggests the possibility of RNA contamination. Contamination of a large amount of RNA affects the labeling reaction. The fluorescence analysis method is described in the following item. At the time of measurement, it is important to confirm that the DNA is completely dissolved. If the measured concentration exceeds 350ng/μL, dilute it two-fold and measure again.

- To confirm degradation of genomic DNA, agarose gel electrophoresis or fully automated high throughput electrophoresis, TapeStation, with genome DNA kit is used. Degradation of genomic DNA not only affects the yield of labeled DNA and efficiency of labeling but also hinders the process of performing random fragmentation with restriction enzymes.
- For quantitative determination of genomic DNA, it is recommended to use a fluorescence analysis method which is specific to double stranded DNA like Qubit Fluorometer. Quantitative values obtained by ultraviolet-visible spectrophotometer have a risk of erroneous determination due to contaminants in DNA as also noted in the above item. In CGH and CGH + SNP microarrays, the amount of DNA is within the recommended DNA amount range depending on each microarray format. It is important that the amount of the DNA to be analyzed is the same as that of the control DNA competitively hybridized to the microarray. Incorrect quantification of DNA not only causes the DNA used for labeling to deviate from the recommended range, but also causes deviation between the amount of control DNA and the amount of DNA to be analyzed. As a result, the assay noise (Derivative Log Ratio Spread (DLRSD) of the data rises, and the detection power of the copy number change region decreases, so it is important to quantify as accurately as possible. Quant-IT assay using Qubit, and PicoGreen reagent by Thermo Fisher Scientific Inc. are used as fluorescence analysis methods. When fluorescence analysis methods are used, it is preferable to prepare a calibration curve at an appropriate frequency and to measure a DNA of known concentration every time so as to prevent a decrease in the quantitative value due to repeated measurement, in order to obtain a measurement value that is as accurate as possible.

(3) Quality check of genomic DNA after restriction enzyme reaction

For DNA labeling procedures of CGH and CGH + SNP microarrays, genomic DNA is first fragmented with *Alu* I and *Rsa* I restriction enzymes. Separate the DNA after the restriction enzyme reaction using 0.8% agarose gel electrophoresis, confirm that most of the fragmented DNA is in the range of 200 bp to 500 bp. Due to the low concentration of DNA, it is desirable to use a combination of high sensitivity detection reagents such as SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific).

(4) DNA quality check after labeling

Labeled DNA is prepared by annealing random primers to both the control DNA fragmented with the restriction enzyme and the DNA to be analyzed, and performing exo-Klenow fragment enzymatic reaction in the presence of Cyanine 3-dUTP or Cyanine 5-dUTP, respectively. In the protocol which omits the restriction enzyme reaction, fragmentation is carried out by performing longer heating when annealing random primers to the genomic DNA. The labeled DNA is isolated by column purification, adjusted to a predetermined capacity depending on each microarray format, and labeled DNA yield and fluorescent dye uptake rate are measured with an ultraviolet visible spectrophotometer.

Measure A_{260}, A_{550}, A_{650} values, calculate the concentration of labeled DNA from A_{260}, the concentration of Cyanine 3 from A_{550}, and the concentration of Cyanine 5 from A_{650}. The labeled DNA yield (μg) is calculated by multiplying the DNA concentration by the above prepared predetermined volume. Fluorescent dye uptake rates are calculated by dividing the concentrations of Cyanine 3 and Cyanine 5 by the concentration of labeled DNA (pmol/ μg), respectively.

Estimated yield is 3-6 μg when labeling with 200 ng of DNA or labeling with 8 x format, 8-13 μg in case of 500 ng, 9-16 μg in case of 1 μg. Estimates of fluorescent dye uptake rates (Cyanine 3 and Cyanine 5) are 15 to 50 pmol/ μg when labeling with 200 ng of DNA or labeling with 8 × format, and 20-60 pmol/ μg for 500 ng, 20-60 pmol/ μg for 1 μg. When using a protocol omitting the restriction enzyme reaction, the fluorescent dye uptake rate is 5 pmol / μg lower than the above value.

