

# **An Approved Guideline for the Quality Management of Specimens for Molecular Methods: The Procurement, Transport, and Preparation of Specimens**

## **Abstract**

This guideline has been drafted to address the growing number of molecular diagnostic methods used in routine medical laboratories worldwide. A variety of biological specimens with varying characteristics can be analyzed by several molecular diagnostic methods. Specimens of different origins require adequate pretreatment before nucleic acid extraction through one of several protocols, depending on the specimen's characteristics and eventual use. The guidelines describe the general principles and basic methods of collection, storage, transport, and preparation of specimens for the molecular analysis of specific sequences for pathogens, somatic cells, and germ line cells. Each section discusses the proper methods to assure appropriate specimen collection and processing, covers inappropriate specimen conditions, their possible causes, and concludes with the presentation of various techniques that help to either eliminate or avoid these errors.

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## **1. Introduction**

This guideline is drafted to address growing number of molecular diagnostic methods used in routine medical laboratories worldwide. Molecular diagnostic methods facilitate the analysis of specific nucleic acid sequences for pathogens, somatic cells, and germ line cells. Prior to analysis, there are many stages of specimen handling, including collection, storage, transport, and preparation. These preanalytic factors of molecular diagnostics greatly impact the quality of an assay and its subsequent results. A variety of biological specimens can be used for molecular diagnostics. They have varying physical, chemical, and biological features, which may determine the quality of the assay, and therefore greatly influence the yield and the quality of the extracted nucleic acids. Each specimen type requires the appropriate treatment prior to nucleic acid extraction, depending on the specimen's characteristics and eventual purpose of use. In order to assure the quality of assay results, it is extremely important to standardize the pre-analytic process, including the condition and treatment of specimens. However, it is difficult to optimize the process of collection, storage, transport and preparation of specimens. One reason for this is due to the number of professional and non-professionals, including patients, who are involved in the critical procurement process: collection, storage, and transport of specimens. Furthermore, the knowledge and skills involved in sample processing can be highly variable. A more critical explanation is that the methods involved in the collection, storage, transport, and preparation of patient samples are not universally common among different facilities and laboratories. Although several guidelines exist on this topic, they are not suitable for practical and standardized use. We therefore identified the need for such a guideline which is both standardized and effective. The descriptions provided herein deal with the current status of specimens that have been subjected to differing methods of molecular diagnosis in various types of medical laboratories. These discussions have varying levels of evidence. Many of the descriptions are derived from both the literature and manufacturer's instructions, and several are based on the experience of experts in this field.

This guideline is intended to use by all professionals who are involved in some aspect of the collection, storage, transport, and/or preparation of patient or tissue specimens. The appropriate handling of specimens ensures the high quality of such assays while also providing more reliable results, thus contributing to better patient diagnostics and an improved quality of healthcare.

## **2. Scope**

This guideline describes the principles and basic methods of sample procurement: namely, the collection, storage, transport, and preparation of specimens for methods of molecular diagnosis to measure specific sequences for pathogens, somatic cells, and germ line cells. Each section discusses the proper methods to assure appropriate specimen conditions, and also discusses the inappropriate conditions of specimens, possible causes of inaccurate results, and also suggests how to avoid these problems.

## **3. Storage and Transport of Specimens for Molecular Methods**

It is desirable that specimens for molecular detection and measurement methods be used immediately following collection, as in other testing protocols. In the healthcare setting,

however, the specimens must sometimes be either refrigerated or frozen for later delivery to the laboratories or facilities where the molecular analyses will be conducted. In addition, tested specimens are stored for a certain time in case there is a need for retesting. During this time, there is a possibility that the target microbes or cell condition may be altered, affecting the analyses and thus the results of gene-based testing. Therefore, it is required that specimens be appropriately stored until tests are completed, and that extracted nucleic acids be stored together with their corresponding specimens.

In many cases, gene-based testing is used in research laboratories and involves the extraction of high-purity nucleic acids (DNA/RNA). In addition, high-purity DNA can be stored at relatively stable conditions, even at an ambient temperature. However, in the field of clinical testing, in which promptness is critical, nucleic acids are not always purified to the highest degree, and are used as measurement tools with relatively low-purity yields. Conversely, RNA stability is highly dependent on the extraction conditions.

Noncompliance with storage conditions, which are specifically set for individual extraction conditions, can greatly affect the test results (values). It is also known that extracted RNA has a very poor stability over long-term storage.

When *in vitro* diagnostic reagents are used as extraction reagents, it is important to extract and store the specimens properly in accordance with manufactures' instructions. When research reagents are used, it is important to follow the optimal extraction and storage conditions specified in the manufacturers' instructions, but the independent evaluation of the optimal storage conditions for nucleic acids can improve RNA yield and purity.

#### **a. Storage temperature for nucleic acids**

For the storage of extracted high-purity nucleic acids, the appropriate storage methods must be chosen depending on the type of nucleic acids and their eventual use. RNA in particular is easily degraded and unstable and must be stored at ultra-low temperatures (-70 °C or below) for both short- and long-term storage.

For high molecular weight genomic DNA, including human genomic DNA, it is recommended that the DNA be dissolved in Tris-EDTA buffer and stored in a refrigerator at 2-8 °C because repeated freezing and thawing can induce DNA strand nicks or breaks. Extracted DNA stored in a refrigerator at 2 - 8 °C is stable for at least a year. When high molecular weight genomic DNA is stored at ultra-low temperatures (-70 °C or below) for a long time, it is recommended that the DNA be divided into sealed aliquots to avoid freeze dehydration and repeated freeze and thaw cycles.

#### **b. Storage containers**

Small polypropylene tubes are recommended as storage containers, because absorbed amounts of nucleic acids by its surface is only minimal. Even so, it is required that the inside of the tubes be coated with silicon or some other protective materials when extremely small amounts of nucleic acids are stored. Ordinary disposable tubes are not considered to be contaminated with DNase (a DNA degrading enzyme) or RNase (an RNA degrading enzyme) since they are thermoformed at high temperature, however, it is nevertheless recommended that tubes be used which are clearly identified to be DNase and RNase free.

This section mainly describes the stability of specimens in storage prior to extraction, and the stability of nucleic acids in storage is addressed only for special cases in reference to the above matters. When viruses, bacteria, and other species are measured, the target gene stability in each specimen differs according to the individual microorganisms. In such cases, handling depends on the properties of the microorganisms that are present.

### **c. Transport of specimens**

When specimens for gene-based testing are sent to other laboratories, due care must be taken to prevent any specimen delay by informing the personnel at destination facility in advance to expect the arrival of these specimens. In addition, in order to prevent misidentification, specimens must be clearly labeled with their own identification information. The clearly identified specimens and the testing order forms should be shipped together. The patient information associated with the specimens should be anonymized or redacted as needed.

It is desirable that specimen transport be completed in as short a time as possible using the appropriate storage conditions for each specimen, as is the case with other testing. However, transport between facilities frequently occurs only over a long period of time. During the period of specimen transport, controlled temperature and storage conditions are essential. Containers made of polystyrene foam or other appropriate materials that provide insulation and physical protection for the specimens are ideal for shipment.

The typical storage temperature conditions used for the specimen transport are frozen (-20 °C or below), refrigerated (4-10 °C), and ambient (17-28 °C) temperatures. For frozen and refrigerated conditions, dry ice and cooling gel, respectively, should be used as the cooling media. For ambient condition shipment, heat storage materials may be used when required. The amount of temperature-controlled packing material should be adjusted by estimating the temperature inside the containers when the specimens are packed and when they are unpacked. When transport takes a long time, as is the case when specimens are shipped abroad, the sender must anticipate this factor, and should consider consigning the transport to carriers that can easily manage the specimen temperature by, for example, replenishing dry ice in the course of transportation.

The recipients of the specimens must store the specimens at the appropriate storage temperatures immediately after confirming the specimen conditions: namely, the storage conditions of the specimens during the transport, including the optimal longer-term storage temperatures and any damage to the specimens, as well as specimen information, including testing order forms.

## **3.1 Storage and Transport of Specimens in Molecular Methods for Pathogens**

### **3.1.1 Serum and Plasma**

To detect viruses, various molecular methods have been widely implemented because it is difficult to culture targeted viruses, and, even when possible, is very lengthy and time-consuming. For the hepatitis virus C (HCV) and the human immunodeficiency virus (HIV), it is recommended that the serum or plasma be separated within 6 hours after blood

collection, and, for hepatitis virus B (HBV), it is recommended that this be performed within 24 hours. It is well-known that virus measurement results are not significantly affected within these time frames. After separation, serum and plasma specimens can be stored for approximately 5 days under refrigeration (2-8 °C), but freeze preservation is recommended for long-term storage. It is desirable to store specimens for DNA measurement at -20 °C or below for HBV and at -70 °C or below for hepatitis virus A (HAV), HCV, and hepatitis virus E (HEV). Other viruses, including the cytomegalovirus (CMV) and the herpes simplex virus (HSV), are reported to be relatively stable in the blood. Based on the above reports, it is considered that virus specimens are relatively stable in serum and plasma.

(1) Inappropriate and unsuitable conditions for testing

Formation of sediments, discoloration, and dehydration due to freezing

(2) Causes

Possible causes include inappropriate storage conditions, such as long-term exposure to high temperature and repeated cycles of freeze and thaw.

(3) Troubleshooting

When specimens were stored under inappropriate conditions, including high temperatures, new blood specimens are required for subsequent tests. If the extracted nucleic acids have been appropriately stored, they can be used for re-testing. The number of freeze and thaw cycles should be recorded and minimized.

(4) Measures to avoid inappropriate situations

It is important for laboratories to specify the storage temperature, the number of days that the specimens can be stored, to label the storage date on specimens, and to store them under appropriate temperature conditions.

### 3.1.2 Urine

Urine specimens (male) are used for nucleic acid testing for *Chlamydia trachomatis* and *Neisseria gonococcus*. It is desirable that urine specimens be stored with refrigeration (2-8 °C) and undergone the process of nucleic acid extraction within four days. The stability of the extracted nucleic acid depends on the extraction conditions. When extracted properly with the standard methods, the DNA remains stable for one week or longer under refrigerated conditions (2-8 °C). For long-term storage, freezing of the specimens is desirable.

(1) Inappropriate conditions not suitable for testing

Urine specimens may have a cloudy appearance due to bacterial growth.

(2) Causes

Possible causes include failure to store the specimens under appropriate conditions, such as leaving specimens for a long time in collection containers under inappropriate storage temperatures.

(3) Troubleshooting

For initial testing, it is required that new blood specimens be acquired. For re-testing,



extracted nucleic acids can be used if they have been stored appropriately. The number of freeze and thaw cycles should be carefully recorded.

(4) Measures to avoid the inappropriate situations

It is important for laboratories to specify the storage temperature and the number of days that specimens can be stored, to label the storage dates on specimens, and to store them under the appropriate temperature conditions. Specimens that were stored under conditions other than indicated should not be used for measurements.

### 3.1.3 Sputum

Sputum specimens have commonly been employed in nucleic acid testing for *Mycobacterium tuberculosis* and strains of *atypical Mycobacterium*. In the detection of *M. tuberculosis*, it is critical to assure the quality of sputum specimens. (Refer to the Guideline for Mycobacterial Examination by Ministry of Health, Labour, Welfare of Japan, 2007.) The sputum quality is assessed by a macroscopic observation according to the classification of Miller & Jones. For nucleic acid testing, sputum specimens are decontaminated with NALC-NaOH and suspended in phosphate buffer. No detailed data have yet been reported on the storage stability of the suspended specimens. It is empirically known that suspensions can be stored for a week under refrigeration (2-8 °C) and for longer periods when frozen. It is recommended that the suspensions be dispensed into aliquots before cryopreservation in order to avoid repeated freeze and thaw cycles. The stability of the extracted nucleic acids is highly dependent on the extraction conditions. When properly extracted with standard methods, DNA is stable for one week or longer under refrigeration (2-8 °C). For long-term storage, freezing of the specimens is optimal.

(1) Inappropriate conditions not suitable for testing

Sputum specimens with poor viscosity may have been stored in inappropriate conditions.

(2) Causes

Possible causes include inappropriate storage conditions, such as long-term exposure to high temperature and repeated cycles of freeze and thaw.

(3) Troubleshooting

For initial testing, the collection of new sputum specimens is required. For re-testing, extracted nucleic acids can be used if they have been stored properly. The number of freeze and thaw cycles should be recorded and minimized.

(4) Measures to avoid inappropriate sample handling

It is important for laboratories to specify the storage temperature and the number of days that the specimens can be stored, to label the storage date on specimens, and to store specimens using the appropriate temperature conditions. Specimens that were stored using conditions other than those indicated should not be used for subsequent measurements.

## 3.2 Storage and Transport of Specimens in Molecular Methods for Somatic Cells

As the human genome analysis progresses, accumulating information is available for clinical applications. With the development of DNA microarray techniques, more comprehensive types of gene analyses (gene expression and genotypic) may be performed. As a result, genetic testing methods have been applied to the field of clinical research. RNA, the target of gene expression analyses in these tests, is unstable and can be degraded by RNase (RNA degrading enzyme) contamination, which is usually derived from specimens and/or the operators (examiners and researchers). Therefore, when handling RNA, careful attention is critical.

RNA is purified to a relatively high-purity level by silica membrane adsorption in most cases, including clinical testing. To assess the purity of purified RNA, conventional methods include the A260/A280 spectrophotometric absorbance ratio and (denaturing) agarose gel electrophoresis. In addition, recent simple methods have been developed and are widely used. Examples include confirming the 28S and 18S size distribution of ribosomal RNA (rRNA), as well as calculating the 28S-to-18S rRNA ratio using microchip gel electrophoresis.

It is generally desirable to store the purified RNA specimens at -70 °C or below, but some facilities store these samples at -20 °C or below. Therefore, personnel should be aware of the performance of the freezer in which specimens are stored and sufficiently consider storage conditions to avoid freeze and thaw cycles and freezing dehydration. It is generally recommended to dispense RNA specimens into aliquots before storage to avoid freeze and thaw cycles. In practice, the specimen yield is extremely low, and such specimens are cryopreserved without dispensing. In such cases, it is also recommended to reverse transcribe a portion of the RNA sample to obtain the more stable cDNA prior to storage. If the purpose of the testing is accurate quantification of gene expression levels, RNA must be promptly extracted from the specimen under conditions at which minimum autolysis of organs or cells occurs. The same is true for cases in which a high detection sensitivity is required, due to an extremely low expression level.

When specimens are to be used only after some time in storage, they should be stored for maximum stabilization of the nucleic acids, depending on purpose of the testing, specimen types, storage methods, the storage period, and the types of nucleic acid.

### 3.2.1 Tissues and Tissue Slice Fragments

Recently, a comparative analysis of gene expression profiles based on the DNA microarray technique has been applied to clinical diagnoses, and some published reports have already described the conditions of fresh tissue specimens used for these examinations. The mRNA expression level for transcription factors and cytokines in bone marrow tissue are significantly altered over time following the specimen collection, therefore requiring special attention for the handling of bone marrow tissue. Recently, commercially available RNA stabilizers which retain specimen RNA have come into use. It has been reported that, although the total amount of recovered RNA and its molecular weight distribution pattern remained unchanged with the addition of the stabilizer, some variations have been found at

the individual gene level. In these tests, it is important to establish certain fixed conditions that are applied from the time of specimen collection to nucleic acid extraction, and to confirm in advance the changes that may be brought about in the target genes under these conditions.

The handling of tissues obtained by biopsy or surgical removal is more complicated. A small mass of tissue or the targeted parts of the tissue are cut into sections and are either processed immediately for the extraction of nucleic acids or fresh-frozen in liquid nitrogen for longer-term storage. In studies involving tissues, special attention should be paid to the specimen storage conditions to avoid RNA degradation.

Cancer tissue sections are also used for molecular techniques. In the examination of cancer cell-specific gene expression, tissue specimens are embedded in a matrix for the preparation of frozen tissue sections, which are used for microdissection (a method to obtain cancer cells alone using microscopy). In implementing microdissection, it is important to properly process tissue specimens in accordance with the treatment methods specific for each instrument.

(1) Inappropriate conditions not suitable for testing

Specimens containing cells that are destroyed due to repeated cycles of freeze and thaw are inappropriate for analyses. When a fixative is used for tissues in pathological examination, nucleic acids may not be sufficiently recovered to a sufficient degree of quantity and quality. When isolated RNA is used as a specimen for polymerase chain reaction (PCR), careful attention is required to avoid cross-contamination from the process of tissue section preparation.

