

Chapter 2

Tissue Sample Preparation for Biomarker Discovery

Yoshiyuki Suehara, Daisuke Kubota, and Tsuyoshi Saito

Abstract

Global protein expression studies, an approach known as “proteomics,” can offer important clues for understanding tumor biology that cannot be obtained by other approaches. Proteomic studies have provided protein expression profiles of tumors that can be used to develop novel diagnostic and therapeutic biomarkers. In this chapter, we describe the strategy and design of proteomic studies, as well as the protocols for tissue sample collection and preparation for biomarker discovery, especially tumor biomarkers, followed by a few examples of our recent proteomic studies.

Key words Proteomics, 2D-DIGE, Tissue samples preparation, Soft tissue sarcomas

1 Introduction

The strategies and protocols used for human tissue sample preparation for developing biomarkers, especially tumor biomarkers, by proteomic approaches are described in this chapter. Proteomics is the study of global protein expression, and the phrase “expression profile” refers to the expression of thousands of individual proteins simultaneously in a given tissue sample (1–3). Unlike studies of a single protein or pathway, proteomic methods enable a systematic overview of the expressed protein profiles, which, in the case of tumors, can ultimately improve the diagnosis, prognosis, and management of patients by revealing the protein interactions affecting the overall tumor progression (1–3). Furthermore, a differential protein expression analysis can be used to compare tumors with normal tissues or high-grade with low-grade malignant tumors, which may implicate a range of protein biomarkers potentially indicative of disease and the prognosis of the disease (1–3).

Recent developments in high-throughput screening techniques, such as array-based comparative genomic hybridization analysis and cDNA microarray technology, now allow for the screening of several thousand DNA and mRNA sequences in tumors at once, thereby making it possible to identify the genes

relevant to the histological diagnosis and also the clinical features of the tumors (1–3). However, DNA sequencing or measurement of the mRNA expression alone cannot detect the posttranslational modifications of proteins, such as phosphorylation, glycosylation, or differences in protein stability, and these factors play important roles in the malignant behavior of tumors (1–4). Global protein expression studies, known as proteomics, have a big advantage over global DNA or mRNA expression studies for detecting the post-translational modifications (1). Furthermore, the results obtained from proteomic studies will be more easily applicable to the clinical field, because the protein expression levels can be easily evaluated and confirmed using antibodies, such as Western blotting and immunohistochemistry, and these tests are both useful and convenient in the clinical setting (1). The proteomic approaches have already been used to develop molecular subclassifications and diagnostic biomarkers for several kinds of cancers (1–3). In addition, our proteomic technologies have identified candidate proteins associated with a differential diagnosis (3–6), predictive prognosis, (7–9) and the response to chemotherapy (10) in bone and soft tissue sarcomas.

With regard to human tissue sample preparation for biomarker discovery, the most important matters are that investigators must take special care to (1) use high-quality samples and/or proteins (avoiding degradation, dephosphorylation, and contamination) and (2) ensure that the sample received an exact and correct diagnosis by experts (histology, grade, and stage) and (3) that the clinical information can be followed completely (time of metastasis, time of death and disease status). To ensure that these three points are all met, we believe that collaboration between researchers and clinicians, including pathologists and surgeons, is critical for studies involving biomarker discovery and development (Fig. 1). Researchers may know the details of the techniques and skills for proteomics and how to extract proteins, while pathologists can collect surgical tissue samples and diagnose them, and surgeons can collect not only the samples but also the required clinical information. Furthermore, clinicians have a better understanding of what may improve the clinical outcome for the patients, and they can suggest the types of biomarkers that can be used to optimize existing therapeutic protocols based on their experience. Therefore, the collaboration of basic and clinical scientists can allow for the evaluation of the results from a more relevant clinical perspective or can therefore help to more effectively select biomarkers that can best benefit patients.

To discover useful biomarkers from surgical tissue samples by global protein expression studies (proteomics), it is necessary to conduct high-integrity and reliable studies which consist of three sets (Fig. 2): (1) a discovery set that tries to identify the candidate biomarkers from the global protein expression profiles of tissue