If the yield of labeled DNA is lower than the above-mentioned target value, it may be lost due to a failure in the column used for labeling DNA purification. If the column elution solution obtained in the purification process of labeled DNA is preserved, purify from it again. If the yield is still low, it is desirable to confirm the set temperature of the equipment used for the labeling procedure and the quality of the genomic DNA before labeling and to carry out labeling again.

When the fluorescent dye uptake rate is lower than the above-mentioned reference value, it is expected that some problems may have occurred in the labeling procedure such as degradation of the dye. It is important to confirm the temperature setting of the equipment used for the labeling procedure. It is desirable to check the quality of the genomic DNA before labeling and re-label it. There is also the possibility of degradation of the labeling reagent depending on repeated freezing and thawing of the fluorescent dye and the storage temperature of various enzymes, so confirm this point again.

Since it affects the power with which copy number changes can be detected, it is extremely important to properly perform quality checks at each of the above-mentioned steps for

efficient analysis of a valuable DNA specimen.

4-1: Quality Check of NGS DNA Library: SureSelect

Quality control of samples and prepared libraries is important since the quality of DNA sample used will affect the final sequencing efficiency when performing DNA target sequencing using NGS. Here, we describe the method of quality checking in NGS library preparation taking Agilent Technologies' target enrichment reagent SureSelect as an example.

Library quality check for DNA target capture sequence (exon sequence, target sequence)

(1) Notes on procedure

- To avoid nuclease contamination of reagents, always wear powder-free laboratory gloves when using the procedure, use the correct solutions, and use a pipette fitted with a pipette tip with nuclease-free aerosol prevention filter.
- The experiment area should be kept clean at all times.
- Avoid rigorous mixing of solutions containing genomic DNA with a Vortex Mixer, and instead mix solutions by gently tapping.
- Avoid repetition cycles of freezing and thawing as much as possible for solutions containing genomic DNA.
- When using a frozen stock solution, work with it as follows.
  - ① Make sure not to heat at ambient temperature or higher and melt the dispensed solution as quickly as possible.
  - ② Briefly mix with a Vortex Mixer for a short time, centrifuge in a centrifuge for 5-10 seconds, and spin down the liquid adhering to the tube wall and lid.
  - ③ Store on ice or in a cooling block until use.
- Perform procedures under conditions of Biosafety Level 1 (BSL 1).

(2) Quality check of genomic DNA

- Check the ratio of A_{260}/A_{280} using an ultraviolet visible spectrophotometer such as NanoDrop. The ratio of A_{260}/A_{280} should be between 1.8 and 2.0.
- To confirm the presence or absence of degradation of genomic DNA, use agarose gel electrophoresis or fully automated high throughput electrophoresis, such as the genomic DNA kit of TapeStation. If the degradation of genomic DNA is ongoing, the amount of recoverable DNA decreases after fragmentation, or genomic DNA not fragmented to the desired length can occur.
- For quantitative determination of genomic DNA, it is desirable to use a fluorescence assay

method which shows selectivity for double-stranded DNA. When quantitating with an ultraviolet-visible spectrophotometer, substances having absorption near 260 nm are quantitatively measured together, so there is a tendency to estimate the quantitative value excessively. When the quantitative value is overestimated, fragmentation becomes uneven, the yield at purification deteriorates, and unexpected by-products are formed. Conversely, if the quantitative value is underestimated, there may be cases where a library of the amount required for hybridization for target capture can't be obtained. Quant-IT assay using Qubit and PicoGreen reagent by Thermo Fisher Scientific Inc. are used as a fluorescence assay method. When using the fluorescence assay method, in order to prevent lowering of the quantitative value by repeated measurements, it is preferable to prepare a calibration curve at an appropriate frequency and measure the DNA of known concentration each time to obtain an accurate measured value.

(3) Quality check of genomic DNA after fragmentation

In library preparation of NGS, genomic DNA is first fragmented in order to obtain an insert size of a certain length according to the purpose of the sequencing.

In order to obtain DNA fragments of the desired length with as little loss as possible, Agilent Technologies' SureSelect's protocol specifies the use of a Covaris ultrasonicator (M&S Instruments) which uses Adaptive Focused Acoustics (AFA) technology that intensively sonicates a sample with high power and stable ultrasound.