(2) Causes

Storage under inappropriate conditions, including failure to use appropriate procedures, such as freezing immediately, can contribute to the degradation of tissue samples. Avoiding repeated cycles of freeze and thaw can minimize this damage.

(3) Troubleshooting

For initial testing, re-extraction from another aliquot of cryopreserved specimen or re-collection of a tissue specimen is required. For re-testing, extracted nucleic acids can be used if they have been stored appropriately.

(4) Measures to avoid inappropriate situations

It is important for laboratories to specify the storage temperature and the number of days that specimens can be stored, to label the storage date on specimens, and to store these under the appropriate temperature conditions. Specimens that were stored under conditions other than those indicated should not be used for measurements.

### 3.2.2 Whole Blood (WBC)

The nucleic acids (DNA/RNA) extracted from blood cells are used for the molecular analysis of leukemic diseases. Whole blood, or the white cell fraction of the blood isolated by density gradient centrifugation using Ficoll or by cell sorters, is primarily used. It has been reported that peripheral blood contains circulating free-DNA and free-RNA, which

appear to originate from degraded blood cells or tissue-derived cells. Recently, the application of these specimens to cancer diagnosis has been investigated. It has been reported that these circulating nucleic acids are stable *in vivo*. Presumably, there are some protective mechanisms for such nucleic acids released into the circulating blood. In contrast, it is known that the nucleic acids are rapidly degraded when they are added to either the serum or plasma *ex vivo*.

(1) Inappropriate conditions not suitable for testing

Specimens in which cells have been destroyed are inappropriate for testing because of their possible influence on nucleic acid extraction.

White blood cells can be destroyed by strong negative pressure in the syringe at the time of collection, and they are particularly fragile when blood is removed from patients undergoing treatment with anti-cancer chemotherapeutic agents.

(2) Causes

Possible causes include storage under inappropriate conditions, such as long-term exposure to high temperatures and repeated cycles of freezing and thawing.

(3) Troubleshooting

Re-extraction from another aliquot of cryopreserved specimen or recollection of a blood specimen is required. For retesting, extracted nucleic acids can be used if they have been stored appropriately.

(4) Measures to avoid inappropriate situations

- 1) It is important for laboratories to specify the storage temperature and the number of days that specimens can be stored, to label the storage date on specimens, and to store them under appropriate temperature conditions. Specimens that were stored under conditions other than those indicated should not be used for the measurements.
- 2) Specimens should be transported in containers that will prevent damage of cells.

### 3.2.3 Urine and Stool

Urine contains DNA that can be used for cancer screening. Colorectal cancer screening using DNA and RNA excreted into the stool has also been explored, but the stool contains an abundance of food-derived components and various bacteria, which are reported to inhibit gene-amplification reactions. Therefore, when stool specimens are used for gene-based testing, sufficient attention is required for the pretreatment of the specimens.

(1) Inappropriate conditions not suitable for testing

Urine may appear cloudy due to bacterial growth.

(2) Causes

Possible causes include storage under inappropriate conditions, such as long-term exposure to high temperatures or repeated freezing and thawing cycles.

(3) Troubleshooting

For initial testing, it is required that new specimens be acquired. For retesting, extracted nucleic acids can be used if they have been stored appropriately. The number of freeze

and thaw cycles should be recorded and minimized.

(4) Measures to avoid inappropriate situations

It is important for laboratories to specify the storage temperature and the number of days that specimens can be stored, to label the storage date on specimens, and to store them under appropriate temperature conditions. Specimens that are stored under conditions other than those indicated should not be used for the subsequent analyses.

### **3.3 Storage and Transport of Specimens in Molecular Methods for Germ Line Cells**

Recently, genes responsible for the so-called single gene disorders have been identified, and, to date, gene-based testing methods using a wide variety of techniques have been used. In addition, genetic testing for single-gene diseases is being performed at various facilities, and, in particular, testing for rare single-gene diseases is implemented primarily at research facilities. This therefore creates the necessity for transporting specimens from medical facilities (consigners) to the research facilities (consignees).

White blood cells are the major specimen used for genetic testing. Oral mucosa is also used, depending on the purpose of the genetic testing. Oral mucosa is collected using special cotton swabs, but the amount of recovery of DNA differs among individuals and depends on the sampling conditions. Therefore, proper specimen collection and transport under appropriate storage conditions is critical. For the transport of these biological specimens, due consideration is required to prevent sample contamination or damage.

#### **3.3.1 Outsourcing of Genetic Testing**

Because genetic testing may reveal important information about the subjects (patients) as well as their family members and relatives, obtaining informed consent is absolutely essential, and genetic counseling must be provided as required. When genetic testing is consigned to other facilities, the appropriate security measures (organizational, human, physical, and technical) must be taken to protect the individual's genetic information. In other words, patients' names should be anonymized, and due attention must be paid to the handling of specimens and the management of test information throughout all stages of the process, from specimen collection to the reporting of test results. In addition, an agreement should be enacted between the concerned participants and subjects prior to testing concerning the post-analytical handling of specimens and test results. The most common specimen used for genetic testing is white blood cells from peripheral blood samples collected from the subjects. After the identity of the subject has been confirmed, qualified medical personnel collect the blood specimens.

Depending on the purpose of the testing, specimens that are collectable by the subjects themselves, such as oral mucosa specimens, may also be tested. Even in such cases, due consideration is required, i.e., a responsible or accredited third person must attend during the specimen collection to identify the subject and his/her specimen. Collected specimens are anonymized and patient information redacted in a traceable fashion, and are stored

under the most appropriate conditions. In order to prevent the loss of specimens or their accompanying information during transport, the specimens and testing order forms should be sent together and strictly managed.

Responsibility for the transportation of the specimens between facilities is assumed directly by the personnel concerned, or it may be consigned to common delivery systems including door-to-door parcel delivery services, unless a special specimen transport system has been established between the facilities. The means of specimen transport have to be selected carefully and must comprehensively consider the safety, stability, reliability, required time, and other factors related to the transport system.

Specimens should be transported with the appropriate storage temperature suitable for the type of specimen and be contained in polystyrene foam shipment boxes, which provide excellent insulation and are resistant to crushing.

The facilities that perform the molecular diagnostics should confirm the receipt of the specimen, acknowledge the content of the testing order form, and perform the analyses based on the information in the order form. The examination processes and the subsequent results should be accurately recorded, stored, and managed so as to be able to respond to inquiries during testing and after the test results are reported. In addition, the information on analytical validity, clinical validity, and clinical utility of the implemented tests must be provided to the consigner of the test, as required.

The results of the tests should be reported only to the consigner. To prevent leakage of the test results to third parties, the report should be placed in a thick envelope so that the results cannot be read from the outside, the envelope should be marked "CONFIDENTIAL," and the envelope should be sent to the facility that requested the test (consigner). When common delivery systems, such as the postal service or door-to-door parcel delivery services, are used, sender should request that tracking information and a delivery record be available upon request.

After the completion of testing and reporting the result, the specimens and the extracted nucleic acids (DNA/RNA) should be properly discarded, retained or sent back to the facility that requested the testing. In the interim, proper storage conditions should be maintained, at least until the next proficiency testing to allow for identification of problems and corrective re-testing, if necessary, in accordance with the agreement with the facility. Upon receipt of the test results, the consigner of the test identifies the subject of the test and uses the results to diagnose that specific subject's condition.

This section summarizes methods to prepare specimens used for molecular methods.

## **4.Preparation of Specimens for Molecular Methods**

### **4.1 Preparation of Specimens in Molecular Methods for Pathogens**

The purpose of molecular methods for pathogens is to identify pathogens responsible for diseases by specifically detecting the genomic DNA or RNA of foreign pathogens.

Therefore, it is important to select the most suitable testing material that is most likely to harbor targeted viruses or bacteria, based on the pathology, disease stage, or manner of

infection. Due to widespread use of *in vitro* diagnostic reagent kits, specimens of the affected lesions, including serum, plasma, sputum, urine, and a swab are the norm for the molecular diagnostics involved in the examination of pathogens.

During the last few years, an increasing number of molecular methods for pathogens using stool specimens have been implemented, because the symptoms of vomiting and diarrhea due to norovirus often occur and spread during the winter season. In addition, specimens are collected from many different sources, depending on the nature of the pathogen and the purpose of the testing. These specimens include peripheral blood, cerebrospinal fluid, swabbing of affected lesions, broncho-alveolar lavage fluid (BALF), and biopsies of affected tissues.

When these specimens are collected, the quality of the specimen may be compromised depending on the collection methods or conditions, and the establishment of standardized preparation methods is ideal. In this section, the following are listed for each of the typical testing materials: 1) findings of inappropriate conditions of specimens that are often seen in our daily testing procedures, 2) the possible causes of these conditions, 3) methods for rescuing specimens under poor conditions, and 4) how to avoid obtaining specimens in poor conditions. In cases new specimens cannot be obtained other than inappropriate conditions of those, a try for use of buffers or enzymes that may reduce inhibitory effects is to be considered.

#### 4.1.1 Serum

The specified amount of peripheral blood is drawn using a vacuum blood collection tube that contains a serum separating gel. The tube is left at an ambient temperature for an hour until coagulation is completed. The tube is immediately centrifuged (at 1,190 x g (gravity acceleration) [2,8 00 rpm for the rotor with a radius of 170 mm], at 4 °C for 10 minutes). If a coagulation accelerator is added to the collection tube, the time required for coagulation is shortened. The collection tube that contains a reagent for neutralizing heparin should not be used, because of its inhibitory effects on nucleic acid amplification.

Only the amount of specimen required for molecular diagnostics for pathogens should be taken from the blood collection tube. Transferring the specimen into another container should be avoided. When specimens are not going to be tested immediately after centrifugation, the separated serum in the original blood collection tube should be directly frozen (-20 °C or below) and stored until use.

##### (1) Inappropriate conditions not suitable for testing

Transparent rust-colored, red-colored, or yellow-green colored serum should not be used for the molecular analyses.

##### (2) Causes

The rust or red color is due to hemoglobin contamination and hemolysis. The yellow-green or green tea color is bilirubin contamination due to jaundice. If vacuum blood collection tubes are used for blood specimen collection, hemolysis-induced hemoglobin contamination is less susceptible to damage from physical forces, including

intensive negative pressure at the time of blood collection. However, this contamination may occur if the specimens were erroneously frozen before centrifugation and then thawed prior to the analyses. It may also occur if the specimens were frozen before allowing the red blood cells to settle to the lower layer of the separating gel, usually due to insufficient centrifugation and low centrifugal load (*g*) or too short a spin time.

(3) Troubleshooting

Bilirubin contamination is attributable to the patient disease status at the time of blood collection, and even re-collection of a blood specimen will not improve this factor. Therefore, efficient nucleic acid extraction methods that can remove impurities should be used. The inhibitory effect of nucleic acid amplification reaction can be overcome by adsorbing the nucleic acids onto filters, membranes, or magnetic particles and washing out impurities, rather than extracting it with phenol/chloroform and precipitating with ethanol or isopropanol. If an internal standard is included with the detection reagents, the inhibitory effect against the amplification reaction can be assessed and can reduce the frequency of false-negatives. When no internal standard is included, the effect of inhibitory substances can be confirmed by evaluating the amplification efficacy after adding a known amount of plasmid DNA to the DNA solution extracted from the specimen or the cDNA.

(4) Measures to avoid inappropriate situations

Serum specimens allow stable nucleic acid amplification using basic operation procedures. Specifically, the collection of a specified blood volume without creating negative pressure in the blood collection tubes, waiting until the coagulation reaction is completed, and centrifuging the tube are the steps required for this process. When freezing blood collection tubes commonly used in nucleic acid testing following serum separation, the color and turbidity of the serum layer immediately after the centrifugation must be noted, and must also be checked for the presence of remaining clots that have not completely sunk into the lower layer of the separating gel before storing these samples in the freezer. If the color of the serum is off the empirically-determined standard range, collection of a new blood specimen should be considered.

#### 4.1.2 Plasma

EDTA should be used for plasma anti-coagulation. Separated plasma, for which ACD (acid citrate dextrose) or CPD (citrate phosphate dextrose) are used, can also be used for nucleic acid amplification tests similarly to those treated with EDTA.

(1) Inappropriate conditions unsuitable for testing

- 1) Fibrin precipitation is suspected as observed by either the presence of a clot or if the specimen has a high viscosity.
- 2) Massive leukocyte contamination may be present according to the turbidity and deposit status.
- 3) Heparin has contaminated the specimen.

(2) Causes



- 1) If EDTA tubes are not immediately and repeatedly inverted and mixed following vacuum blood collection, blood coagulation gradually progresses and fibrin precipitation occurs some time after the plasma separates. In mild cases, the plasma may be slightly viscous. Similar conditions are observed when blood is drawn without the use of anti-coagulation agents and are immediately transferred into EDTA tubes, because the coagulation system has already been activated by this time.
  - 2) Due to insufficient centrifugal conditions in terms of  $g$  (gravitational acceleration) or the time of centrifugation, lymphocytes are present at a low density and platelets are distributed within the lower layer of the plasma. When the plasma fraction is pipetted, some of these leukocytes may be aspirated together with the plasma. This is especially problematic when plasma levels of RNA from viruses such as HIV, which propagate through the infection of T lymphocytes, are monitored over time, since the incorporation of lymphocytes accurately quantifies RNA levels.
  - 3) In essence, EDTA, ACD, and CPD are the anti-coagulants that can be used for the preparation of plasma specimens for molecular diagnostics. However, nucleic acid amplification tests, including PCR, are frequently inhibited when blood is drawn using heparinized vacuum tubes or heparinized syringes. In addition, patients with IVH (intravenous hyperalimentation), retained catheters, or who on artificial hemodialysis are often heparinized for the prevention of blood clot formation. In such cases, depending on the concentration, heparin in the blood may directly affect nucleic acid amplification reactions. Even a trace amount of heparin contamination (0.05 units per reaction) inhibits DNA amplification in PCR.
- (3) Troubleshooting
- 1) In a plasma specimen, virus particles may unevenly distribute and become entrapped among fibrin fibers, and as a result accurate quantification may be compromised. Recentrifugation of the plasma after plasma homogenization (making the viral particles distribute as evenly as possible by stirring the plasma with disposable pipette tip or homogenization) is followed by carefully pipetting the surface layer to obtain the specimens for nucleic acid extraction.
  - 2) Centrifuge the plasma again under appropriate conditions (at  $1,710 \times g$  (gravity acceleration) [3,000 rpm for the appropriate rotor, with a radius of 170 mm], at  $4^\circ\text{C}$  for 10 minutes or longer), and pipette the plasma from the surface layer carefully so as not to disturb the pellet with jarring or shock. Because the major part of the related analysis using plasma specimens lies in the amplification of nucleic acids, this strength of centrifugation is sufficient to precipitate blood cells without affecting the results of the test.
  - 3) Even if some inhibitory effects are detected in the nucleic acid amplification reaction, it is difficult to attribute these effects to heparinization unless we observe the blood collected in heparinized vacuum tubes or with other possible situations. However, if an internal standard is included in the reaction mixture, it may actually

demonstrate the inhibitory effect against the amplification reaction. When no internal standard is included, the effect of inhibitory substances can be confirmed by evaluating the amplification efficiency, after adding a known amount of plasmid DNA to the reaction mixture together with DNA extracted from the specimen or transcribed cDNA.

When heparinized plasma is used as the specimen, viral nucleic acids should be purified to eliminate the effect of heparin as much as possible using nucleic acid extraction reagents based on filters, membrane, or magnetic particle methods, since heparin, unlike blood cells, cannot be removed by repeated washes and centrifugations. In addition, treating extracted nucleic acids with heparinase is sometimes effective in removing heparin.

The simplest and easiest action to be taken is to confirm resolution of the inhibitory effect by diluting the nucleic acid specimens, although it should be noted that there is a risk that targeted nucleic acid levels are also diluted to a level below the detection limit.

(4) Measures to avoid inappropriate conditions

- 1) EDTA vacuum tubes should be used for blood collection; the tubes should be gently and repeatedly inverted 10 or more times to prevent blood coagulation.
- 2) A complete buffy coat layer is formed by centrifugation (at  $1,710 \times g$  (gravity acceleration) [3,000 rpm for a rotor with a radius of 170 mm], at 4 °C for 10 minutes or longer) with a low-speed, cooled centrifuge. The blood collection tubes should be removed from the centrifuge gently to avoid vibration or jarring, and the minimum required volume of the specimen from the middle layer of the plasma fraction should be removed by pipetting, avoiding contamination of blood cells from the surface layer.
- 3) EDTA-containing vacuum tubes should be used at all times. Avoid sharing blood specimens with those for cellular immunology or chromosomal analyses, for which blood specimens are collected in heparinized tubes. When the subject is heparinized in a clinical setting, a new blood specimen should be collected after confirming that the effect of the heparinization has been eliminated. When blood is drawn through an IVH catheter, the first several few milliliters (2-3 times the catheter volume) should be discarded due to the potential effect of heparin, and blood drawn after that should be used as the specimen.