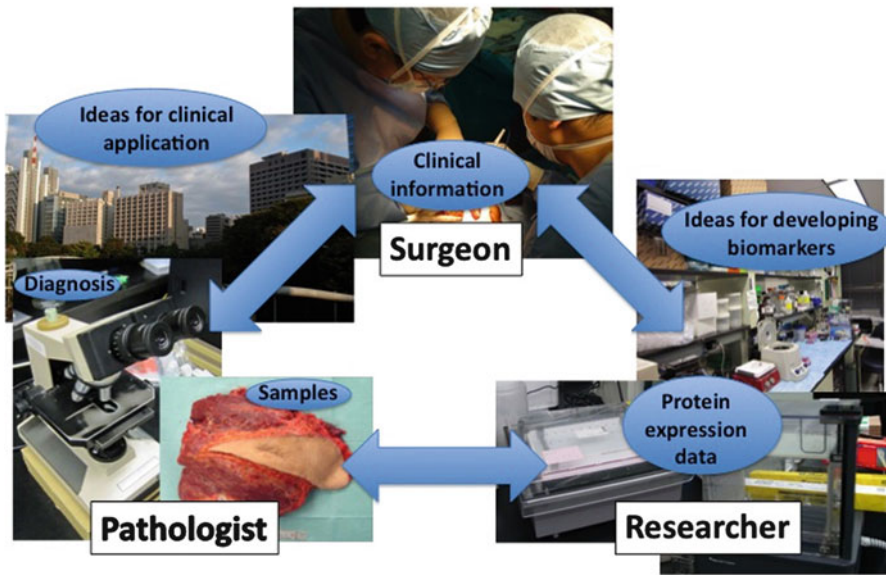


Fig. 1 Collaboration between basic researchers, pathologists, and surgeons for developing biomarker. The collaboration between researchers and clinicians, including pathologists and surgeons, is critical during biomarker discovery development. While researchers may know the details of the techniques and skills for proteomic studies, the pathologist can collect surgical tissue samples and diagnose the samples, and surgeons can collect samples and the required clinical information. Furthermore, clinicians often have a better understanding of what may improve the clinical outcome for the patients

samples (in our studies, we usually use two-dimensional difference gel electrophoresis (2D-DIGE) and mass spectrometry (MS) for these studies), (2) a confirmation set that is used to confirm the protein expression differences identified in the discovery set using other proteomic tools (in our studies, we usually use a Western blot analysis), and (3) a validation set that is used to verify the power and ability of a biomarker on a large scale using numerous samples, to develop a biomarker for clinical applications (in our studies, we generally use immunohistochemistry and Western blot analyses).

The first procedure involving the discovery set is to acquire protein expression profiles from tissue samples by 2D-DIGE, to list the candidate protein spots, and so on based on the expression data, and to identify proteins by MS. Second, the confirmation set is to substantiate the protein expression levels in the results of the discovery set. A Western blotting analysis employing specific antibodies against the candidate proteins is a useful and efficient confirmation tool for this step. Finally, the purpose of the validation set is to prove and establish the abilities and capabilities (as well as limitations) of biomarkers, in order to develop useful