For DNA fragmented to the target length in the Covaris ultrasonciator, confirm the size of the fragmentation and confirm the quantitative value of fragmentation with a microchip type electrophoresis system Bioanalyzer or TapeStation (Agilent Technologies). As an example, when performing all exon analysis with Illumina's NGS, the target peak size of fragmentation is 150-200 bp. It should be a single peak with a peak top between 150-200 bp in the electrophoretic diagram of the Bioanalyzer or TapeStation and with the same peak shape as the figure described in the protocol. It is also desirable to measure yields. If a yield remarkably far from the expected initial starting amount is obtained, it is necessary to start over from the quantification of genomic DNA.

(4) Quality check of DNA library with adapter after adapter addition, PCR and purification

DNA fragmented with the Covaris ultrasonicator goes through the steps of end repair, A-tail addition, adapter addition, PCR amplification, bead purification, and hybridization to capture and concentrate the sequence of interest. In this case, it is important to quantify the DNA library added by the adapter and perform hybridization in the amount specified in the protocol in order to obtain a high capture efficiency.

In the SureSelect protocol, use a Bioanalyzer or TapeStation for size confirmation and

quantification. As an example, when exon analysis is performed with Illumina's NGS, the DNA library peak size after adapter addition should be about 250 bp-275 bp. It should be a single peak with a peak top between 250 and 275 bp in the electrophoretic diagram of the Bioanalyzer or TapeStation. Then, confirm that there are no other unnecessary peaks and shoulder, and that the peak observed has the same peak shape as the figure described in the protocol. Further, the obtained peak is to be quantified. For whole exon analysis, a DNA library with adapter of 750 ng is required for hybridization.

(5) Quality check of DNA library with adapter after capture, concentration and amplification

By hybridization with a biotinylated RNA probe designed in the target region of interest, the captured adapter-attached DNA library is concentrated by streptavidin beads. Then, through the process of PCR and bead purification, which adds an index bar code for analyzing multiple samples in one lane, it becomes a library for final sequencing with NGS. Since this final library has a low concentration, a high-sensitivity DNA kit of a Bioanalyzer or TapeStation is used for size confirmation and quantification. As high sensitivity kits are also sensitive to contamination, day-to-day maintenance of equipment used, such as electrode washing, is important for obtaining stable results. It should yield a single peak with a peak top between 300-400bp in the electrophoretic diagram of the bioanalyzer or TapeStation. Confirm that there is no PCR nonspecific amplification product and that it has a peak shape resembling the figure described in the protocol. Typical yields (concentrations) are between 0.7 ng/μL and 1.2 ng/μL when whole exon analysis is performed with Illumina's NGS.

In addition, when adding a barcode to each sample and multiplexing multiple samples in one lane and performing sequencing, it is recommended to quantify the library concentration by quantitative PCR. Since quantitative PCR can selectively quantify only the library to which the adapter is added at both ends, the accuracy of the quantitative value is high. A kit for quantification of NGS libraries from Agilent Technologies Inc., Illumina Inc., and Thermo Fisher Scientific Inc. can be used.

The target sequence by NGS is an analytical method which still requires considerable cost and time, though running costs have drastically decreased in recent years. In order to efficiently obtain the maximum sequence results from valuable DNA specimens, it is extremely important to properly perform the quality checks at each of the above-mentioned steps. In preparing DNA libraries for each NGS manufacturer, the quality checking method at each step is detailed in each protocol, so refer to it.

4-2: Quality Check of NGS DNA Library: HaloPlex

Quality control of specimens and prepared libraries is important since the quality of DNA

specimens used will affect the final sequencing efficiency when performing DNA target sequencing using NGS. Here, we describe the method of quality checking in NGS library preparation taking Agilent Technologies' target enrichment reagent HaloPlex as an example.

【Library quality check for DNA target capture sequencing】

Notes on procedure and quality check of genomic DNA are basically the same as SureSelect (for details see appendix 4-1 NGS DNA library quality check section).

(1) About ECD control

HaloPlex is a method to create an NGS sequence library by capturing arbitrary sequences of human targets of 5 Mb or less in total with high selectivity and efficiency by combining hybridization, ligation and PCR techniques. It has several features such as the required amount of DNA can be as small as 225 ng, the ability to amplify more than 200,000 amplicons in a single tube, easy change of the target region, and a short experiment time. It is expected to be used in desktop type next-generation sequencers such as Illumina's MiSeq.