#### 4.1.3 Sputum

Sputum specimens are collected either through expectorating from the bronchus at deep coughing by the patients themselves or through aspirating with a suction catheter. Sputum specimens are mainly used for the diagnosis of infectious diseases caused by tubercle bacilli, atypical mycobacteria, and others.

(1) Inappropriate conditions unsuitable for testing

- 1) Specimens comprised most partly of saliva, as judged by macroscopic observation of non-viscous (S(M), according to Miller and Jones' classification) sputa.

- 2) Specimens that have a large amount of blood with the sputa.
- (2) Causes
    - 1) Possible causes include insufficient expectoration by the patient or limited sputum secretion.
    - 2) Possible causes include hemorrhages from affected respiratory tracts, such as the bronchus.
  - (3) Troubleshooting
    - 1) When too little sputum is contained in the specimen, targeted pathogens, including acid-fast bacilli and non-tuberculous mycobacteria, may not be detected and may give false-negative test results. Therefore, the collection of alternative specimens, including pooled sputum, gastric juice, broncho-alveolar lavage fluid (BALF), and bronchial scratching, should be considered.
    - 2) To eliminate red blood cells and hemoglobin, the specimen should be left for approximately 15 minutes after the addition of 5-10 times its volume of sterile, distilled water to induce hemolysis and centrifuged thereafter to obtain the precipitate, which is used for nucleic acid extraction. However, it should be noted that normal sputum is highly viscous and does not precipitate in this state, even after centrifugation (at 1,190 x g (gravity acceleration) [2,500 rpm for the rotor with a radius of 170 mm], at 4 °C for 10 minutes) in many cases. After centrifugation, as much supernatant as possible should be gently removed by aspiration. This supernatant should be placed in another container rather than being discarded. Dissolve the remaining fraction of sputum, which contains cellular components and bacteria surrounded by mucus, in NALC (N-acetyl-L-cysteine) or semi-alkaline protease (SAP) (trade names: "Sputazyme," "Presolve," and others) to eliminate viscosity. Then, the sputum must be washed with physiological saline or PBS, centrifuged (at 1,190 x g (gravity acceleration) [2,500 rpm for the rotor with a radius of 170 mm], at 4 °C for 10 minutes), and used for nucleic acid extraction. If specimen pre-treatment procedures are indicated in a package insert for the reagents, follow these instructions. If the color of precipitate after washing and centrifugation is nearly white, it is possible that inhibition of the reaction by hemoglobin will not occur in the following procedures.
  - (4) Measures to avoid inappropriate situations

When a large amount of blood is mixed in the sputum, hemoglobin contamination of the centrifugal precipitate of sputum is unavoidable, and the subsequent inhibition of the nucleic acid amplification reaction in the PCR and TMA analyses is a serious consideration. In such cases, it is desirable to collect additional sputum specimens, but it is always necessary to take measures suitable to the subject's disease state, especially those patients with respiratory diseases. This is particularly applicable to cases in which only saliva was collected. If subjects have difficulty in expectorating sputum by themselves, sputum collection by aspiration with suction catheters or utilization of gastric juice as an alternative specimen should be considered.

#### 4.1.4 Stool

Recently stools have been increasingly used as the specimen for molecular methods for detection of pathogens, primarily for diagnosing viral diarrhea. Because specimens are collected at the peak of the diarrheal symptoms, most of these are watery in nature. To evaluate the patient as an infection source to prevent secondary infections, the testing is repeated several times following the first diagnosis until patient recovery to confirm whether a virus is present. Stool specimens are also used for identification of enterohemorrhagic *Escherichia coli* or the detection of verotoxin genes, although these examinations are less frequent.

##### (1) Inappropriate conditions unsuitable for testing

The stool specimen may have been deteriorated in the quality if it is not frozen (at -20 °C or below) within one day of sample collection. However, it is difficult to technically detect deterioration of specimen quality prior to analysis. There is always a possibility that the detection sensitivity for the targeted pathogen of the nucleic amplification acid testing has been reduced, because the deterioration of quality cannot be easily recognized before testing.

##### (2) Causes

Leaving specimens in ambient temperature for a long time leads to the expansion of a variety of non-targeted bacteria, lowering the detection sensitivity for the targeted pathogen and increasing the frequency of non-specific amplification events, eventually resulting in false-negative results. It is also possible that viral nucleic acids are degraded by proteases and nucleases produced by bacteria, thereby leading to false-negative test results.

##### (3) Troubleshooting

If the bacteria and viruses in the stool cannot be stabilized by rapid cryopreservation within a few hours of defecation, it is practically impossible to regain the original state of protein and nucleic acid quality. In this circumstance, it is impossible to estimate the extent of quality deterioration.

##### (4) Measures to avoid inappropriate situations

It is essential to properly explain the requirements for nucleic acid testing to subjects, and that the subjects understand that stool specimens must be stored at refrigerated temperatures for as short a time as possible, must be refrigerated as soon as possible, and must be frozen for longer-term storage. In particular, if a stool specimen is collected outside the medical facilities, the time of collection, the storage period, and the temperature condition during the storage should be recorded.

#### 4.1.5 Urine

Most molecular methods for pathogens in urine specimens diagnose sexually transmitted diseases (STD), such as infections by gonococci, chlamydia, and other pathogens. Typically, the initial morning voiding urine is used as a specimen. The initial voiding urine at birth is

often used for the diagnosis of cytomegalovirus infection in the fetus or neonate.

(1) Inappropriate conditions not suitable for testing

Urine specimens suspected of hematuria or bilirubinuria and those with large amounts of precipitates should not be tested.

(2) Causes

Hematuria is attributed to the inflammation of urethra or bladder, which are affected by many pathogens. Precipitates are also formed when the specimens are refrigerated.

(3) Troubleshooting

To eliminate contamination with blood cell components prior to extracting and purifying nucleic acids, the use of low-speed cooled centrifugation (at 430 x g (gravity acceleration) [1,500 rpm for a rotor with a radius of 170 mm], at ambient temperature for 5 minutes) is effective. However, cryopreserved urine specimens are occasionally contaminated with hemoglobin, which is unavoidable because hemolysis occurs during the thawing process. When the detection target is a virus, then centrifuged supernatant can be used for analysis, but when the target is bacteria, then careful attention should be paid to the supernatant, because improper centrifugation may precipitate bacterial cells. Rather, it is more important to make the specimen homogeneous by repeatedly inverting the specimen collection tubes. Because deposits are frequently formed due to specimen refrigeration, the specimen must be dissolved as much as possible by incubation at 37 °C for 30 minutes before pipetting and the subsequent analysis. To minimize the effects of impurities, it is advisable to select nucleic acid extraction reagents which utilize filters, membranes, or magnetic particles. In addition, in order to monitor the actual inhibitory effect of impurities on nucleic acid amplification reactions, the internal standards included with the detection reagent kits, if any, should be used. If such standards are not available, it is desirable to conduct an addition-recovery test independently by using plasmid DNA of known copy numbers close to the detection limit. When the effect has been confirmed, the nucleic acid amplification reaction may be recovered by diluting the nucleic acid specimen. It should be noted that this procedure contains some risk, in that the targeted nucleic acid may be diluted to a level below the limit of detection, thereby causing false-negative test results.

(4) Measures to avoid inappropriate situations

Because the major objective of this testing is to diagnose infectious diseases, it is important to collect urine specimens by selecting the appropriate timing so that the highest probability exists for pathogenic genome DNA and RNA. It is best to collect initial voiding in the morning, so as not to obtain false-negative results by the washout of pathogens by urination over the course of the day. In such cases, the specimen may be purulent with a large amount of white blood cells, red blood cells, and exfoliated epithelial cells. When extracting nucleic acids, contamination of genome DNA and RNA derived from these human cells, which are not the target of detection in this testing, should be prevented. However, a certain level of contamination is inevitable because collection of pathogens affected in the human cells is a primary concern in this testing.

#### 4.1.6 Whole blood (WBC) and Bone Marrow

After a specified volume of blood is drawn into blood collection tubes containing an anti-coagulant (EDTA and others), the tubes should be immediately inverted and its contents (the specimen) gently mixed. If nucleic acids are not extracted on the day the specimens are collected, they should be refrigerated (2-8 °C). When blood collection tubes containing anti-coagulants (EDTA and others) are used, there is no need to add a stabilizer. However, in the case of tests to detect pathogens that are responsible for bacteremia or sepsis, the long-term storage of specimens at an ambient temperature may reduce detection sensitivity. Therefore, when cells for such tests are stored for a long time, they should be stored at ultra-low temperatures (-70 °C or below). In the case of bone marrow specimens (bone-marrow aspirate), a specified volume should be stored in a special storing solution (cell culture medium containing FBS) with refrigeration (2-8 °C).

##### (1) Inappropriate conditions unsuitable for testing

- 1) In specimens in which fibrin clots are observed, centrifugal collection of bacteria is hampered by the presence of fibrin, and sampling from the fluid fraction is prevented; this may result in a loss of accuracy in quantitative measurements. Fibrin clots can be recognized by the poor fluidity of the tube contents or during pipetting operations.
- 2) In analyses using PCR, heparin in the blood may cause poor or no amplification in PCR, leading to “undetectable” or false-negative results because heparin is a potent inhibitory substance for PCR. (Even a trace amount of heparin contamination (0.05 units) inhibits DNA amplification in PCR.)

Because heparin contamination cannot be detected visually, label information on the collection tube should be confirmed carefully. In particular, careful attention must be paid to bone marrow specimens, because they are often collected using heparin as an anti-coagulant.

- 3) When the quantity of bacteria responsible for bacteremia or sepsis is near the detection sensitivity limit, the bacteria may not be detected.

##### (2) Causes

- 1) The possible causes of fibrin deposition include failure to use tubes containing anti-coagulants (EDTA and others) for specimen collection and failure to invert the tubes and mix the contents immediately after the collection.
- 2) The main cause of heparin contamination is considered to be blood collection using heparin as an anti-coagulant. This applies to the case in which blood specimens collected for other analytical purposes are used for molecular methods. It is also of concern that blood specimens collected from patients who are receiving heparin as a part of either hemodialysis or thrombolytic therapy may be contaminated with heparin.
- 3) The quantity of bacteria in the specimens depends on the pathological condition of the patients at the time of specimen collection.

- (3) Troubleshooting
  - 1) Before extracting nucleic acids, the fibrin clots in the specimen must be physically crushed until they start dissociating and fibrin is sufficiently dispersed throughout the specimen. Thereafter, the specimen and the fibrin should be tested. In the DNA extraction process, addition of DTT (dithiothreitol) and the protein-digesting enzyme Proteinase K promotes fibrin degradation.
  - 2) The effects of heparin can be reduced by using nucleic acid extraction kits, which rely on nucleic acids adsorption to filters, membranes, or magnetic particles with a subsequent washout of any impurities. However, it is impossible to completely remove impurities. Heparin contamination can be resolved by completely digesting the sample with heparinase.
  - 3) In some of the specimens that contain a small quantity of the bacteria responsible for bacteremia or sepsis, treatment with proteinase K may improve the DNA extraction efficiency and enable detection in some cases.
- (4) Measures to avoid inappropriate conditions
  - 1) Fibrin deposition can be prevented by repeatedly inverting blood collection tubes containing anti-coagulants (EDTA and others) and by sufficiently mixing the contents immediately after specimen collection.
  - 2) Heparinized blood collection tubes should not be used in nucleic acid testing. In heparinized patients, it is desirable that blood specimens be collected after heparin has been eliminated from the blood. However, when heparin contamination is unavoidable, as in cases in which the patients are on heparin therapy, this information should be provided to the staff involved in testing by writing, “the patient is heparinized” on the testing order form. This also applies to bone marrow specimens. When nucleic acids are extracted from bone marrow specimens, care must be taken to thoroughly wash the cells to remove any heparin that may have been present during specimen collection.
  - 3) Because the quantity of pathogens responsible for bacteremia or sepsis in the specimen depends on the condition of the patient at the time of specimen collection, the test detection sensitivity should therefore be considered when test results are interpreted.

#### 4.1.7 Pleural Effusion, Ascites, Pericardial Fluid, Pancreatic Fluid and Broncho-Alveolar Lavage Fluid (BALF)

Each specimen should be refrigerated (2-8 °C) after collection or should be frozen (-70 °C or below) for long-term storage.

- (1) Inappropriate conditions unsuitable for testing
  - 1) When a large amount of cell components, including hemoglobin, is present in the specimens due to hemolysis, there is a concern that the detection sensitivity will be lowered due to the inhibition of PCR amplification. The extent of incorporation of blood components can be confirmed by measuring the hemoglobin level, counting

the number of white blood cells, or by semi-quantitation according to the color of the specimen.

- 2) In specimens with fibrin clots, the centrifugal collection of bacteria is hampered, and accurate sampling cannot be performed, which may result in a lack of quantitative performance in cases of quantitative analysis. Fibrin clots can be detected by observing the fluidity of the tube contents or during pipetting operations.
- 3) Purulent specimens contain vastly different bacteria types, and there is a possibility that DNA extraction by alkaline heat treatment may lead to undetectable results. This purulent condition can be confirmed macroscopically or microscopically.

(2) Causes

- 1) Incorporation of blood components at the time of specimen collection.
- 2) Incorporation of purulent discharge at the time of specimen collection.

(3) Troubleshooting

- 1) In specimens contaminated with blood components, the inhibitory effect of hemoglobin from red blood cells after PCR amplification may be eliminated by hemolysis by the addition of sterile distilled water to the specimen, followed by centrifuging and subsequent extraction of DNA from these precipitates. Alternatively, PCR can be performed using the extracted diluted DNA solution to reduce the concentration of the inhibitory substances.
- 2) Before extracting nucleic acids, fibrin clots in the specimen must be physically crushed until they start dissociating and sufficiently disperse fibrin throughout the specimen. Then, the whole specimen containing the fibrin is used in the analyses. In the process of DNA extraction, the addition of DTT (dithiothreitol) and proteinase K promotes fibrin degradation.
- 3) In order to prevent bacterial growth in the specimens after collection, instructions for the storage conditions of the specimens should be strictly followed. The volume of pretreatment solution used for nucleic acid extraction should be increased to remove impurities with surfactants and protein denaturing agents.

(4) Measures to avoid inappropriate situations

- 1) Incorporation of blood cell components at the time of specimen collection should be avoided.
- 2) Containers containing sodium citrate or EDTA should be used for the collection of pleural effusions, since pleural effusions sometimes coagulate after collection due to fibrin deposition.
- 3) Specimens should be collected when purulent discharge are not present.

#### 4.1.8 Lymph Nodes and Solid Tissues (Biopsy or Surgery)

Tissue specimens harvested by biopsy or surgery should be frozen immediately at ultra-low temperatures (-70 °C or below) to prevent nucleic acid degradation due to tissue autolysis. At this time, it is desirable to dissect only the affected lesions alone by removing them from



the harvested tissue. For detection of tubercle bacilli, affected lesions can be confirmed by morphological examination techniques.

(1) Inappropriate conditions unsuitable for testing

- 1) For large or oversized specimens, the proportion of the affected lesion (the target of the test) to the whole specimen is smaller. The affected lesion can be diluted by the presence of other tissues, which may reduce the detection sensitivity, eventually leading to false-negative results. For tests for tubercle bacilli, the affected lesions can be confirmed by morphological examination via microscopy.
- 2) There is a concern that red blood cell contamination causes poor amplification in PCR, which leads to false-negative results, in detection of *Helicobacter pylori* (*H. pylori*) using gastric biopsy tissues and HCV (hepatitis C virus) and HBV (hepatitis B virus) using hepatic biopsy tissues. The status of red blood cell contamination can be confirmed by visually observing the rust color or by measuring the hemoglobin concentrations.

(2) Causes

- 1) Tissue other than the affected lesion has not been removed from the whole tissue harvested as the specimen.
- 2) The harvested tissue (specimen) contains blood.