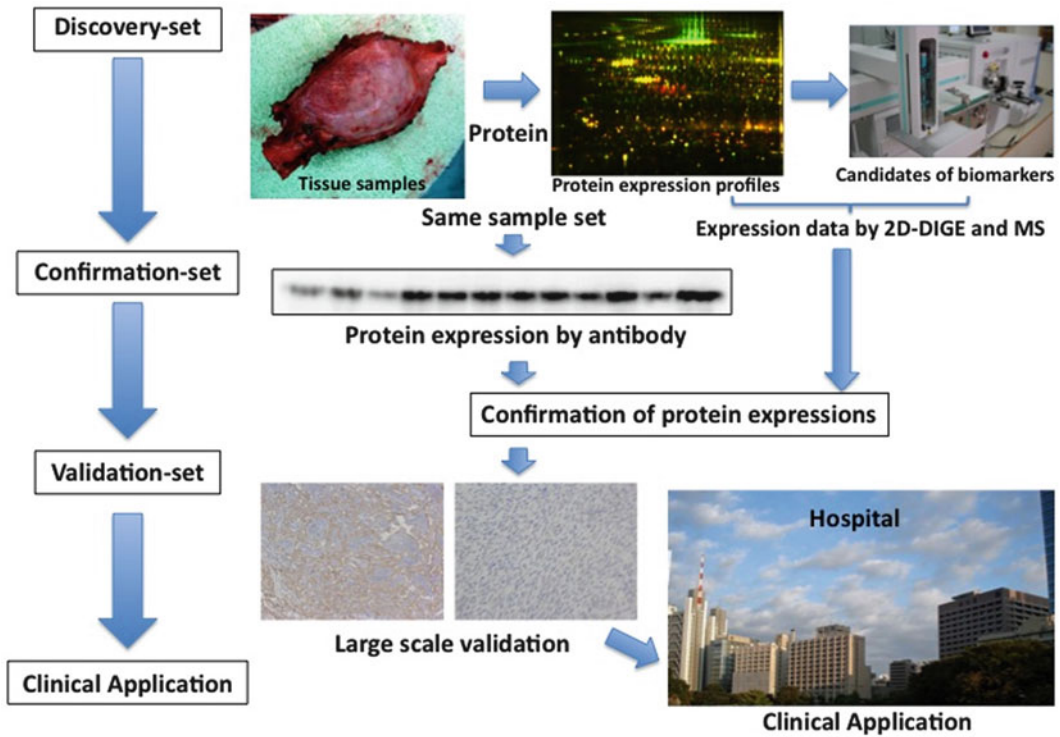
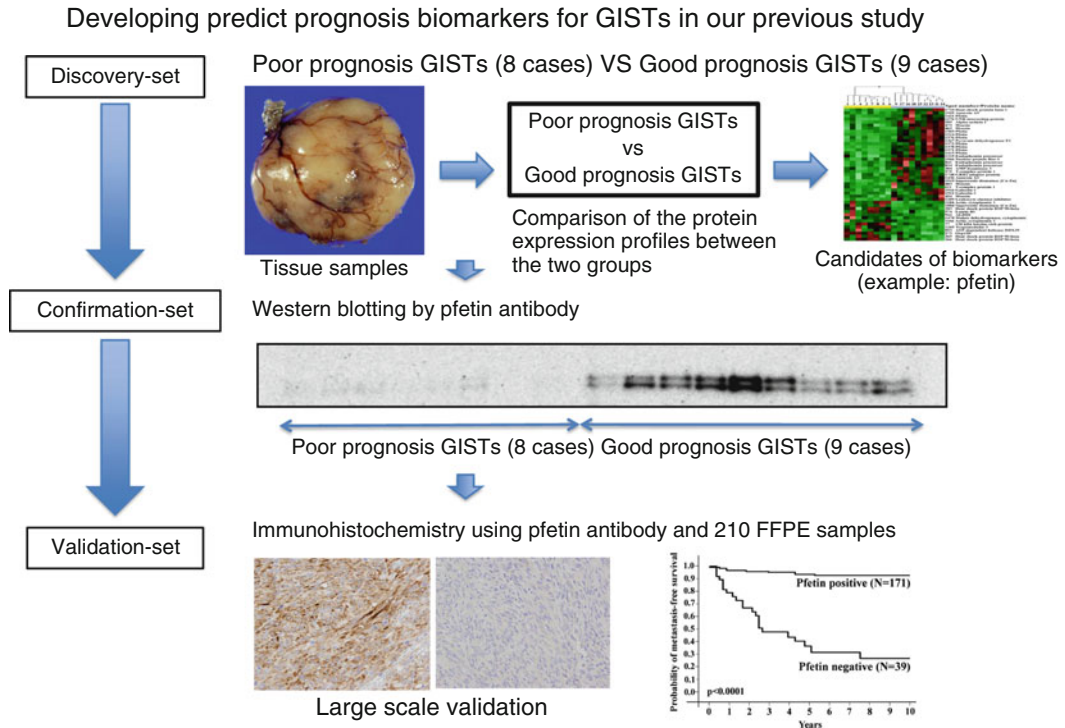


Fig. 2 The strategy for developing biomarkers using tissue samples. To discover and identify useful biomarkers using surgical tissue samples by proteomics studies, it is necessary to conduct high-integrity and reliable studies which consist of three sets: (1) a discovery set, (2) a confirmation set, and (3) a validation set. In our study, the discovery set is used to try to identify candidate biomarkers from global protein expression profiles of tissue samples using 2D-DIGE and MS. The confirmation set is used to try to confirm the protein expression of the discovery set by other techniques, such as a Western blot analysis. The validation set is used to verify the power and ability of a biomarker for predicting the disease using a large number of samples (e.g., FFPE samples), and often involves immunohistochemistry

biomarkers for clinical applications. As the validation set should include a large number of samples, we usually use immunohistochemistry for the confirmation of the expression changes. The reasons for this are (1) that the hospitals usually stock and store many more formalin-fixed paraffin-embedded (FFPE) than frozen tissue samples and (2) pathologists use FFPE samples to diagnose the patient's disease as part of their routine clinical assessment.