In HaloPlex, restriction enzymes are used to fragment genomic DNA, so no equipment for physical fragmentation like the Covaris ultrasonicator is needed. Dedicated Enrichment Control DNA (ECD) is supplied with reagents as whether this restriction enzyme treatment progresses as expected, being an important point in quality control. For the experiment, it is recommended that quality control is performed by including this ECD for one specimen.

(2) Confirmation of restriction enzyme treatment

HaloPlex has been devised to improve the accuracy of mutation calls by covering the target region with multiple amplicons, and genomic DNA is treated with 8 kinds of 16 restriction enzymes determined. Whether 8 pairs of restriction enzyme treatment have been performed without problems is confirmed by analyzing ECD samples treated with restriction enzymes of each set and undigested ECD samples with TapeStation or Bioanalyzer.

The ECD sample contains genomic DNA and a PCR product of 800 bp, and this PCR product contains the cleavage sites of all restriction enzymes used in the HaloPlex kit. In undigested DNA control, a band of genomic DNA larger than 2.5 kbp and a band of PCR product of 800 bp are observed. In restricted enzyme digested ECD samples, smear bands derived from genomic DNA are observed between 100 and 2500 bp, and three major overlapping bands around 125, 225, and 450 bp. These three bands correspond to restriction enzyme digestion fragments derived from the 800 bp PCR product, and the exact size after cleavage with 8 different RE master mixes are different. Minor bands other than the three major bands of about 125, 225, and 450 bp can be detected in the lane of the restriction enzyme digested ECD sample. If three major bands are found, the defined enzymatic digestion can be

considered successful.

(3) Quality check of final amplified DNA library

Restriction enzyme-treated DNA fragments are hybridized with a HaloPlex probe designed for the target region. By hybridization with a HaloPlex probe, the target DNA fragment forms a cyclic structure. Only the target DNA having the digested fragments of restriction enzymes at both ends is ligated to form a cyclic structure. This target circular DNA is eluted, PCR is carried out, bead purification is carried out, and the final library for the sequence is completed. In addition to hybridization, only the defined restriction enzyme-treated fragment is ligated, and only DNA having a cyclic structure is PCR amplified, enabling capture to be performed with extremely high selectivity.

Confirm and quantify the library size using the Bioanalyzer or TapeStation for the completed final library. In the example using Illumina's NGS device, the amplicon consists of a 50 to 500 bp target DNA insert and a 125 bp sequencing motif. As a result, the amplicon size ranges from 175 bp to 625 bp, confirming that most amplicons are distributed in the range of 225 bp to 525 bp. Use amplicons between 175 bp and 625 bp for quantification. Even if peaks with sizes smaller than 175 bp are observed, they are not included in the subject of quantification.

HaloPlex has also been reported to be applied to FFPE samples, and its flexibility and high capture efficiency is expected to make it possible for new applications to be obtained by rapid and comprehensive analysis of tens to hundreds or thousands of exon sites of target genes. In order to stably bring out more reliable results, the quality check in these experiment steps is very important.

4-3: Quality Check of NGS DNA Library: Ion AmpliSeq ™

When performing target sequencing using NGS, the quality of the DNA used will affect the final sequence efficiency and the quality of the sequence. For this reason, quality control of specimens is very important. Here, we describe the quality checking method in NGS library preparation using multiplex PCR, taking Ion AmpliSeq ™ as a target enrichment method provided by Thermo Fisher Scientific Inc., as an example.

Ion AmpliSeq ™ is a target enrichment method using multiplex PCR, enabling the simultaneous amplification of up to 6,144 primer pairs from 1 to 100 ng of genomic DNA in one tube. By adjusting the length of the amplification product at the primer designing stage, it is also possible to deal with FFPE samples in which decomposition of nucleic acids occurs more frequently.