(3) Troubleshooting

- 1) The morphological examination of tissue specimen is useful in distinguishing the targeted cellular components from other components, so that they can be separated and removed for testing. Laser microdissection allows accurate collection of targeted cellular components from the prepared frozen tissue section on the glass slide, but requires special equipment. The simplest and easiest method is to manually collect targeted cellular components with a razor, excluding unnecessary cellular components as much as possible from the paraffin embedded tissue section on the glass slide.
- 2) The inhibitory effect of contaminated impurities following PCR amplification can be reduced by using nucleic acid extraction kits based on a purification method in which nucleic acids are adsorbed to filters, membranes, or magnetic particles, and impurities are washed out and removed.

(4) Measures to avoid inappropriate situations

- 1) At the time of specimen collection, affected lesions should be isolated from the unaffected areas and stored. Even when accurate differentiation under visual observation is difficult, it is important to remove tissues other than the target tissue as much as possible.
- 2) It is inevitable that solid tissues harvested by biopsy or surgery will contain blood components.

#### **4.2 Preparation of Specimens in the Molecular Methods for Somatic Cells**

Molecular methods for somatic cells can clarify temporary genetic information that changes

with medical conditions, such as DNA mutations and gene expression changes in cancer cells. These methods are used for the diagnosis of malignant tumors in general, including cancer, leukemia, and sarcoma, utilizing various approaches for the major purposes of, i) differentiating between malignant and non-malignant tumors, ii) evaluating the degree of malignancies, iii) detecting an early stage of malignant tumor cells, and iv) differentiating between metastatic and multiple primary cancers.

The targets of molecular methods for somatic cells are those that represent pathological changes, such as cancer cells. Therefore, the major specimens are blood cells and solid tissue, but others which may contain pathological cells are also used: pleural effusions, ascites, pericardial fluids, pancreatic fluid, sputum, broncho-alveolar lavage fluid (BALF), and urine (sediment). When specimens are collected, it is not possible to separately collect the targeted pathogenic cells. As a result, most of the specimens contain normal cells surrounding the target ones. If a large quantity of normal cells is incorporated in the specimen, there is a concern that the target cells are diluted so that the test could give false-negative results due to a failure to detect mutations in the cancer cells. Therefore, the test results must be carefully interpreted. In cases new specimens cannot be obtained other than inappropriate conditions of those, a try for use of buffers or enzymes that may reduce inhibitory effects is to be considered.

Recently, there has been an increase in the number of molecular methods for somatic cells utilizing formalin-fixed, paraffin-embedded, tissue blocks which were used as specimens for histopathological examinations. Because these tissue blocks can be stored for a long time after histopathological examination, their use provides advantages for medical experts in a retrospective study of the pathology and parallel use with morphological examinations. It also provides many advantages for patients because it eliminates the need of additional tissue collection every time a new test method is developed. On the other hand, a significant percentage of the tests utilizing formalin-fixed, paraffin-embedded, tissue blocks ends up giving “undetectable” results, because the DNA in most of the tissue blocks is fragmented due to DNA degradation caused by formalin fixation or remaining DNase activity in the tissue, or because amplification is reduced by remaining impurities due to inappropriate DNA extraction techniques. Therefore, some modification to be considered for improvement in DNA or RNA detection from such tissue blocks, including using methods that enable high-purity nucleic acid (a column method is preferable for RNA) extraction, evaluation of the quality of the extracted DNA, or a focus on short DNA fragments as the targets of detection. Since the formalin fixation protocols greatly affect DNA fragmentation and frequently cause false-negative results, it is desirable to establish a standardized fixation method that does not affect pathological examination (morphological examination and diagnosis) and that is less likely to cause DNA fragmentation. RNA integrity number (RNA), which is used for evaluation of RNA quality, constantly gives a low value for formalin-fixed, paraffin-embedded, tissue.

In order to perform Southern blotting or to create a DNA genomic library using DNA extracted from fresh tissues, blood cells, or cultured cells, DNA with high molecular weight

and high-purity must be prepared. Since high molecular weight DNA is easily damaged by physical forces, including shear force, the DNA must be extracted from a fresh specimen or a freshly frozen specimen, without adding physical stirring.

#### 4.2.1 Lymph Nodes and Solid Tissues (Biopsy or Surgery)

Specimens harvested by biopsy or surgery should be frozen immediately at an ultra-low temperature (-70 °C or below) and stored to prevent degradation of nucleic acids due to autolysis of the tissue. At this time, it is desirable to dissect the affected lesion from the harvested tissue before storage.

To accurately quantify gene expression, the pretreatment of specimens for measurement should be implemented immediately (within 2 hours after specimen collection). Otherwise, the specimen should be treated with an appropriate method to stabilize the RNA and should be stored until the RNA is extracted. The method of RNA stabilization depends on the purpose of the test, the specimen type, and the storage period. When RNA is extracted with guanidine isothiocyanate at a concentration of 5 ml/L to prevent denaturing of the specimen, the specimen can be stored for approximately one week at ambient temperature. The resulting extracted RNA should be stored at an ultra-low temperature of -70 °C or below. Repeated freezing and thawing of extracted RNA should be avoided, because this can contribute to the degradation of the RNA and thus may cause false-negative results.

##### (1) Inappropriate conditions unsuitable for testing

- 1) The specimens that contain a large amount of cellular components may give false-negative results due to the dilution of targeted cells with non-targeted cells. The proportion of the affected lesion in the specimens can be confirmed by the morphological examination of the frozen tissue sections.
- 2) When the tissues are either necrotic or apoptotic, the DNA in the tissue has already been degraded and may not render good quality DNA for subsequent testing. Necrotic and apoptotic conditions can be confirmed by morphological approaches utilizing H&E (hematoxylin-eosin) staining and the TUNEL (TdT-mediated dUTP-biotin nick end-labeling) method, respectively. Electrophoretic examination of DNA extracted from necrotic tissues shows a smeared pattern, because the DNA is completely degraded, and the DNA from apoptotic tissues gives a ladder-like pattern, because the DNA is degraded into segments according to the size of nucleosomes (185 bp).
- 3) Tissues embedded in embedding media specific for the preparation of the frozen section may not render appropriate testing results as in the PCR procedure, where embedding media can have an inhibitory effect on PCR.

##### (2) Causes

- 1) When it is difficult to macroscopically differentiate between a targeted affected lesion and non-targeted tissues at the time of specimen collection by biopsy or surgery, it is sometimes unavoidable that the specimens contain tissues other than the target tissue.

- 2) The necrosis process or the apoptosis process may have already started in the specimen tissue .
  - 3) The specimen is embedded in media specific for the preparation of the frozen tissue section.
- (3) Troubleshooting
- 1) The detection sensitivity of these methods should be confirmed so that targeted gene mutations can be specifically detected, even when the overall proportion of targeted cells is low. Otherwise, the frozen tissue sections should be prepared and the targeted cellular components collected using morphological examination and microscopy to prevent the incorporation of non-target cellular components. The method of laser microdissection allows the accurate collection of targeted cellular components from prepared frozen tissue sections on glass slides, but specialized equipment is required. The simplest and easiest method is to manually collect the targeted cellular components with a razor, excluding unnecessary cellular components as much as possible from the tissue section on the glass slide. When cellular components are collected based on morphological observations and are tested for the purpose of medical treatment, such as selecting patients for certain treatments, the confirmation of target lesions by pathological experts is imperative.
  - 2) In order to detect degraded DNA following necrosis or apoptosis, it is necessary to devise appropriate measures such as setting primers to detect shorter target DNA fragments.
  - 3) To remove the embedding media used for the preparation of the frozen section, the specimens should be washed adequately with PBS (phosphate-buffered saline) before the extraction of nucleic acids, as these fragments are soluble in PBS.
- (4) Measures to avoid inappropriate situations
- At the time of specimen procurement, the affected lesions for target detection should be stored after being isolated from the unaffected area. Even when accurate differentiation under visual observation is difficult, it is important to remove tissues other than the detection target as thoroughly as possible.
- When the tissues to be tested are already in the necrotic or apoptotic state *in vivo*, their effect on the measurement cannot be avoided. Therefore, specimens in these states should not be used for testing.
- When the tissue is scheduled for a molecular method, avoid the use of embedding media for the preparation of the frozen tissue section. When embedding media has been used for the preparation of the frozen tissue section, it is necessary to inform the involved professionals who are responsible for performing the molecular analyses.

#### 4.2.2 Formalin-Fixed Paraffin Embedded Tissue

Specimens used for pathological examination should be appropriately fixed by immersion in a 10-20% formalin solution immediately after collection by biopsy or surgery. After

fixation, the tissue is immediately embedded in paraffin to prepare a tissue block. When formalin-fixed paraffin-embedded blocks are to be used for molecular methods, tissue slices 5-10- $\mu$ m thick must be cut using a microtome. To prevent cross contamination among specimens, due consideration is required, including exchanging the microtome blade after the preparation of each specimen.

Generally, the recommended fixing solution is 10% neutral buffered formalin, and the standard fixing time at ambient temperature is between roughly 18 and 36 hours and 3 to 12 hours, for surgically and biopsy obtained specimens, respectively. It has been reported that fixation in 10% neutral buffered formalin delays progression of nucleic acid fragmentation compared with fixation in 10-20% non-buffered formalin. In addition, in a paraffin-embedded tissue block stored at ambient temperature or at low temperatures (4 °C), most nucleic acids can be preserved without further degradation for a long time. Tissue slices can be used in 10 days after cutting without further degradation of RNA.

(1) Inappropriate conditions unsuitable for testing

- 1) Formalin fixation treatment is accompanied by the fragmentation of nucleic acids, and therefore this treatment is generally not suitable for a molecular method that requires high molecular weight DNA, such as Southern blotting. It may even affect the results of measurement using PCR, which targets low molecular weight nucleic acids. The results should be interpreted carefully, because inaccurate amplification may occur using PCR. Simple methods to determine the test target for a specimen, such as excessive or insufficient fixation, include morphological examination utilizing H&E staining and immunostaining, which both allow a certain level of estimation. The RNA purity with the A260/A280 over 1.8 may provide a likelihood of the success in amplification. In addition, methods to directly evaluate the fragmentation status of DNA include direct confirmation by electrophoresis, evaluation of the PCR amplification of a known length of DNA fragments (housekeeping genes, including  $\beta$ -globin and the vitamin D receptor), and evaluation of the amplification speed using real-time PCR. To evaluate RNA fragmentation, methods include investigating the RNA purity and the A260/A280 ratio or directly measuring the RNA confirmation using denatured agarose gel electrophoresis. Furthermore, the integrity of the extracted RNA can be evaluated by the ribosomal RNA (rRNA) and housekeeping gene expression levels, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and  $\beta$ -actin.
- 2) Specimens that contain a greater amount of cellular components (normal and non-tumor cells) than the target cells may give false-negative results due to the dilution of the target cells by normal cell populations. The proportion of target cells in the specimens can be confirmed using a morphological approach, including histopathological examination using H&E staining.

(2) Causes

- 1) Nucleic acids in tissues can be fragmented by such factors as inappropriate handling of the tissue before fixation (such as letting a long time pass before immersing the

tissue in the fixation solution), autolysis by intrinsic nucleolytic enzymes during formalin fixation, protein crosslink formation by formalin fixation, and the effect of acidification due to the formation of formic acid in the fixation solution. The extent of fragmentation attributable to formalin fixation is greatly influenced by the types of fixation solution, time, and the temperature.

- 2) Paraffin-embedded tissue blocks used as a specimen may contain a greater amount of cellular components (normal and non-tumor cells) compared to target cells.

(3) Troubleshooting

- 1) In order to detect nucleic acids that were degraded into small molecules due to the formalin fixation treatment, appropriate measures include setting primers to detect shorter lengths of target DNA fragments. When using specimens in which the nucleic acids are severely fragmented, it is important to implement a molecular method only after fully understanding the characteristics of the specimens in order to obtain highly reliable results. In particular, special attention is required when a previously paraffin-embedded tissue block is used for more recent tests, such as in a retrospective study.

- 2) The detection sensitivity of the molecular method should be high enough to specifically detect targeted gene mutations, even when the proportion of targeted cells is low. Otherwise, a method to visually collect targeted cellular components from the section under morphological observation is useful to minimize the incorporation of cellular components other than the target cell population. The method of laser microdissection allows the accurate collection of targeted cellular components from the paraffin-embedded section on the glass slide, but special equipment is required for these experiments. The simplest and easiest method is to manually collect the targeted cellular components with a razor, excluding unnecessary cellular components as much as possible from the paraffin-embedded section on the glass slide. When collected cellular components are based on morphological observations and are used in tests for the purpose of medical treatment, such as selecting patients for certain chemotherapy treatments, confirmation by pathological experts is imperative.

(4) Measures to avoid inappropriate situations

- 1) When a tissue is scheduled to be analyzed by molecular methods besides pathological examination, it is important to separately secure these specimens by cryopreserving a portion of the affected lesion at ultra-low temperatures ( $-70^{\circ}\text{C}$  or below) to avoid the fragmentation of nucleic acids. When formalin-fixed tissues are used for molecular examination, it is desirable to perform the pre-fixation treatment and formalin fixation at a low temperature in the shortest possible time. If the specimen is also used for pathological examination, it is important to consider balancing the requirements for specimens for pathological examination and for the subsequent molecular analyses. For example, specimens for pathological examination should be fixed for a certain period of time at an ambient temperature

because of the requirements to maintain tissue morphology and structure, as well as for reasons of occupational safety. Therefore, in such cases, a certain level of fragmentation of nucleic acids is unavoidable, but it is possible to avoid completely defective specimens by noting the following points: As a prefixation treatment, large masses of tissue, such as those obtained by surgery, should be sliced into 4-5 mm thicknesses so that the fixation solution can easily penetrate throughout the tissue, thereby shortening the time required for fixation. In the fixation procedure, the size of the tissue and the time for formalin penetration are key issues in avoiding the fragmentation of the nucleic acids. It has been reported that 10% formalin usually penetrates the tissue at the rate of about 1 mm per hour under ambient temperature, which can be referenced to set the fixation time for specimens obtained by both biopsy and surgery.

- 2) If multiple numbers of paraffin-embedded tissue blocks are available for an identical lesion, those with the largest overall proportion of affected cells should be selected as the specimen based on morphological examination utilizing H&E staining. Even when only one paraffin-embedded tissue block is available, the existence of affected cells should be confirmed by morphological examination utilizing H&E staining or other methods prior to the molecular analysis.

#### 4.2.3 Whole Blood (WBC) and Bone Marrow

After a volume of blood is drawn into the blood collection tubes containing anti-coagulant (EDTA), the specimen should be inverted and mixed immediately. If nucleic acid extraction will not be performed on the same day of specimen collection, the samples must be refrigerated. If the purpose of the testing requires the separation of the cell components of the collected blood, the required procedure should be performed. For bone marrow specimens, an indicated volume should be stored in special storing solution (cell culture medium supplemented with FBS) under refrigeration (2-8 °C).

In case of gene expression analysis for accurate quantification, total RNA containing mRNA should be extracted immediately (within two hours) after collection of the specimen. When RNA extraction cannot be implemented immediately, the specimen should be treated with an appropriate method to stabilize the RNA and stored until the RNA is to be extracted. The method that is chosen to stabilize the RNA depends on the purpose of the test, the specimen type, and the storage period. When guanidine isothiocyanate at a concentration of 5 ml/L is used to prevent denaturing of the specimen, the specimen can be stored for about a week under ambient temperature. The extracted RNA should be stored at an ultra-low temperature of -70 °C or below. Repeated freezing and thawing of extracted RNA should be avoided, because this can contribute to RNA degradation and thus may cause false-negative results.

When DNA extraction is performed for gene mutation analysis, the specimen can be stored at ambient temperature for as long as three days without causing any significant effect. However, it is desirable that DNA extraction be performed within 24 hours for molecular

methods that examine high molecular weight DNA, as is the case with Southern blotting, and otherwise within three days, as for PCR. For long-term storage, the specimens should be stored at ultra-low temperature (-70 °C or below) after the appropriate procedures, including separation of the buffy coat.

When DNA is extracted from whole white blood cells without separating blood cell components, the specimen may be frozen and stored immediately after collection. However, the amount of DNA recovered from this sample will decrease. Freezing and thawing cause the degradation of nucleic acids, and these processes should not be repeated. For long-term storage, specimens should be stored at an ultra-low temperature (-70 °C or below).

When specimens are collected from patients who have received pretreatment for hematopoietic stem cell transplantation (bone marrow transplantation) and who have extremely low white blood cell counts, it should be noted that several times the indicated volume of blood may be needed to secure the required number of white blood cells for the analyses.