To provide an example of such studies, we used 2D-DIGE to investigate the tissue profiles and identify novel prognostic biomarkers in patients with gastrointestinal mesenchymal tumors (GISTs), which is one of the mesenchymal tumors of the gastrointestinal tract and has been shown to exhibit a broad spectrum of clinical behaviors (Fig. 3) (7). As our discovery set, we conducted



Suehara, Y. Clin Cancer Res 2008 14: 1707-1717

Fig. 3 The development of prognostic biomarker for GISTs (7). We previously performed a study to investigate the protein expression profiles and identify novel prognostic biomarkers in GISTs. As our discovery set, we conducted a proteomic study of GISTs using 2D-DIGE, and extracted proteins from surgical tissues samples. To identify the protein expression profiles that correlated with the prognosis of GISTs, we compared the protein expression profiles between two groups (eight poor prognosis patients vs. nine good prognosis patients) using 2D-DIGE. We identified that the pftin protein was more highly expressed in samples from the good prognosis group. As our confirmation set, we confirmed the pftin expression using the Western blot analyses. As our validation set, these results were validated based on immunohistochemical studies of the pftin expression in 210 FFEP samples. There were statistically significant differences in the overall survival of GISTs patients between those who were pftin positive and those who were negative ($p < 0.0001$)

a quantitative expression study of the proteins in GIST samples using 2D-DIGE. These protein samples were extracted from frozen tissues of GIST surgical samples. To identify the protein expression profiles that correlated with the prognosis of the GISTs, 17 GIST samples were divided into two groups (eight poor prognosis patients and nine with a good prognosis). We then compared the protein expression profiles between the two groups using 2D-DIGE. We identified that the pftin protein had different intensities in the two groups of samples, with higher intensity expression in the good prognosis group. As our confirmation set, we confirmed that the pftin expression was higher in the good prognosis group than in the poor prognosis group using a Western blot analysis.

Table 1
Optimal samples for proteomics research to develop biomarkers

Sample type	Tool	Frequency in use
Frozen tissue sample	2D-DIGE, MS, ELISA, WB	Discovery set > confirmation set > validation set
Formalin-fixed paraffin-embedded (FFPE)	IHC (includes TMA)	Validation set > confirmation set > discovery set

2D-DIGE two-dimensional difference gel electrophoresis, *MS* mass spectrometry, *ELISA* enzyme-linked immunosorbent assay, *WB* Western blot, *IHC* immunohistochemistry, *TMA* tissue microarray

Furthermore, as our validation set, the results were validated based on the immunohistochemical studies of the pftet expression in 210 FFEP samples of GIST.

For proteomics studies intended to develop biomarkers for useful clinical applications, we employ frozen tissue samples as well as FFPE samples (Table 1) (1, 3–11). The frozen tissue samples are most suitable for acquiring the global protein expression profiles in the discovery set, as the tissue samples can provide high-quality proteins, which yield more accurate protein profiles. The extracted proteins are also used for the confirmation set. In the validation set, frozen tissue samples can sometimes be employed; however, it is generally difficult to prospectively collect a sufficiently large number of frozen tissue samples for large-scale analyses required for validation. On the other hand, FFPE samples are accumulated during routine clinical work for diagnosis, and a sufficient number of samples are likely to be stored to allow for a retrospective analysis. Therefore it is easier to collect FFPE than frozen tissue samples making FFPE samples the most commonly used type of sample for the validation set.

In the studies of biomarker discovery for humans, it is necessary to employ human tissue samples, especially patient samples, which can be obtained from resected surgical or biopsy materials (Fig. 4). In order to obtain high-quality proteins, it is necessary to recover the surgical material promptly from the operating room, and to collect the tissue samples from the fresh surgical materials before fixation with formalin. If tissues cannot be recovered promptly, the materials should be preserved at an optimal temperature (we recommend 4 °C). As, naturally, the clinical diagnosis for patients has first priority in the samples, it is necessary to collect representative parts of the materials after the pathologist has already secured sufficient information and samples for the diagnosis. It is also helpful to work with the pathologist to ensure that high-quality and representative samples of the tissue are collected. Therefore, investigators should be in close collaboration with pathologists.

With respect to sampling, all studies have to be permitted by the institutional review board. When collecting tissue samples from