(1) Notes on procedure

· To avoid nuclease contamination into reagents, always wear powder-free laboratory gloves, use an appropriate solution, and use pipettes fitted with pipette tips with nuclease-free aerosol prevention filters.
· The experiment area shall always be kept clean.
· Avoid rigorous mixing of solutions containing genomic DNA with a Vortex Mixer. Mix the liquid by gently tapping with your finger.
· Avoid repetition cycles of freezing and thawing as much as possible for solutions containing genomic DNA.
· When using a frozen stock solution, work with it as follows:
  ① Make sure not to heat at ambient temperature or higher and melt the dispensed solution as quickly as possible.
  ② Briefly mix with a Vortex Mixer for a short time, centrifuge for 5 to 10 seconds in a centrifuge, and remove the liquid adhering to the tube wall and lid.
  ③ Store on ice or in a cooling block until use.
· Perform procedure under conditions of Biosafety Level 1 (BSL 1).

(2) Quality check of genomic DNA

· Check the ratio of A_{260}/A_{280} using an ultraviolet visible spectrophotometer such as NanoDrop. The ratio of A_{260}/A_{280} should be between 1.8 and 2.0.
· Measurement with an ultraviolet-visible spectrophotometer tends to overestimate the amount of DNA because the substance is quantitatively measured in the vicinity of 260 nm. When the quantitative value is excessively estimated, there is a possibility that there will be insufficient amplification products to make a library due to insufficient amount of genomic DNA as a template for PCR. For this reason, it is desirable to use quantitative determination of genomic DNA by using a fluorescence assay method showing selectivity for double-stranded DNA. As the fluorescence assay method, Qubit ™ dsDNA HS Assay Kit using Qubit ™ and PicoGreen from Thermo Fisher Scientific Inc. are used. When using the fluorescence assay method, in order to prevent the quantitative value from decreasing due to repeated measurements, it is recommended to prepare a calibration curve at an appropriate frequency and to measure the DNA of known concentration each time to obtain as accurate measured value. Otherwise, since 1 to 100 ng of genomic DNA corresponds to 300 to 30,000 copies, it is also possible to quantify the amount of DNA to be used by quantitating the copy number using TaqMan® RNase P Detection Reagent Kit (Thermo Fisher Scientific) using real time PCR.
· Use genomic DNA kit of agarose gel electrophoresis or microchip electrophoresis to confirm the presence or absence of degradation of genomic DNA. If the genomic DNA is degraded,

amplification products enough for a library can not be obtained due to insufficient amount of genomic DNA as a template for PCR.

- In particular, it is important to confirm the decomposition degree of nucleic acids of FFPE sample where decomposition of nucleic acid is seen more frequently and the yield of nucleic acid is expected to be so small that it can not be analyzed by electrophoresis. For this reason, it is desirable to adopt a method of estimating the extent of DNA degradation by real-time PCR using a plurality of primer sets designed to have different lengths at the same site.

(3) Quality check of amplified DNA library

In the Ion AmpliSeq ™ technology, in order to sequence the target region, an adaptor for emulsion PCR and sequencing reaction is added after amplification of the target region by multiplex PCR.

The success or failure of multiplex PCR and adapter addition reaction is judged by whether enough adapter-added library DNA has been amplified.

For confirmation of the amount of this library DNA, use the Agilent ™ High Sensitivity DNA Kit of Microchip type electrophoresis (Bioanalyzer ™) and calculate from the obtained peak area, or by the Ion Universal Library Quantitation Kit of real time PCR. Quantitation of this library DNA affects subsequent amplification by emulsion PCR, so it is necessary to perform it as precisely as possible.

However, in the case of homogenizing the amount of library DNA by the Ion Library Equalizer ™ kit using the magnetic bead method, this process can be omitted.

The success or failure of the reaction of the library DNA with this Ion Library Equalizer ™ kit is judged based on the sequence result.

As described above, success or failure of preparation of library DNA by Ion AmpliSeq ™ depends largely on the quality of genomic DNA. Therefore, the quality check of genomic DNA is important for stable, reliable results.

4-4: Quality Check of NGS RNA Library: TruSeq RNA Access

When sequencing RNA using NGS, the quality control of the specimen is important because the decomposition degree and the amount of the RNA specimen used affect the final sequencing efficiency. Here, Illumina's target enrichment reagent and TruSeq RNA Access are taken as examples, and a method of quality checking in NGS library preparation with RNA as input is described.