(1) Inappropriate conditions unsuitable for testing

- 1) Specimens that contain a greater amount of cellular components (normal cells and non-tumor cells) than the target cells may give false-negative results due to the dilution of the targeted cells by normal cell populations. The proportion of targeted cells in the specimens can be confirmed by the hemogram and myelogram analyses.
- 2) In specimens with fibrin clots, white blood cells are not evenly distributed in the specimen, which may result in a lack of quantitative performance in the case of quantitative analysis. Fibrin clots can be detected by observing the fluidity of the specimen in the blood collection tubes or during pipetting.
- 3) If heparin exists in the specimens, poor PCR amplification will occur, resulting in “undetectable” or false-negatives, because heparin inhibits PCR. Because heparin contamination cannot be visually detected, the information on the tube label should be carefully checked. Heparin is often used as an anti-coagulant when collecting bone marrow specimens, so careful attention is required for such specimens.

(2) Causes

- 1) It is difficult to separately collect targeted cells at specimen collection, and in many cases, contamination with normal cells is unavoidable.
- 2) The possible causes of fibrin deposition include the failure to use tubes that contain anti-coagulants (EDTA and others) when collecting blood specimens, failure to use a special storage solution (cell culture media supplemented with FBS) for the storage of bone marrow, and insufficient tube inversion and mixing immediately after sample collection.
- 3) The main cause of heparin contamination is considered to be the use of heparin as an anti-coagulant during blood collection. This also applies to cases in which blood specimens collected for other analytical purposes are used for molecular examination. Heparin contamination is also a concern when blood specimens are collected from patients who are receiving heparin in hemodialysis or thrombolytic



therapy.

(3) Troubleshooting

- 1) Detection sensitivity of the method should be confirmed to be high enough to detect targeted gene mutations, even when the proportion of targeted cells is low.
- 2) Before extracting nucleic acids, fibrin clots in the specimen must be physically crushed until they start dissociating and sufficiently dispersing fibrin throughout the specimen. The whole specimen and the fibrin can be used for the subsequent analysis. In the process of DNA extraction, the addition of DTT (dithiothreitol) and proteinase K promotes fibrin degradation.
- 3) The effect of heparin can be reduced by using nucleic acid extraction kits in which nucleic acids are adsorbed onto filters, membranes, or magnetic particles, and then impurities are washed out and removed. However, it is impossible to completely remove impurities. The effect of heparin can be resolved either by completely digesting the heparin by the addition of heparinase, or by washing the white blood cell fraction with physiological saline at least twice after fractioning of white blood cells.

(4) Measures to avoid inappropriate situations

- 1) In the case of a specimen in which the proportion of targeted cells is low, the fractionation of targeted cells should be performed by density gradient centrifugation using Ficoll, by the antibody method, or by the use of a cell sorter. When leukemia has progressed, the mRNA from the tumor cells dominate the specimen, so the mRNA derived from contaminating normal lymphocytes is generally minimized.
- 2) Fibrin deposition can be prevented by repeatedly inverting the blood collection tubes (containing EDTA or other anti-coagulants) and sufficiently mixing their contents immediately after collecting blood specimens, and by using a special storage solution (cell culture media supplemented with FBS) when bone marrow specimens are collected.
- 3) In principle, heparinized blood collection tubes should not be used for molecular methods. In heparinized patients, it is desirable that the specimen be collected after the effects of heparin have been eliminated from the blood. However, when heparin contamination is unavoidable, for example, when patients are on heparin therapy, this information should be provided to the professionals performing the molecular analysis by stating, "the patient is heparinized" on the testing order form. This also applies to bone marrow specimens. When nucleic acids are extracted from bone marrow specimens, a thorough washing of the cells is required to remove any heparin that may have been present during specimen collection.

4.2.4 Pleural Effusion, Ascites, Pericardial Fluid, Pancreatic Fluid, Broncho-Alveolar Lavage Fluid (BALF), Urine Sediment and Sputum

Each specimen should be stored under refrigeration (2-8 °C) after collection. If the purpose

of the testing requires separated collection of blood cell components, the requisite procedure(s) should be performed.

For accurate quantification of gene expression analysis, total RNA and mRNA should be extracted immediately (within two hours) after the collection of the specimen. When RNA extraction cannot be performed immediately, the specimen should be treated with an appropriate method to stabilize the RNA and should be stored until RNA extraction. The method that is chosen to stabilize the RNA depends on the purpose of the test, the specimen type, and the storage period. When guanidine isothiocyanate is used at a concentration of 5 ml/L to prevent specimen denaturing, the specimen can be stored for approximately one week in an ambient temperature. The extracted RNA should be stored at an ultra-low temperature of -70 °C or below. Repeated freezing and thawing of extracted RNA should be avoided, because this can contribute to RNA degradation and thus cause false-negative results.

When DNA extraction is performed for gene mutation analysis, it is desirable that DNA extraction be performed within 24 hours for the analyses that examine high molecular weight DNA, as is the case with Southern blotting, and otherwise within three days, as is the case with PCR. For long-term storage, specimens for DNA tests should be stored as follows:

- Pleural effusion, ascites, pericardial fluid, pancreatic fluid, broncho-alveolar lavage fluid (BALF): After centrifugation (760 x g (gravitational acceleration) [2,000 rpm], at an ambient temperature, for 10 minutes), the precipitates are washed with PBS. After an additional centrifugation, the supernatant is discarded and only the precipitates are stored at ultralow temperatures (-70 °C or below).
  - Urine Sediment: After centrifugation (6,000 x g (gravitational acceleration) [8,200 rpm], at an ambient temperature, for two minutes), discard the supernatant and store only the precipitates at an ultralow temperature (-70 °C or below).
  - Sputum: Store at an ultra-low temperature (-70 °C or below).
- (1) Inappropriate conditions unsuitable for testing
- 1) Specimens that contain a greater amount of cellular components (normal cells and non-tumor cells) than the target cells may give false-negative results due to the dilution of the targeted cells by normal cell populations. The proportion of targeted cells in the specimens can be visually confirmed with morphological examination.
  - 2) In specimens in which fibrin clots are observed, centrifugal collection of cells is hampered and sampling from consistent fluid is prevented, which may result in a lack of quantitative performance for quantitative analysis. Fibrin clots can be detected by observing the fluidity of the specimen in the tubes or during pipetting operations.
- (2) Causes
- 1) Because collection of pleural effusion, ascites, pericardial fluid, pancreatic fluid, sputum, broncho-alveolar lavage fluid (BALF), and urine sediment are not methods to directly harvest the affected lesion, contamination by cells not in the target area,

including normal cells, is unavoidable in many cases.

- 2) Possible causes include the contamination of blood components at the time of specimen collection.
- (3) Troubleshooting
  - 1) The detection sensitivity of these methods should be confirmed to be high enough specifically detect the targeted gene mutations, even when the proportion of targeted cells is low.
  - 2) Before extracting nucleic acids, fibrin clots must be physically crushed in the specimen until fibrin begins to disaggregate and sufficiently disperses throughout the specimen. Then, the specimen is sampled together with the fibrin. In the process of DNA extraction, the addition of DTT (dithiothreitol) and proteinase K promotes fibrin degradation.
- (4) Measures to avoid inappropriate situations
  - 1) In case of specimens in which the proportion of targeted cells is low, of targeted cells should be fractionated by density gradient centrifugation using Ficoll, by the antibody method, or by using a cell sorter.
  - 2) Because pleural effusions and others may coagulate after collection due to fibrin deposition, they should be collected in a container that contains sodium citrate or EDTA.

#### 4.2.5 Cultured Cells

After collecting cultured cells in the centrifuge tubes, they should be centrifuged (300 x g (gravitational acceleration) [1,250 rpm for a rotor with a radius of 170 mm], at an ambient temperature, for five minutes). The precipitated cells should be stored at an ultra-low temperature (-70 °C or below) and used for the extraction of nucleic acids.

To analyze the gene expression for accurate quantification, total RNA should be extracted immediately (within two hours) after the collection of the specimen. When RNA extraction cannot be implemented immediately, the specimen should be treated with an appropriate method to stabilize RNA and then should be stored until the RNA is extracted. The methods for stabilizing RNA differ by the purpose of the test, the specimen type, and the storage period. When guanidine isothiocyanate (5 ml/L) is used to prevent specimen denaturing, the specimen can be stored for approximately one week with in ambient temperature. The extracted RNA should be stored at ultra-low temperatures of -70 °C or below. Repeated freezing and thawing of extracted RNA should be avoided, since it may contribute to the degradation of RNA and thus cause false-negative results.

##### (1) Inappropriate conditions unsuitable for testing

It is necessary to consider the effects of the anti-freezing agent DMSO (dimethyl sulfoxide) contained in the cryopreservation solution of the cultured cells. DMSO is sometimes used to enhance the specificity of the PCR reaction, but when DMSO remains in the solution of extracted nucleic acids (DNA/RNA), it may lower the amplification efficiency, or the fluorescent substance included in DMSO may raise the

background level in the measurement of DNA concentration.

(2) Causes

Possible causes include residual DMSO in extracted nucleic acids. DMSO is an anti-freezing agent that is added for the purpose of preventing freezing of the cultured cells during cryopreservation.

(3) Troubleshooting

Sufficiently wash cultured cells with PBS and others to remove the anti-freezing agent (DMSO) before nucleic acid extraction.

(4) Measures to avoid inappropriate situations

When the anti-freezing agent (DMSO) is used, the information should be provided to the staff engaged in the molecular method by describing this issue in the testing order form.

### **4.3 Preparation of Specimens in Molecular Methods for Germ Line Cells**

Molecular methods for germ line cells can reveal permanent genetic information that does not change over a long period of time, such as genetic testing for single-gene diseases. This is classified into the following categories: (1) genetic testing for confirmatory diagnoses performed for patients (or the proband), carrier testing, presymptomatic testing, disease susceptibility testing, pharmacogenomic testing, prenatal testing, and neonatal screening; (2) genotype testing to clarify the HLA type of the donor and recipient (patient) for the purpose of transplantation; and (3) DNA testing for the determination of parentage paternity and personal identification.

In molecular methods for germ line cells, blood specimens are mainly used, but oral (buccal) mucosa, hair, and nail specimens are sometimes used because their collection is less invasive. In addition, in the field of forensic medicine, blood stains and umbilical cords that are kept as tokens of one's birth are also used for to provide specimens for evidence and to identify an individual, respectively.

When the test is performed for an individual who underwent either hematopoietic stem cell transplantation (bone marrow transplantation) or organ transplantation, it is potential that the specimen contains blood or tissue of the donor. Thus, before the collection of a specimen, it should be confirmed whether or not the person has undergone organ transplantation. If the individual has received organ grafts, tissues that do not contain donor-derived tissues should be used for testing.

In order to perform Southern blotting or to create a DNA genomic library using DNA extracted from blood, high molecular weight and high-purity DNA must be prepared. Since high molecular weight DNA can be easily damaged by physical forces, including shear force, DNA must be extracted from a fresh specimen or from a freshly-frozen specimen without physical stirring.

Because genetic testing can reveal genetic information about the subject (patient) as well as the subject's relatives, obtaining informed consent is essential prior to performing the tests. Generally, blood and other specimens are collected at medical facilities, but some

specimens, including oral mucosa and hair, can be collected by the subjects themselves. However, in order to ensure personal identification, it is required that specimen collection be conducted in the presence of a responsible or accredited third person.

#### 4.3.1 Whole Blood (WBC)

After the specified volume of blood is drawn into blood collection tubes that contain an anti-coagulant (EDTA and others), the specimen should be inverted and mixed immediately and refrigerated if nucleic acid extraction is not performed on the same day of specimen collection. The specimens can be stored at an ambient temperature for as long as three days without any significant effects. However, it is desirable that DNA extraction be performed within 24 hours for molecular methods that require high molecular weight DNA, as is the case with Southern blotting, and otherwise performed within three days, as for PCR. When blood specimens are cryopreserved after collection, they may be frozen immediately, but it is important to avoid repeating the freezing and thawing process, which contributes to the degradation of nucleic acids. For long-term storage, blood specimens should be stored at ultra-low temperatures ( $-70^{\circ}\text{C}$  or below). In cases new specimens cannot be obtained other than inappropriate conditions of those, a try for use of buffers or enzymes that may reduce inhibitory effects is to be considered.

When specimens are collected from patients who have received pretreatment for hematopoietic stem cell transplantation (bone marrow transplantation) and who have extremely low white blood cell counts, it should be noted that several times the specified volumes of blood specimens may be needed to secure the required number of white blood cells for the test. Blood specimens should be collected by qualified medical personnel only after the subject has been carefully identified.

##### (1) Inappropriate conditions unsuitable for testing

- 1) In specimens with fibrin clots, fibrin prevents the even distribution of the white blood cells throughout the specimen. Fibrin clots can be detected by observing the fluidity of the specimen in the blood collection tubes or during pipetting operations.
- 2) In the PCR analyses, if heparin is present, PCR amplification will be poor and thus an “undetectable” result will be obtained, because heparin is an inhibitory substance for PCR. Because heparin contamination cannot be visually detected, the information on the tube label should be carefully checked.

##### (2) Causes

- 1) Potential causes of fibrin deposition include failure to use tubes containing anti-coagulant (EDTA and others) and improper inversion and mixing of the tubes immediately after the specimens are collected.
- 2) The main cause of heparin contamination is considered to be the use of heparin as an anti-coagulant agent during blood collection. This also applies to the case in which blood specimens collected for other analytical purposes are used for genetic testing. Heparin contamination is also a concern when blood specimens are collected from patients who are receiving heparin for thrombolytic therapy.

(3) Troubleshooting

- 1) Before extracting nucleic acids, fibrin clots in the specimen must be physically crushed until they begin dissociating and sufficiently disperse fibrin throughout the specimen. Thereafter, the whole specimen containing the fibrin are analyzed. During the process of DNA extraction, the addition of DTT (dithiothreitol) and proteinase K promotes fibrin degradation.
- 2) It has been stated in the literature that the effects of heparin can be reduced by using nucleic acid extraction kits, in which nucleic acids are adsorbed onto filters, membranes, or magnetic particles, and impurities are washed out and removed. However, it is impossible to completely remove the impurities. The effect of heparin can be resolved either by completely digesting the heparin with heparinase or by washing the white blood cell fraction with physiological saline at least twice after fractioning of white blood cells.

(4) Measures to avoid inappropriate situations

- 1) Fibrin deposition can be prevented by repeatedly inverting the blood collection tubes that contain anti-coagulant (EDTA and others) and sufficiently mixing the contents immediately after collecting blood specimens.
- 2) In principle, heparinized blood collection tubes should not be used in genetic testing. For heparinized patients, it is desirable that the specimen be collected after the heparin effects have been eliminated from the blood. However, when heparin contamination is unavoidable, for example when patients are on heparin therapy, information should be provided to the medical professionals performing the molecular analyses by stating, “the patient is heparinized,” on the testing order form.

#### 4.3.2 Oral Mucosa and Fluid

Oral mucosal specimens are collected by rubbing the inside of the cheek several times with a swab. Subsequently, the swab should be air dried immediately and stored at an ambient temperature. After oral mucosa collection, if the swab is left in moist conditions, such as stored in a sealed plastic bag or a test tube without drying, the DNA in the oral mucosa will be rapidly degraded due to DNases in the sample.

Oral mucosal specimens can be collected by the subjects themselves, but in order to ensure personal identification, the specimen collection must be conducted in the presence of a responsible or accredited third person after confirming the subject’s identity.

(1) Inappropriate conditions unsuitable for testing

Regarding specimens collected just after eating, drinking, or breast-feeding, the effects of the incorporation of foods or breast milk on DNA extraction or test results are of concern.

(2) Causes

If oral mucosa specimens are collected while food, drink, or breast milk is in the oral cavity, there is the possibility that they will be collected together with the oral mucosa

by the swab.

(3) Troubleshooting

By using molecular methods that can specifically detect human DNA, the effect of DNA derived from food and drink can be eliminated. Regarding the effect of breast milk, the extent of incorporation of maternal DNA can be confirmed by personal identification by genetic testing using the extracted DNA.

(4) Measures to avoid inappropriate situations

Because the effects of eating, drinking, or being breast-fed disappear in about 30 minutes, specimens should not be collected until 30 minutes after eating, drinking, or being breast-fed. In addition, the oral cavity should be rinsed with water before specimen collection to remove any remaining effects of food, drink, or breast milk.

#### 4.3.3 Hair

Hair specimens should be collected by removing the hair with tweezers. The specimens should be dried and stored at an ambient temperature. Genomic DNA in hair exists mainly in the root cells of the hair, and extremely small amounts exist in the shaft of the hair. Therefore, for the detection of genomic DNA, the root cells of the hair should be confirmed to be attached. The amount of recoverable DNA is much less from fallen hair than from removed hair. The use of fallen hair as a specimen should also be avoided, so that the origin of the specimen is attributable to an individual.

Mitochondrial DNA can be detected from the shaft of the hair, but yields hetroplasmic results more often than that from other tissues. Thus, the results should be carefully interpreted.

Hair specimens can be collected by the subjects themselves, but, in order to ensure personal identification, it is required that specimen collection be conducted in the presence of a responsible or accredited third person after confirming the subject's identity.

(1) Inappropriate conditions unsuitable for testing

- 1) Hair specimens to which hair dressing or hair dye has been applied may give poor PCR amplification. A recent history of using hair dressings and hair dyes should be checked with the test subject, and hair dye should also be confirmed by measuring the amount of dye compound and by microscopic examination.
- 2) There is a possibility for poor PCR amplification due to the effects of melanin remaining in the extracted DNA.

(2) Causes

- 1) Some components in hair dressings may inhibit the PCR reactions. In addition, DNA in the hair shaft may be fragmented by the oxidative effects of hair dyes.
- 2) Possible causes include insufficient melanin removal in the process of DNA extraction.

(3) Troubleshooting

- 1) Before extracting DNA, hair specimens should be washed with ethanol to remove any remaining hair dressings. In order to detect DNA that has been degraded into

small molecules, it is necessary to devise appropriate measures such as setting primers to detect shorter DNA fragments.

- 2) The amount of specimen (hair) used in one batch of the DNA extraction reaction can be reduced to improve the efficiency of melanin removal during the DNA extraction procedures. For protein removal during DNA extraction, methods that are suitable for melanin removal should be used; for example, the NaI method has been reported to be more suitable for melanin removal than the phenol method. In order to reduce the inhibitory effect of melanin on PCR, extracted DNA should be diluted and used for PCR.
- (4) Measures to avoid inappropriate situations
- 1) The subjects of the test should be carefully interviewed concerning their recent use of hair dressings and hair dyes, and the subjects should provide information to the staff through the testing order form.
  - 2) If no DNA is detected, a new specimen should be obtained.

#### 4.3.4 Nails

Before collection, carefully wash the areas between the nail and the finger, remove fingernail scrapings and dirt to the maximum extent possible, and use nail polish remover to remove all nail polish. Cut a specimen from the excess white part at the tip of the nail (free edge of the nail) with a nail clipper and store the specimen at ambient temperature. To avoid mixing nail specimens, each patient must use a different, clean nail clipper.

Nail specimens can be collected by the subjects themselves, but in order to ensure personal identification, it is required that collection be conducted in the presence of a responsible or accredited third person after confirming the correct person's identity.

- (1) Inappropriate conditions unsuitable for testing  
If large pieces of the nail are used for DNA extraction, they may not be completely dissolved by the protein-digesting enzymes during the treatment process, and the amount of recovered DNA may be reduced.
- (2) Causes  
Possible causes include incomplete dissolution of the specimen, since the hard fibrous protein (keratin), which is one of the main components of the nail, is not easily dissolved by treatment with protein -digesting enzymes.
- (3) Troubleshooting  
If the specimen cannot be dissolved by the protein-digesting enzymes during the DNA extraction process, the DNA extraction should be continued using only the dissolved portion.
- (4) Measures to avoid inappropriate situations  
Specimens used for DNA extraction should be cut into small pieces (around 1 mm x 1 mm) in advance to improve the digestion efficiency by protein-digesting enzymes.

#### 4.3.5 Dried Blood Spots



Blood should be dripped on a white filter paper, air dried, and stored under an ambient temperature. In cases of specimens that will be used in the field of forensic medicine, the blood may be stained on various materials. If it is possible to collect a blood spot alone from any of the materials, peel off the blood spot or collect blood components using swabs that have been moistened with distilled water and dry them for storage under an ambient temperature. If blood is absorbed in the material and it is impossible to collect blood components alone, collect the stained material and dry and store it under an ambient temperature. The conditions before and after the collection of the blood spot must be recorded.

(1) Inappropriate conditions unsuitable for testing

In the case of specimens used in the field of forensic investigations, blood which is inseparably mixed with some substances may be a cause of poor amplification in PCR or a band shift in electrophoresis.

(2) Causes

In forensic investigations, blood is generally found where it has stained various materials. Possible causes include that the specimen includes some substances that cannot be removed in the DNA extraction processes.

(3) Troubleshooting

The efficiency in impurity removal in DNA extraction differs depending on the substance the DNA is mixed with. Thus, it is necessary to consider and select appropriate DNA extraction methods for different specimens.

(4) Measures to avoid these situations

In forensic investigations, blood that has already stained material is used in these tests. Accordingly, contamination with unknown substances is unavoidable. When swabs are used for sampling, special swabs that do not affect DNA extraction should be used.

#### 4.3.6 Umbilical Cord

This section describes a dried umbilical cord kept as a token of one's birth, and that was used as a specimen for identification of an individual by genetic testing. The origin of the umbilical cord is the fetus, but because it is a colloidal connective tissue, little DNA can be collected from the umbilical cord itself. Instead, blood-derived cells in the umbilical cord are used for DNA extraction. Information attached with the umbilical cord should be used as the specimen collection record.

(1) Inappropriate conditions unsuitable for testing

Blood spots that are considered to be of maternal origin are found on the surface of the umbilical cord.

(2) Causes

Possible causes of maternal blood found on the surface include incomplete removal of maternal blood at the time of umbilical cord collection. However, this is an uncontrollable situation because, at the time the umbilical cord was collected, there was no intention that it would be used for DNA extraction.

(3) Troubleshooting

Whether there are blood spots on the surface of the umbilical cord or not, the outer surface of the umbilical cord should be washed with PBS or physiological saline prior to DNA extraction. In case DNA types of more than one individual are detected by DNA testing, DNA testing of the mother should be performed to identify the source of the detected DNA type.

(4) Measures to avoid inappropriate situations

It should be assumed that an umbilical cord that is kept as a token of one's birth is contaminated with tissues of maternal origin. The outer surface of the umbilical cord should be washed with PBS or physiological saline before DNA extraction procedures, even if no contamination is visually recognized.

## **5. Collection of Specimens for Molecular Methods**

This section summarizes methods to collect specimens for molecular methods.

### **5.1 Collection of Specimens in Molecular Methods for Pathogens**

#### **5.1.1 Viral Infection**

##### **5.1.1.1 Hepatitis Virus**

The following types of hepatitis viruses are known: hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV). HDV requires the co-existence of HBV for replication. Other than these viruses, the EB virus (EBV), cytomegalovirus (CMV), and herpes simplex virus (HSV) also cause hepatitis. HAV and HEV are primarily orally transmitted, while HBV, HCV, and HDV are transmitted through the blood.

If viral hepatitis is suspected, typically serum or plasma separated from blood collected with EDTA is used as a specimen. It is desirable that specimens for molecular methods for pathogens be stored at temperatures of -20 °C or below for the measurement for HBV DNA, or at temperatures of -70 °C or below for the measurement of RNAs of HAV, HCV, and HEV. It is of critical importance to avoid repeated freezing and thawing cycles. To confirm that viruses are completely eliminated after anti-viral therapy, an ultra-sensitive nucleic acid amplification test may be performed using tissue from a liver biopsy as the specimen. As the activity markers, such techniques as detection of negative-strand RNA and cccDNA (covalently closed circular DNA) for HCV and HBV, respectively, in the hepatic cells have been attempted in some investigational studies.

Acute hepatitis by HAV and HEV is transient, and does not usually have a chronic course. Serum collected in the acute phase is used as the specimen. Stool is also used as an important specimen, since the viruses that cause these diseases characteristically tend to be excreted in the stool for a long time after they are eliminated from the blood.

##### **5.1.1.2 Virus Causing Acute Respiratory Infection**

Several viruses responsible for acute respiratory infections include influenza virus, RS virus, parainfluenza virus, coronavirus, enterovirus (non-polio), rhinovirus, and adenovirus.

The major specimens collected to identify causal viruses are listed below. The collection methods of specimens for nucleic acid testing conform to those for virus isolation.

1) Collection of throat swab specimens

Tubes containing 2 ml of PBS (-) with 0.5% BSA and cotton swabs with wooden shafts that can be snapped with the fingers should be prepared. After rubbing the patient's throat with the tip of the swab, the cotton part should be dipped into the solution in the tube, the cotton part rinsed by vigorous agitation, and the solution squeezed out of the cotton part of the swab by pressing it against the inside of the tube, after which the swab should be discarded. Alternatively, the swab with shaft can be left in the solution in the tube after the shaft is snapped with the fingers.

2) Collection of nasal wash solution

The subject's head should be tilted back by approximately 45 degrees. The tip of the rubber bulb syringe containing 3-5 ml of physiological saline should be inserted into the nostril, and a squeeze of the bulb will inject the physiological saline into the nostril, then upon release it will immediately collect the physiological saline in the nostril back into the bulb due to the negative pressure. This solution is used as the specimen for the testing. This procedure requires the cooperation of the subject and skillful technique, and it has some other disadvantages in that the physiological saline injected into the nostril may not be recovered sufficiently or the virus concentration may be reduced if some of the physiological saline remained in the bulb, thereby not being injected into the nostril.

3) Collection of nasopharyngeal discharge

A vinyl catheter with a trap should be prepared between the vacuum source and the tip. This catheter should be inserted into the innermost boundary of the nostril and the nasopharyngeal discharge aspirated. The nasopharynx is the place where the Eustachian tubes open, and infants and young children have extensive nasopharyngeal discharge in this area. The isolation rates of influenza virus and RS virus are higher in nasopharyngeal discharge specimens than in throat swab specimens, which make nasopharyngeal discharge a favorable specimen for nucleic acid testing. There is an alternative method for collection of nasopharyngeal discharge, which utilizes a swab with fine, flexible shaft, but not a hard one for the throat. The swab should be carefully inserted along the lower edge of the nostril by imagining a plane that connects the nostril opening to the acoustic opening, reaching the innermost section of the nostril. The swab is left there for several seconds, and then removed.

4) Collection of throat wash solution

The patient gargles with a minimal volume of PBS or physiological saline, and this solution is used as the specimen. Only a minimal amount of solution should be used,

otherwise it is difficult to extract viral nucleic acids from a large specimen volume, which may affect the test results. Specimens should be collected in the acute phase and tested immediately after the collection. For storage, freezing is required.

#### 5.1.1.3 Virus Causing Diarrhea

Viruses responsible for acute, infectious diarrhea include rotavirus, adenovirus, calicivirus, astrovirus (norovirus and sapovirus), parvovirus, coronavirus, enterovirus, and influenza virus. Gastroenteritis accompanying rotavirus and influenza as well as Calicivirus (oyster-related food poisoning) infections are most commonly seen during the winter, while enteroviral infections occur more frequently during the summer. Adenovirus and sporadic Calicivirus are detected in the stools of patients with diarrhea all the year.

Many of these viruses replicate in the intestinal mucosal cells and bring about severe diarrhea and vomiting. An extremely large number of virus particles are excreted into the stool (several hundred million particles per 1 g of stool). For the detection of pathogens by nucleic acid testing, stool specimens collected at the peak of the symptoms are used, but vomitus is sometimes used as the specimen in cases of norovirus infections. Because the number of viruses contained in the vomitus is lower than that of the stool, it is difficult to detect pathogens by immunoassay, and requires the highly-sensitive PCR.

#### 5.1.1.4 Encephalitis Virus

Viral encephalitis is classified into two types: primary encephalitis, which is developed by direct viral infection of the brain tissue, and secondary (post-infection) encephalitis, which develops secondarily to the infection of other organs of the body. Primary encephalitis includes those by herpes simplex virus (HSV) encephalitis and Japanese encephalitis, and secondary encephalitis includes those caused by measles (rubeola), epidemic parotitis (mumps), and rubella.

To diagnose these viral encephalites, pathogenic examinations, including the isolation of viruses and the detection of viral nucleic acids, are important tools. In the case of acute encephalitis, cerebrospinal fluid (CSF) is useful as a specimen, because the testing target is the affected brain tissue lesion. CSF should be collected in the acute phase prior to treatment. Sufficient care is required to avoid blood contamination during the collection. If the patient dies, the brain parenchyma is used as the affected lesion in the tests.

#### 5.1.1.5 Aseptic Meningitis Virus

Viruses responsible for aseptic meningitis are represented by enteroviruses, including echovirus and Coxsackie B virus. Aseptic meningitis is prevalent during the summer in Japan, as are other diseases caused by enteroviral infection, but can also occur during the fall and winter. Some meningitis cases are caused by other viruses, including the mumps and herpes simplex viruses. The major symptom of meningitis caused by enterovirus is fever, which is sometimes accompanied by headache, nausea, and vomiting. Generally, however, the patient prognosis is good. The serotype of prevalent enterovirus and the

degree of the epidemic usually differs every year. The major route of infection is fecal-oral transmission, and the incubation period is typically 4-6 days.

Stools, spinal fluid, and oral or throat swabs are used as specimens for molecular methods for pathogens, as in the case with virus isolation. Recently, many reports have described viral identification methods that use homology searches based on the base sequence of the virus. In these methods, specimens with virus are inoculated and cultured in medium, and nucleic acids of the obtained virus are amplified and are used for the determination of the base sequence.

Specimens should be collected as soon as possible after the onset of the symptoms and tested immediately. If stored, specimens should be frozen. However, test methods for enteroviruses have not been standardized. Depending on the purpose of the test, appropriate specimens and appropriate procedures should be selected.

#### 5.1.1.6 Epstein-Barr virus: EBV

EBV is a virus of the *γ-herpesvirinae* subfamily that primarily infects B lymphocytes and certain epithelial cells; after that, it persists in a latent state. EBV is transmitted primarily by the saliva. Primary infection during early adolescence and after may lead to infectious mononucleosis, virus-associated hemophagocytic syndrome, and chronic active EBV infection (CAEBV). In addition, EBV is considered a tumor virus, as it is involved in the development of nasopharyngeal cancer, Hodgkin's lymphoma, NK/T lymphoma, gastric cancer, and opportunistic lymphoma.

Various specimens, including serum, blood, spinal fluid, and throat swabs, are used to confirm the presence of EBV by molecular methods for pathogens. Quantification of EBV DNA in peripheral mononuclear cells is useful for the diagnosis of CAEBV. In addition, affected tissues are also used as specimens. When a significant amount of the EB viral genome is confirmed in the tested area, the clonality of the infected cells is analyzed by Southern blotting.

#### 5.1.1.7 Cytomegalovirus: CMV

CMV belongs to the *β-herpesvirinae* subfamily, and, after the primary infection, it remains permanently latent in the spinal cord, peripheral mononuclear cells, and granulocyte-macrophage precursor cells. When the primary infection occurs, it infrequently causes infectious mononucleosis-like syndrome, but usually the infection is not apparent. However, a primary infection during pregnancy is placentally transmitted to the fetus and may lead to the cytomegalic inclusion disease, which is associated with splenohepatomegaly, jaundice, hemorrhagic lesions, microcephaly, cerebral calcification, and chorioretinitis. In many such cases, the prognosis is poor. Even in the case of non-apparent infection, the infant may develop hearing loss and intelligence disorder later, which requires long-term follow-up. In addition, such infectious diseases as interstitial pneumonia, hepatitis, encephalitis, retinitis, and enteritis due to CMB are issues of great concern in immunocompromised hosts, including children with primary immunodeficiency

diseases, patients who have had a kidney transplantation or hematopoietic stem cell transplantation (bone marrow transplantation), and those with AIDS or malignant tumors. Many specimens are useful for diagnosing a patient with CMV including serum, plasma, cerebrospinal fluid, ocular aqueous humor, and amniotic fluid, and have great diagnostic significance by providing good indicators of CMV infection activity. Recently, it has been described that the quantification measurement of CMV DNA in peripheral blood is useful for the diagnosis of post-transplantation and congenital CMV infections. Other CMV specimens include urine specimen collected within the first three weeks of life for the diagnosis of congenital CMV infection, broncho-alveolar lavage fluid (BALF) and pulmonary tissue for the diagnosis of interstitial pneumonia, hepatic tissue for the diagnosis of CMV hepatitis, peripheral white blood cells and serum for the determination of activity of systemic CMV infection, cerebrospinal fluid for the diagnosis of encephalitis, and ocular aqueous fluid for the diagnosis of retinitis.