【Library quality check for RNA target capture sequencing (target sequence)】

(1) Notes on procedure

· To avoid nuclease contamination into reagents, always wear powder-free laboratory gloves and use an appropriate solution, and a micropipette fitted with pipette tips with nuclease-free aerosol prevention filters when operating.
· The experimental area shall be kept clean and cleaning with RNase remover is also performed.
· Avoid rigorous mixing of solutions containing RNA with a Vortex Mixer. Mix the liquid by gently tapping the tube.
· Avoid repetition cycles of freezing and thawing as much as possible in solution containing RNA. Store at -80 ° C.
· When using a frozen stock solution, work with it as follows.
  ① Melt on ice.
  ② Briefly mix with tapping, and centrifuge for 5 to 10 seconds with a centrifuge, to spin down the liquid adhering to the tube wall and lid.
  ③ Store on ice or in a cooling block until use.
· Experiments are carried out under conditions of Biosafety Level 1 (BSL 1).

(2) RNA quality check

· Check the ratio of A_{260}/A_{280} using an ultraviolet visible spectrophotometer such as NanoDrop. It is desirable that the ratio of A_{260}/A_{280} is about 2.0.
· Use RNA kit of microchip type electrophoresis (e.g. Agilent TapeStation, Bioanalyzer) to confirm degradation of RNA. Apart from the RIN value, the degree of degradation is evaluated by the ratio (DV 200: percentage of RNA fragments > 200 nt fragment distribution value) of RNA fragments leaving a length of 200 bases or more. If RNA degradation is severe, increase the amount of RNA used and make adjustments to ensure that the library preparation is reliable. By these adjustments, decomposed RNA with RIN value of about 2.0 can be flexibly used for library preparation.
・ For RNA quantification, it is desirable to use a fluorescence assay that shows selectivity for RNA. When quantitating with an ultraviolet-visible spectrophotometer, substances having absorption near 260 nm are quantitatively measured together, so there is a tendency to overestimate amounts of RNA. When the amount is overestimated, there are problems such as poor library yield, biased gene expression profiles, and unexpected by-products. Quant-IT assay using Qubit and RiboGreen reagent by Thermo Fisher Scientific Inc. are used as the fluorescence assay method. When a fluorescence assay is used, it is necessary to prevent lowering of the quantitative value by repeated measurements. For that purpose, prepare a calibration curve at an appropriate frequency and measure the RNA of known concentration every time to obtain a measurement value that is as accurate

as possible.

(3) Quality check of library with adapter after addition of adapter, PCR, and purification

RNA goes through steps of heat fragmentation, reverse transcription, A-tail addition, adapter addition, PCR amplification, bead purification, and then hybridization step to capture and concentrate the sequence of interest. The amount of RNA and fragmentation time should be adjusted according to the degree of degradation. In order to obtain high capture efficiency, it is an important point as well as the reagent targeted for DNA to quantify the library added the adapter and perform hybridization in the amount specified in the protocol.

In the TruSeq RNA Access protocol, use a Bioanalyzer or TapeStation for size confirmation and quantification. The library peak size after adapter addition is about 250 bp - 275 bp. In the electrophoretic diagram of the Bioanalyzer or TapeStation, confirm that there is a single peak, with the expected peak shape, size length of the peak top, and size at the peak tail. For quantification at this point, a fluorescence assay method or the like which shows selectivity to dsDNA can also be used.

Prior to capture, up to 4 samples can be pooled at this stage and work efficiency can be improved. In the case of pooling, it is desirable to refer to the degree of degradation of RNA measured prior to library preparation and to mix specimens with close decomposition degrees.

The preparation of the library after capture is the same as that of SureSelect (For details, see appendix 4-1 NGS DNA library quality check section).

TruSeq RNA Access is designed on the premise of application to FFPE samples. Due to its high flexibility, applications such as comprehensive gene expression analysis and fusion gene detection are expected even for highly degraded RNA specimens. Even when RNA with high degree of degradation is used, bias in the gene expression profile is unlikely to occur, enabling more useful results to be produced. In order to stably bring out more reliable results, the quality check in these experimental steps is very important.

(5) Bibliography

1. Literature

1) Dubbink HJ, Deans ZC, Tops BB, et al. Next generation diagnostic molecular pathology: critical appraisal of quality assurance in Europe. Mol Oncol. 2014; 8: 830-9.

2) Chang F, Li MM. Clinical application of amplicon-based next-generation sequencing in cancer. Cancer Genet. 2013; 206: 413-9.