When the nucleic acid amplification test is used for the diagnosis of CMV infection, it is important (i) to take the immune status of the patient into consideration, (ii) to use specimens taken from the infected site as possible, and (iii) to implement quantitative measurements as required.

#### 5.1.1.8 Human Parvovirus B19

Human Parvovirus B19 is a causal virus for infectious erythema, which is accompanied by a rash on the cheek. Seven to 10 days before the development of the rash, a slight fever and common cold-like symptoms are observed in many cases, during which period the viremia is occurring. The prognosis is generally good, but in patients with hemolytic anemia, infection with this virus may cause severe anemic crisis, for which special caution is needed. Otherwise, it is known that this virus causes persistent infection in patients with arthritis, rheumatoid arthritis, thrombocytopenia, granulocytopenia, hemophagocytic syndrome, and immune disorders. In addition, it should be noted that infection during pregnancy has been linked to fetal anomaly (hydrops fetalis) and spontaneous abortion.

Because culture methods for this virus have not been established yet, nucleic acid testing is performed to prove the presence of the pathogen. To perform nucleic acid testing by dot blotting or Southern blotting, it is required that a blood specimen be collected at the period of viremia. The PCR technique allows detection of viral DNA from the serum specimens, at least during the period when the IgM against the virus is detectable. Compared with other viruses, this virus is difficult to inactivate or remove by heat treatment or filtration, and the potential contamination of various plasma-derived products cannot be excluded. In response to each of the cases mentioned above, it is required that biological materials that contain the greatest amount of the virus be collected at the appropriate time. Serum is the standard specimen for the test, but, depending on the clinical symptoms, other specimens including joint fluid, peripheral white blood cells, bone marrow fluid, amniotic fluid, umbilical blood, and blood preparation products can be used.

#### 5.1.1.9 Human papilloma virus: HPV

HPV is currently classified into more than 100 types, based on the base sequence of the HPV genome DNA, and each type can be classified into an epidermal or mucosal type, based on the site of infection. The mucosal type is further classified into the high-risk and the low-risk groups. The high-risk group infects the mucosal epithelia of genitourinary organs and the head and neck area, and is involved in the development of malignant lesions in these areas. The low-risk group is involved in the development of benign lesions, including Condyloma acuminatum and papillomas.

Because HPV culture methods have not yet been established, the virus must be detected by nucleic acid testing. In the high-risk group, specimens are collected together with the cytology specimen at the time of cervical cancer screening by rubbing the cervical wall with a special sampling tool. In Europe and the U.S., liquid-based cytology (LBC) has recently become the mainstream of the screening and is regarded as the most favorable method for specimen preparation, that is, scraped cells are stored suspended in alcohol fixative, and a cytological examination is performed using cytospin specimens prepared from the suspension. In order to examine the involvement of human papillomavirus in the development of cervical cancer, the analyses using PCR and *in situ* hybridization are implemented, using tissue sections excised from cancerous areas. For the diagnosis of the low-risk group, which includes causal viruses for verruca vulgaris and Condyloma acuminatum, PCR and *in situ* hybridization are performed using biopsied skin tissues to confirm the presence and types of the virus.

#### 5.1.1.10 HTLV-I (Human T-Lymphotropic Virus Type I)

HTLV-I is a virus responsible for adult T-cell leukemia/lymphoma (ATLL), HTLV-I associated myelopathy/tropical spastic parapalysis (HAM/TSP), and uveitis of the eye. After the virus infects human T cells, its genome is integrated into the host cell genome in the form of provirus DNA, at which point the infected person becomes a carrier of HTLV-I. It is transmitted through provirus DNA in lymphocytes. One out of 1000 HTLV-1 carriers develops ATL every year. Approximately three out of 100,000 carriers develop HAM every year. The routes of infection are mainly from mother to the newborn infant through breast milk, but other routes include transfusion and, extremely rarely, between husband and wife (mostly from male to female).

Carriers are generally detected by the measurement of serum HTLV-I antibody level, but when the result is unclear and difficult to judge, provirus DNA testing by PCR is performed using genomic DNA extracted from peripheral white blood cells. Highly sensitive provirus DNA testing with PCR is the only tool that can be used to determine whether infants have been infected through breast milk, especially during the period after maternal antibodies have disappeared and until their own antibody titer has risen. However, caution is required in interpreting the results because there can be false-positive results due to contamination as well as false-negative results due to insufficient collection of peripheral white blood cells or inhibition of amplification. In order to examine the cause of such diseases as HAM and

uveitis, which are induced by an increase in the number of infected T cells and in the amount of provirus DNA in some local regions, it is desirable to obtain specimens from sites that reflect the affected lesions. Spinal fluid and ocular aqueous fluid can also be used as specimens. In order to diagnose ATL, it is required to detect, by Southern blotting, proviral HTLV-I DNA that was inserted into the chromosomal genomic DNA of neoplastic T lymphocytes and to prove monoclonal propagation of infected T cells based on its band pattern. The specimens used for this purpose include DNA extracted from abnormal cells in the peripheral blood, affected lymph nodes, or skin tissues.

#### 5.1.1.11 HIV (Human Immunodeficiency Virus)

HIVs (HIV-1 and HIV-2) are the viruses responsible for AIDS (acquired immunodeficiency syndrome). HIV is present in body fluids, including blood, seminal fluid, and vaginal secretions, as well as the tissues or organs of infected people. As the major routes of infection, sexual contact with infected persons, contact with blood contaminated with HIV, and infected mother-to-child transmission are currently known.

Nucleic acid testing to determine the pathogen includes quantification of HIV RNA using nucleic acid amplification techniques, proviral DNA detection, subtype identification, and detection of drug-resistant genes. The quantification of HIV RNA in the blood of patients infected with HIV is useful for the purpose of understanding patients' pathological conditions, monitoring the effects of antiviral chemotherapy, and estimating the progression of the disease. Serum or plasma is used as a test specimen. For the collection of plasma specimens, sodium citrate or EDTA is used as an anticoagulant. The use of heparin should be avoided because heparin inhibits the PCR amplification reaction. In addition, because HIV infects low-density lymphocytes, if the plasma fraction is collected when the buffy coat formation by centrifugation is incomplete, there is a risk for incorporating lymphocytes that have not fully sedimented. Therefore, careful attention is needed when the quantity of viruses is closely monitored. The HIV proviral DNA test is useful for the diagnosis of neonates born from mothers who are HIV antibody-positive, and genomic DNA extracted from peripheral white blood cells is used as the specimen for this test, as in HTLV-1 testing. The plasma HIV RNA level is an indicator for viral replication, and higher RNA levels are linked to the possibility that the number of CD4 positive lymphocytes decreases earlier and that the condition progresses to AIDS development. However, this kind of viral activity cannot be evaluated by a proviral DNA test alone. For drug resistance testing, viral RNA extracted from viral particles in the blood is used.

It is extremely important to identify infected persons in the earlier stages of AIDS development. The period during which the presence of the viruses cannot be detected, even though they are present and can be transmitted to other people through transfusion or other mechanisms, is referred to as the "window" period of infection. While this period is 22 and 18 days for the antibody test and the simultaneous antigen and antibody detection tests, respectively, it is calculated to be 11 days for the nucleic acid amplification test (NAT), thus suggesting that the HIV RNA test and the HIV provirus DNA test are useful for early



diagnosis.

## 5.1.2 Bacterial Infection

### 5.1.2.1 Tuberculosis and Other Mycobacteria

Accurate and rapid diagnosis and differentiation of tubercle bacilli and nontuberculous mycobacteria are each extremely important for selection of the treatment, estimation of the prognosis, and prevention of group infection and nosocomial infection. The smear test allows rapid detection but has low sensitivity and is unable to differentiate between tubercle bacilli and nontuberculous mycobacteria. Culture tests enable the identification of bacterial species and drug resistance testing, but take a long time before the identification can be made. Molecular methods for pathogens, when combined with conventional methods, are extremely useful for rapid diagnosis. Test methods include direct detection from specimens, identification of isolated bacterial species, and identification of drug-resistant genes.

\Sputum is a major specimen, but an enormously diverse array of other specimens is also used. These specimens include broncho-alveolar lavage fluid (BALF), aspirated bronchial mucus, gastric juice, stools, spinal fluid, plural effusion, ascites, urea, purulent materials, abscess walls, granulation tissues, and organs. For highly precise detection, it is important to select the appropriate specimen and to control the quality of the specimen. The selected specimen should be that which most closely reflects the lesion. Sputum is extensively used, and high quality sputum, which reflects a lesion in the lower respiratory tract, must be collected. The purulent portion should be collected while avoiding saliva incorporation. Precautions include collecting the specimens at the time of awakening in the early morning after sufficient gargling, if possible, and collecting specimens before anti-tuberculosis treatments are started. In order to collect high-quality sputum, appropriate instructions for patients and collaboration among the concerned medical staffs are important. At the time of collection, the medical staffs should take certain measures to prevent occupational infection, such as wearing a mask and gloves. Because pleural effusion and other specimens may coagulate after collection due to fibrin deposition, they should be collected in a container that contains sodium citrate or EDTA. The incorporation of blood should be avoided, because it is an inhibitory substance. Specimens collected in sterile containers should be completely sealed to avoid dehydration and leakage to the outside.

### 5.1.2.2 *Legionella*

Legionnaires' disease or legionellosis is a collective term for the infectious diseases caused by bacteria belonging to the genus *Legionella*. These bacteria are roughly classified into the Pontiac fever type, for which the prognosis is generally good, and the pneumonia type (*Legionella pneumoniae*), which often cause severe symptoms. The Pontiac fever type presents common cold-like symptoms including fever, chills, muscle ache, joint ache, fatigue, and headache, but most patients recover within several days. Conversely, the pneumonia type progresses rapidly. After an incubation period of 2-10 days, symptoms begin with general fatigue, headache, and muscle ache in the early stages. Thereafter,

respiratory disease symptoms appear several days later, including a fever exceeding 39 °C, dry or sometimes wet coughing, chest pain, purulent sputum, and difficulty in breathing. These symptoms become severe within 48 hours. Neurological symptoms, such as limb tremors and clouded consciousness, may appear.

In molecular diagnosis of pathogens, such respiratory tract-derived specimens as sputum collected before the administration of antibacterial drugs, broncho-alveolar lavage fluid (BALF), endotracheal aspirate, pleural effusion, and lung tissues are used as with bacterial isolation cultures, but pericardial fluid, spinal fluid, and blood can also be used as the specimens. Because a large amount of blood cells and other cells present in the specimens can inhibit the amplification reaction, in general it is desirable to use broncho-alveolar lavage fluid (BALF), pleural fluid, or endotracheal aspirate. For the identification of the source of infection, specimens from the environment, including (concentrated) water from cooling towers and bath water, and hot-water systems are also checked.

#### 5.1.2.3 *Mycoplasma pneumoniae*

The major diseases caused by this bacterium are bronchitis and pneumonia. Together with chlamydial pneumonia, mycoplasma pneumonia represents a large proportion of primary atypical pneumonia cases, at a frequency of approximately 30-40%. It occurs relatively frequently in younger people, but it has been observed in almost all age groups. In Japan, it used to be called the “Olympic Games pneumonia,” since its outbreak occurred every four years in the country, but now such periodic outbreaks no longer occur. Its complications include skin rash, hemolytic anemia, arthritis, otitis media, meningoenzephalitis, peripheral neuropathy, pericarditis, and constrictive pericarditis. When it is accompanied by central nervous system complications, the disease may become severe.

The specimens that are most commonly used are throat swabs. However, if there are symptoms of complications, other specimens may also be used for the tests, including pleural fluid, spinal fluid, and cellular tissues. When collecting specimens with throat swabs, the cells should be scraped from the posterior wall of the throat using sterile swabs. Because this bacterium adheres to mucosal cells in the throat, it is important to scrape off as many mucosal cells as possible using swabs. This bacterium is susceptible to dryness and refrigeration, so the specimens must not be allowed to dry out. For long-term storage, a storage temperature of -70 °C or below is recommended.

#### 5.1.2.4 *Chlamydia pneumoniae/psittaci*

*C. pneumoniae* is a causal bacterium for bronchitis and pneumonia, but its links with bronchial asthma and coronary artery atheroma have also been reported. The prevalence of antibodies in people increases drastically after the age of 4-5 years, and 50-60% of the adult population is antibody positive. *C. psittaci* causes severe pneumonia (parrot fever, from bird) for which fetal cases have been reported. Based on antibody survey results, it is estimated that approximately 100 people develop this disease in Japan every year. Specimens used for the detection of *C. pneumoniae* include nasopharyngeal/throat swabs,

sputum, broncho-alveolar lavage fluid, and autopsied tissues. Among these, the nasopharyngeal/throat swab is the most commonly used. The specimen is scraped from the nasopharynx, or posterior wall of the throat, by rotating sterile swabs. For nucleic acid testing, the swab is put in the tube as it is and transported at a temperature of 4 °C or is frozen with dry ice. As for *C. psittaci*, human specimens used for nucleic acid testing are similar to those used for *C. pneumonia* testing, while, for obtaining avian specimens, cloacal swabs and feces are collected from living birds, whereas specimens are collected from the liver, spleen, lungs, and intestinal tracts of dead birds that are suspected as the source of infection.

#### 5.1.2.5 *Chlamydia trachomatis*

*Chlamydia trachomatis* is a causal bacterium for sexually transmitted diseases (STD), and approximately 40% of patients with STDs are infected with this bacterium. This bacterium causes urethritis, cervicitis, uterine adnexitis, and intrapelvic infection, as well as neonatal conjunctivitis and pneumonia by birth canal infection. However, many adult patients are asymptomatic, and several percentages of healthy pregnant women are positive for this bacterium.

Specimens from women are collected using uterine cervical swabs. After wiping off the cervical mucus, the cervical cells are scrubbed by turning a sterile swab. After scrubbing, snap the shaft of the swab, leave the swab in the sterile test tube, seal the tube, and transport the specimen at a temperature of 4 °C or under frozen condition with dry ice for nucleic acid testing. The range of infection is wide for females, and there is a possibility that no bacteria will be detected from the uterine cervix even though the patient has a peritoneal infection. In such cases, it is necessary to detect the abnormality from the symptoms and findings from pelvic examination. In the case of male patients, specimens should be taken by scrubbing with a sterile swab inserted into the urethra. However, since nucleic acid testing has a high level of sensitivity, the bacteria can be sufficiently detected even from morning first-voiding urine. The urine specimens should be stored at a temperature of 4 °C and centrifuged as soon as possible, or otherwise cryopreserved at a temperature of -70 °C. When pharyngeal infection is suspected, a specimen should be collected with a throat swab. As for neonatal pneumonia and inclusion conjunctivitis, use nasopharyngeal swabs and eye mucosa swabs, respectively.

#### 5.1.2.6 *Neisseria gonorrhoeae*

*Neisseria gonorrhoeae* is a causal bacterium for STD, and it mainly infects the urethra and the uterine cervix in male and female patients, respectively. Because this bacterium requires carbon dioxide to survive, it cannot survive in most environments. The main route of infection is human-human as an STD. Infection with this bacterium is not restricted to genitourinary organs and pharynx, but is disseminated hematogenously and can cause arthritis, hepatitis, pericarditis, and meningitis, which are referred to as disseminated gonorrheal infections (DGIs).

Specimens to detect this bacterium include purulent urine and discharge from patients with acute urethritis, as well as prostate discharge, urine voided after prostate massage, and vaginal discharge from chronic patients. In suspected DGI patients, bacteria can be detected from blood (especially collected from eruption), knee joint fluid, pharyngeal mucus, and rectal mucus. Thus, specimens should be collected, considering the infectious pathology of this bacterium. Some methods or kits for throat swab testing show the cross-reaction with intraoral bacteria of the *Neisseria* family, so careful attention is required for the diagnosis of gonorrheal pharyngeal infection.

#### 5.1.2.7 MRSA (Methicillin-Resistant *Staphylococcus aureus*)

MRSA is *Staphylococcus aureus* that has become resistant to antibiotics. Although it is sometimes isolated from medical staff, they remain as healthy carriers, and do not develop symptoms. Patients who are susceptible to the development of this disease include elderly people, immunodeficient patients, including premature babies and those with leukemia, those who have undergone gastrointestinal or cardiac surgery, and those with underlying medical conditions, including diabetes, those who are receiving prophylactic antibiotics, are catheterized, or are hospitalized for a long time. When antibacterial agents are administered, substitution of microbial flora is induced. When this happens, MRSA that are colonized to the nasal cavity or throat of the patients can propagate abnormally, sometimes leading to such infectious diseases as sepsis, respiratory infections, or MRSA enteritis. In these patients, bacterial testing is conducted using specimens of blood, sputum, or stools. Otherwise, to identify the carrier state, the presence of nosocomial bacteria, including MRSA and *Pseudomonas aeruginosa*, should be regularly checked. MRSA can be differentiated by confirming the presence of the *mecA* gene by PCR.

### 5.1.3 Fungus Infection

#### 5.1.3.1 Pathogenic Fungi

Among fungal diseases, molecular methods are most often used for the diagnosis of deep-seated mycoses, for which ordinary isolation and culture of the causal fungi is difficult. Most of the deep mycoses observed in Japan, including candidiasis, cryptococcosis, aspergillosis, and zygomycosis, are caused by microbial flora in humans and animals or saprophytes in the environment, which are of low pathogenicity. Therefore, blood, spinal fluid, or biopsied tissues are used as specimens for the detection of these microbes. These specimens are normally germ-free, and usually yield negative results in healthy individuals. If nucleic acids of a certain fungus are detected in these specimens, the subject would be diagnosed as having an overt infection due to the fungus. Most appropriate specimens vary according to the type of disease, for example blood for fungemia, spinal fluid for fungal meningitis, sputum for pulmonary infections, and biopsied liver tissue section for hepatic abscesses. Various patterns of these deep mycoses and of superficial mycoses and deep dermatomycoses can be diagnosed by the use of appropriate specimens, including tissue sections and scrapes of a local lesion and purulent materials.

### 5.1.3.2 *Pneumocystis jiroveci*

Pneumocystis pneumonia is a type of pneumonia caused by the yeast-like fungus *Pneumocystis jiroveci*. In the past, it was called “carinii pneumonia,” since it was regarded as caused by *Pneumocystis carinii*, which used to be classified in the protozoan category. It is rarely observed in people with normal immune functions, and is an opportunistic infection that occurs in immunocompromised people due to acquired immunodeficiency syndrome (AIDS), chemotherapy, long-term internal steroid use, and other causes. Specimens used to detect this fungus include biopsied lung tissues, BALF, and sputum. Many people have non-apparent infections with this fungus. Therefore, even if this fungus is detected by highly-sensitive PCR testing, it does not necessarily mean the person has pneumonia. The results must be interpreted carefully. This fungus strongly adheres to the alveolar epithelium with its pseudopodia, and does not easily come out in the sputum. This makes it difficult to detect the fungus by the conventional staining method. In such cases, nucleic acid testing provides useful and supplementary information.

## 5.2 Collection of Specimens in Molecular Methods for Somatic Cells

### 5.2.1 Solid Tissue Tumors

#### 5.2.1.1 Pancreatic carcinoma

It is known that nearly 90% of pancreatic cancers have a K-ras mutation, most of which are point mutations at codons 12, 13, and 61, especially concentrated around codon 12. Therefore, this method has long been used as a test to complement imaging diagnostics and pancreatic fluid cytology, which have been used for the diagnosis of pancreatic cancer. However, because the highly sensitive detection system also detects similar mutations in non-tumorous lesions, including epithelial hyperplasia of the pancreatic duct, chronic pancreatitis, and pancreatic cysts, such molecular methods cannot be necessarily said to have high clinical significance. Therefore, for clinical diagnosis the results of this testing must be interpreted carefully, and clinical information and the results of other tests must also be considered. Pancreatic fluid collected by endoscopic retrograde cholangio-pancreatography (ERCP) is usually used for the K-ras mutation analyses. In contrast, surgically removed pancreatic cancer tissues are rarely used. In addition to pancreatic fluid, other specimens that are used for the testing are bile, lavage fluid from the opening of the duodenal papilla, pancreatic duct brush cytology specimens, and fine-needle biopsied pancreatic cancer tissue.

#### 5.2.1.2 Lung Carcinomas

The EGFR gene and K-ras gene mutation analyses for lung cancer have been used as an item for gene-based testing to identify malignant tumors. Currently, EGFR mutation analysis for lung cancer is widely utilized to identify the best mode of chemotherapy, to select a treatment method, and as an indicator for drug efficacy assessment. Approximately 40% of pulmonary adenocarcinomas have mutations in the EGFR gene. The mutations are

concentrated around exons 18, 19, 20, and 21, which encode the tyrosine kinase active site of the EGFR protein. Mutations in exons 18, 20, and 21 are primarily point mutations, and the mutation in exon 19 is mainly a deletion. Regarding the relationship between mutations and such molecular-targeted therapeutic agents as Gefitinib and Erlotinib, mutations in exons 18, 19, and 21 are related to chemotherapeutic sensitivity, while mutations in exon 20 are related to chemotherapeutic resistance.

Meanwhile, K-ras mutations in lung cancers primarily show point mutations in codons 12 and 13, which differ from EGFR mutations. However, it is known that drug efficacy is greatly reduced when these mutations are detected. In lung cancers, molecular-targeted therapies are applied for unresectable or recurrent cancers. Thus, surgical specimens collected at the time of resection of the primary lesion or biopsied specimens are primarily used as the specimens for the molecular methods. However, when surgical collection of specimens is difficult, pleural effusions, BALF, pericardiac fluid, exfoliated cells for cytology, or formalin-fixed paraffin-embedded tissue can be used instead.

#### 5.2.1.3 Colon Carcinomas

For many years, the analyses of point mutations and loss of heterozygosity (LOH) in p53 and K-ras genes in colorectal cancer have been conducted using various techniques in colorectal carcinogenesis models, in the evaluation of risk of hepatic metastasis, or as an index for evaluating malignancies. Freshly resected cancer tissues are the most common specimens for this analysis, but in most cases, the purpose of the analysis is investigational rather than diagnostic. Furthermore, investigational attempts to detect these mutations as biomarkers in colorectal cancer cells in patient stool samples have been conducted.

However, this method cannot compete with the fecal occult blood test in terms of cost effectiveness at present.

However, in addition to the fact that molecular-targeted therapy has come to be used for colorectal cancer, it has been reported that the clinical effects of such molecular-targeted therapeutics as Cetuximab and Panitumumab differ significantly, depending on the presence or absence of K-ras mutations.

Surgically-resected cancer tissues and endoscopically-resected biopsy tissues have been used as alternative specimens for mutational testing. However, in the future, formalin-fixed paraffin-embedded tissue is expected to be the only specimen for these analyses in many cases. Therefore, there are important challenges for the future, including the improvement in analytical methods, more careful interpretation of test results, and the development and establishment of new technologies.

#### 5.2.1.4 Gastrointestinal Stromal Tumors (GIST)

C-kit mutation analysis is a gene-based testing method related to gastrointestinal stromal tumor (GIST). Since the publication of a report entitled “c-kit mutation in GIST,” this test has been widely utilized for making judgments on the application of therapeutic agents, for selecting treatment methods, and as an indicator for drug efficacy, as with “EGFR

mutations in lung cancers.” Sixty to eighty percent of GIST cases have a c-kit mutation, most of which (80-90%) lie in exon 11 in various mutational forms, including deletions, point mutations, and insertions. Multiple insertions in exon 9 and point mutations in exons 13 and 17 have also been observed.

Meanwhile, PDGFR $\alpha$  mutations are detected in approximately half of the GIST cases lacking a c-kit mutation, and various gene alterations such as deletions, point mutations, insertions in exons 12 and 18, and point mutations in exon 14 have been found. GIST cases with either of these mutations show good response to some molecular-targeted therapeutics, including Imatinib and Sunitinib. In particular, Imatinib is known to show extremely high clinical efficacy in GIST cases with c-kit mutations in exon 11, and it is suggested that the efficacies differ according to the site and type of genetic alterations. It is also known that the clinical efficacy of this drug is best in GIST cases with c-kit mutations in exons other than exon 11, and the PDGFR $\alpha$  mutation is not as common as that in GIST cases with c-kit mutation in exon 11.

As unresectable or recurrent cases are the target of chemotherapy, specimens used for mutation analysis are primarily comprised of small amounts of biopsied tissues or formalin-fixed paraffin-embedded tissues. Because the site and type of genetic alterations vary widely, as mentioned above, improved analytical methods/techniques should be devised, and great care should be taken when the specimens are collected, because the analysis is difficult without a sufficient amount of high quality DNA.

#### 5.2.1.5 Breast and Stomach carcinoma

Tests for HER2 breast or stomach carcinoma include immunohistochemistry (IHC) which measures the production of the HER2 protein by the tumor, and fluorescence or bright field *in situ* hybridization (BRISH) which uses fluorescence or dinitrophenyl (DNP)-labeled probes, respectively, to look at the number of HER2 gene copies in a tumor cell. A result of HER 2 positive is important, as it indicates that the cancer can be treated with trastuzumab, in combination with other chemotherapy drugs. In metastatic cases with resistance to trastuzumab and HER2 positive, lapatinib can be used.

In preparation for formalin-fixed paraffin-embedded tissues, the recommended fixing solution is 10% neutral buffered formalin, and the standard fixing time at ambient temperature for surgically obtained specimens is between 6 and 48 hours (depending on the size in cases of biopsied tissue). Tissue slices in 6 weeks after cutting can be used.

#### 5.2.2 Hematological Neoplasms

##### 5.2.2.1 Leukemias

Highly sensitive detection using real-time RT-PCR of chimeric mRNA formed by chromosomal translocation is frequently used for routine testing of hematopoietic organ tumors, with *BCR/ABL* chimeric mRNA testing as a typical example. In these diseases, because tumorigenesis occurs at the level of hematopoietic stem cells, bone marrow fluid would be an ideal specimen to ensure the detection of MRD following treatment. However,

considering the burden on the subjects, a highly invasive bone marrow puncture every time the test is conducted should be avoided, and detection of MRD using peripheral blood as the test specimen is more desirable and less invasive for the patient. In *BCR/ABL* chimeric mRNA testing, almost the same level of results are obtained for CML from both bone marrow and peripheral blood specimens, but it must be noted that peripheral blood specimens are not suitable for acute lymphoblastic leukemia (ALL) following treatment.

#### 5.2.2.2 Malignant Lymphomas

Detection of immune-related gene rearrangements by PCR or Southern blotting to diagnose malignant lymphomas, acute lymphoblastic leukemias, and chronic lymphocytic leukemias, has been used.

The primary treatment for malignant lymphoma until recently was surgical resection or chemotherapeutic reduction of tumor-forming lesions of lymph node and extra-nodal tissues. Currently, in the cases of follicular lymphoma with *BCL2/IGH* translocation, the effect of the molecular-targeted antibody Rituximab is a promising adjunct to the standard anti-cancer chemotherapeutic regimen (CHOP chemotherapy). In particular, lymphoma cells of this type characteristically flow into the peripheral blood stream. Thus, peripheral blood can be used as the specimen for the detection of MRD in the efficacy evaluation.

### 5.3 Collection of Specimens in Molecular Methods for Germ Line Cells

#### 5.3.1 Genetic Testing

In a broad sense, molecular methods for germ line cells are defined as “the tests to detect changes in the germline DNA sequence or in the heritable genome sequence, and it includes genetic testing for definitive diagnosis, diagnosis of pathogenic conditions, pre-clinical diagnosis, carrier diagnosis for single gene disorders, a test to determine an individual’s drug response, a test to determine an individual’s susceptibility to diseases (genetic predisposition or diathesis), and a test to determine common parentage in order to identify individuals.”

Among these genetic tests, pre-clinical diagnosis and carrier diagnosis may be implemented for asymptomatic individuals, and the results can be used to predict the individuals’ future health conditions and have implications for the subjects and for their family members.

Therefore, when genetic tests are conducted, personal genetic information must be carefully handled with the ethical, legal, and social challenges in mind. In addition, the genotypic determination test results are permanently maintained as a part of the patient’s medical records, usually without confirmatory retesting. Thus, it is important to provide the appropriate levels of various supports to the subjects and their families before conducting the tests and after the test results are returned. When various kinds of information are provided as the core of the support, cooperation with experts such as medical geneticists or certified genetic counselors will help meet the needs of the subjects, and these professionals can help the patients in an informed manner.

As previously mentioned, for the implementation of genetic tests the specimens should be



collected with due consideration of the characteristics of the test. In addition, various analytical techniques are used in genetic testing, including genomic and gene analysis (nucleic acids; DNA/RNA), various chromosomal analytical technologies (e.g., the CGH array method, the FISH method, and the SKY method), and genetic biochemical methods. Other than DNA extracted from blood (white blood cells), other specimens are used for genetic testing, including skin fibroblasts, amniotic fluid cells, villous cells, umbilical cord blood, B lymphoblast-like cell line, oral mucosa, and oral fluid. The choice of the specimen depends on the purpose of the test. In the field of genetic biochemistry, serum is also used as a specimen.

This manual has covered the genetic tests that use nucleic acids (DNA/RNA), and it focuses on tests “to clarify the genetic information in the genome and mitochondria that the individual inherently possesses and will essentially not change for lifetime (information that can be unraveled by germline genetic analysis), including genetic testing related to single gene diseases, multifactorial disorder, efficacy / adverse effect / metabolism of drugs, and personal identification.”

### 5.3.2 Single Gene Disorders

Hereditary disease testing is a typical genetic test for single gene diseases and inborn errors in metabolism. When these hereditary disease tests are conducted, the scientific validity of the tests (analytical validity, clinical validity, and clinical utility) and the ethical, legal, and social issues associated with the testing must be upheld. Therefore, when specimens for the tests are collected, the subject’s human rights and concerns must be considered. The main materials used as specimens for hereditary disease testing include peripheral blood (white blood cells), cultured cells, and oral mucosa.

- (1) Hereditary disease testing is implemented when the patient is suspected to have either of the following genetic diseases.
  - (i) Duchenne muscular dystrophy
  - (ii) Becker muscular dystrophy
  - (iii) Fukuyama-type congenital muscular dystrophy
  - (iv) Dystrophic epidermolysis bullosa
  - (v) Familial amyloidosis
  - (vi) Congenital long QT interval syndrome
  - (vii) Spinal muscular atrophy
  - (viii) Congenital cerebral white matter atrophy
  - (ix) Mucopolysaccharidosis type I
  - (x) Mucopolysaccharidosis type II
  - (xi) Gaucher’s disease
  - (xii) Fabry’s disease
  - (xiii) Pompe’s disease
  - (xiv) Huntington’s disease
  - (xv) Spinal and bulbar muscular atrophy

- (2) The genetic disease tests listed from (i) to (viii) of (1) are performed by the PCR method, by DNA sequencing, the FISH method, or by Southern blotting. Those listed from (ix) to (xiii) are performed by enzyme activity measurements, by DNA sequencing, or the culture method. That listed in (xiii) of (1) is performed by the PCR.
- (3) These tests must be conducted in compliance with the requirements specified in “Guideline for the Appropriate Handling of Personal Information by Medical and Nursing Care Related Operators,” issued by MWHL in December 2004, and “Guideline on the Genetic Testing,” issued by concerned societies in August 2003.

### 5.3.3 A pharmacogenomic test

Invader *UGT1A1* molecular assay is a pharmacogenomic test that identifies patients with polymorphism associated with an increased risk of neutropenia after use of Camptosar (irinotecan). This was approved for marketing and medical care charge in the health insurance scheme by MHLW in June and November 2006, respectively.

## 6. References

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## 7. Appendix A

### Terms and Abbreviations Used in this Report

- 1) SNPs (single nucleotide polymorphisms)
- 2) PGx (pharmacogenomics)
- 3) HAV (hepatitis A virus)
- 4) HBV (hepatitis B virus)
- 5) HCV (hepatitis C virus)
- 6) HDV (hepatitis D virus)
- 7) HEV (hepatitis E virus)
- 8) EBV (epstein-barr virus)
- 9) CAEBV (chronic active EBV infection)
- 10) CMV (cytomegalovirus)
- 11) HSV (herpes simplex virus)
- 12) HPV (human papilloma virus)
- 13) HTLV-I (human T-lymphotropic virus type I)
- 14) HIV (human immunodeficiency virus)
- 15) MRSA (methicillin-resistant *Staphylococcus aureus*)
- 16) ERCP (endoscopic retrograde cholangio-pancreatography)
- 17) LOH (loss of heterozygosity)
- 18) TUNEL: TdT-mediated dUTP-biotin nick end-labeling
- 19) GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
- 20) DTT: dithiothreitol
- 21) *G* (gravitational acceleration)
- 22) EMR (endoscopic mucosal resection)
- 23) MRD (minimal residual disease)
- 24) BALF (broncho-alveolar lavage fluid)
- 25) CSF (cerebrospinal fluid)
- 26) DGI (disseminated gonorrhoeal infection)

## 8. Appendix B

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