3) Hagemann IS, Cottrell CE, Lockwood CM. Design of targeted, capture-based, next generation sequencing tests for precision cancer therapy. Cancer Genet. 2013; 206: 420-31.

4) Aftimos PG, Barthelemy P, Awada A. Molecular biology in medical oncology: diagnosis, prognosis, and precision medicine. Discov Med. 2014; 17: 81-91.

5) Collins FS, Varmus H. A new initiative on precision medicine. N Engl J Med. 2015; 372: 793-5.

6) Garraway LA, Verweij J, Ballman KV. Precision oncology: an overview. J Clin Oncol. 2013; 31: 1803-5.

7) Li T, Kung HJ, Mack PC, Gandara DR. Genotyping and genomic profiling of non-small-cell lung cancer: implications for current and future therapies. J Clin Oncol. 2013; 31: 1039-49.

8) Reddington K, Tuite N, Minogue E, et al. A current overview of commercially available nucleic acid diagnostics approaches to detect and identify human gastroenteritis pathogens. Biomolecul Detect Quantificat. 2014; 1: 3-7.

9) Organization for Economic Cooperation and Development. Guidelines for Quality Assurance in Molecular Genetic Testing. 2007< http://www.oecd.org/dataoecd/43/6/38839788.pdf>

10) Gargis AS, Kalman L, Berry MW et al. Assuring the quality of next-generation sequencing in clinical laboratory practice. Nat Biotechnol. 2012; 30: 1033-6.

11) Aziz N, Zhao Q, Bry L, et al. College of American Pathologists' Laboratory Standards for Next- Generation Sequencing Clinical Tests. Arch Pathol Lab Med. 2015; 139: 481-93.

12) Chiu RWK, Chan KCA, Gao Y, et al: Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. Proc Natl Acad Sci USA. 2008; 105: 20458-63.

13) Palomaki GE, Kloza EM, Lambert-Messerlian GM, et al. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. Genet Med. 2011; 13: 913–920.

14) Barrett AN, Zimmermann BG, Wang D, et al. Implementing prenatal diagnosis based on cell-free fetal DNA: Accurate identification of factors affecting fetal DNA yield. PLoS ONE. 2011; 6:e25202

15) Kosaka Nobuyoshi Editor, Circulating MicroRNAs: Methods and Protocols (2013)

16) Plebani M. The detection and prevention of errors in laboratory medicine. Ann Clin Biochem. 2010; 47: 101–110.
17) Hawkins R. Managing the pre- and post-analytical phases of the total testing process. Ann Lab Med. 2012; 32: 5–16.
18) International Organization for Standardization: ISO 15189 （3rd. Ed.）. Medical laboratories-- Requirements for quality and competence. English-Japanese version. Japan Standards Association. Tokyo. 2013, 1-50.
19) ISO/TS 20658 Medical laboratories examinations -- Requirements for collection, transport, receipt,and handling of samples.
<http://www.iso.org/iso/catalogue_detail.htm?csnumber=68763>
20) Standardisation and improvement of generic pre-analytical tools and procedures for *in-vitro* diagnostics. <http://www.spidia.eu/>

2. Guidelines etc.

The Member Lists of the Technical Committee on Standardization for Gene-based Testing and its Working Group (As of January, 2017)

The Technical Committee on Standardization for Gene-based Testing

Chairperson	Hayato Miyachi Professor of clinical laboratory studies based on basic medical examination, Tokai University School of Medicine
Vice-chairperson	Masayoshi Tsutsumi SRL Inc.
Member	Kiyoaki Watanabe Tokyo Clinical Diagnostic Medicine Center
Member	Kunihiro Ueda Kobe Tokiwa University President
Member	Tadashi Kawai International Clinical Pathology Center
Member	Kiyoshi Kitamura University of Tokyo Professor of International Research Center of Medical Education
Member	Shinji Kosugi Professor, Graduate School of Medicine, Kyoto University
Member	Fumio Takada Professor, Kitasato University Graduate School of Medical Sciences
Member	Kenjiro Kosaki Associate Professor, Division of Medical Genetics Department of Pediatrics, Keio University School of Medicine
Member	Toshihisa Ishikawa President, NGO Personalized Medicine & Healthcare
Member	Masato Maekawa Professor, Laboratory Medicine, Hamamatsu University School of Medicine
Member	Akira Haneda Professor, Department of Public Health, Chiba University Graduate School of Medicine
Member	Sakae Itoga Deputy Engineer, Chiba University Medical School Attached Hospital
Member	Yoshinao Oda Professor, Department of Anatomic Pathology, Graduate School of Medical Sciences Kyushu University
Member	Shinobu Masuda Professor, Division of Oncologic Pathology, Nihon University School of Medicine
Member	Tomohiro Nakayama Professor, Division of Laboratory Medicine, Nihon University School of Medicine
Member	Naohiro Noda National Institute of Advanced Industrial Science and Technology
Member	Hiroki Nakae NPO Japan bio Measurement & Analysis Consortium
Member	Koichi Yamagata Sysmex Corporation
Member	Yoshiaki Tazawa Roche Diagnostics Corporation

Observer Hiroshi Yoshikura Honorary staff of the former National Institute of Infectious Diseases

Observer Yumako Yoshida Regional Medical Care Planning Division, Health Policy Bureau, MHLW

Observer Moe Arita Bio-Industry Division, Commerce and Information Policy Bureau, METI

Observer Yuhei Fukuda Bio-Industry Division, Commerce and Information Policy Bureau, METI

The Working Group

Chairperson Hayato Miyachi* Professor of clinical laboratory studies based on basic medical examination, Tokai University School of Medicine

Vice-chairperson Masayoshi Tsutsumi* SRL Inc.

Member Mayu Takeda* Gifu University of Medical Science

Member Yutaka Hatanaka* Hokkaido University Hospital

Member Sakae Itoga Chiba University School of Medicine affiliated hospital

Member Hiroki Nakae* Biochip Consortium for Specified Nonprofit Organizations

Member Naohiro Noda National Institute of Advanced Industrial Science and Technology

Member Mitsuharu Hirai Arkray Co., Ltd.

Member Hideaki Tazoe Arkray Co., Ltd.

Member Yasuhiro Izumisawa* Abbott Japan Co., Ltd.

Member Yasutaka Yuki Eiken Chemical Co., Ltd.

Member Satoko Nakajo* SRL Inc.

Member Yoshiyasu Ogawa* LSI Mediation Co., Ltd.

Member Daisuke Koga Otsuka Pharmaceutical Co., Ltd.

Member Kumiko Nojima** Sakura Finetek Japan Co., Ltd.

Member Yoko Hano Sysmex Corporation

Member Masashi Hamamura* Sysmex Corporation

Member Kazuhiro Suzuki** Shimadzu Corporation

Member Rie Takeda Sekisui Medical Co., Ltd.

Member Tomoyoshi Shiroshita Sekisui Medical Co., Ltd.

Member Atsuo Mori** Nippon Gene Co., Ltd.

Member Makoto Nagano* BML Inc., Ltd.

Member Kazumi Kenmochi BML Inc., Ltd.

Member Tadachika Yamazaki BML Inc., Ltd.

Member Akio Matsushita Fuso Pharmaceutical Co., Ltd.

Member	Kazumi Sawakami Precision System Science Co., Ltd.
Member	Naoto Sawada* Ho Logic Japan KK
Member	Noriyuki Okita* Roche Diagnostics Corporation
Member	Toru Ogawa* Roche Diagnostics Corporation
Member	Kazuo Sakai** RIKEN Genesis Corporation
Observer	Yuji Sekiguchi National Institute of Advanced Industrial Science and Technology
Observer	Masaki Sato Eiken Chemical Co., Ltd.
Observer	Kazuhiro Okano* QIAGEN Corporation
Observer	Masayuki Ishikura** QIAGEN Corporation
Observer	Taro Ito** Sakura Finetech Japan Co., Ltd.
Observer	Katsura Uchida ** Sekisui Medical Co., Ltd.
Observer	Masamitsu Shikata Shimadzu Corporation

* Document authors ** Document review collaborators

Secretariat	Hideo Kato Director General, Japan Non-Profit Organization Japan Laboratory Standards Council

Acknowledgment

A part of this research was supported by AMED under Grant Number JP19kk0305005s0103.