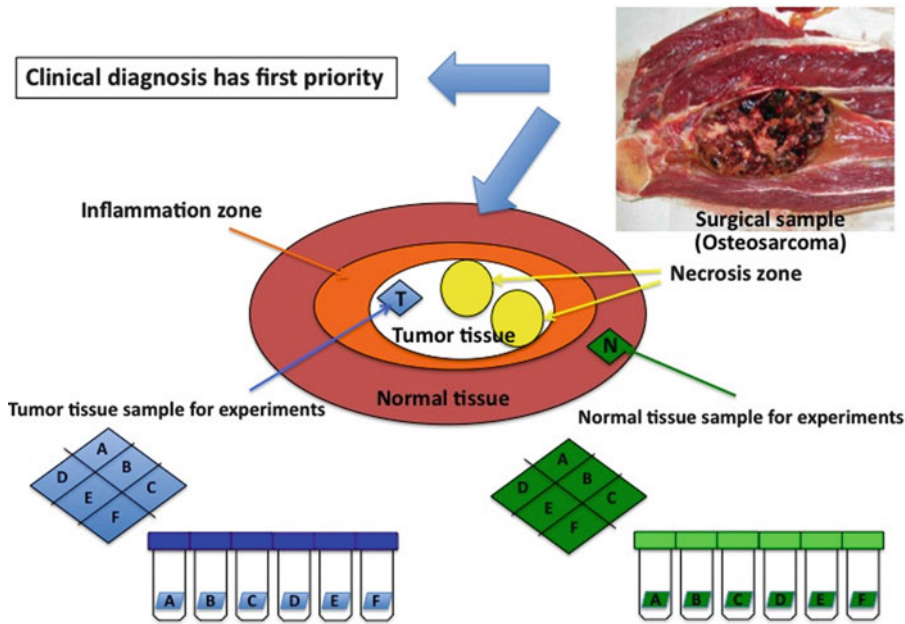


Fig. 4 Tissue sample acquisition from surgical samples and sample storage. Naturally, the clinical diagnosis for patients has first priority with regard to the tissue samples. During sampling, researchers have to collect representative parts of the materials and different tumor locations, and avoid acquiring samples from normal tissues, necrotic areas, and reactive (inflammation) zones. The samples should be cut into 5 mm blocks, and divided samples should be placed in individual tubes with detailed labels

surgical materials, it is necessary to collect representative tumor sections, and to avoid acquiring samples from normal tissue, or necrotic and reactive (inflammatory) zones. Sampling errors are the most frequent cause of noise contamination of data, and interfere with the analyses and identification of proteins specifically expressed in the tumor. If stored samples that were collected previously will be used, it is necessary to check the sample quality and morphology before using the tissue for any analysis. Additionally, when developing biomarkers, it is sometimes necessary to compare tumor samples and normal samples. Therefore, if permission is given by the patient and the institution, then both normal and tumor samples should be collected and stored. For sample storage, the samples should be cut into 5 mm blocks, and divided samples should be placed in the each labelled tube separately. When the tissue samples have contamination due to either blood or other materials, then all samples should be rinsed in clean PBS prior to storage. The tubes should be stored in liquid nitrogen or -80°C until they are used. Additionally, special care should be taken to avoid contamination during all of the steps in each of the procedures.

Table 2
Proteomics for developing biomarkers

Purpose of experiment	Tool(s)	Sample source(s)
To acquire global protein expression profiles	2D-DIGE, MS	Frozen tissue sample
To confirm expression of identified proteins	ELISA, WB	Frozen tissue sample
To confirm expression of identified proteins	IHC (includes TMA)	Formalin-fixed paraffin-embedded (FFPE)

2D-DIGE two-dimensional difference gel electrophoresis, *MS* mass spectrometry, *ELISA* enzyme-linked immunosorbent assay, *WB* Western blot, *IHC* immunohistochemistry, *TMA* tissue microarray

FFPE samples are usually managed and stored by a pathologist. Pathologists usually have detailed information about the FFPE samples. Therefore, supervision by a pathologist is necessary when an investigator has to choose a representative FFPE specimen from all the available tumor specimens. Unstained slides of the representative sample at an optimal thickness (we recommend 3–5 μm sections) should therefore be prepared to conduct more efficient validation studies.

To obtain global protein expression profiles (discovery studies), proteomic studies employ electrophoresis, mass spectrometry, and protein microarrays for the characterization of proteins (Table 2). These proteomic tools have their own individual advantages and limitations, affecting their ability to assess the protein profile. With regard to proteomic analyses to identify biomarkers as part of cancer research, electrophoresis (specifically 2D-DIGE) and MS (especially surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and liquid chromatography–mass spectrometry (LC/MS)) have mainly been used to obtain protein expression profiles. In our studies, we primarily employ 2D-DIGE, because this technology is the most frequently used method for examining protein expression profiles in proteomic studies of biomarkers (1, 3–11). When a specific target has already been identified (in the confirmation and discovery studies), proteomic studies employing a Western blot analysis, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry are common. These tools use specific antibodies against the target protein(s). In our studies, 2D-DIGE, MS, WB, and ELISA all detect proteins that are extracted from frozen tissue samples, while immunohistochemistry is used mainly for FFPE samples.

The procedure used for protein extraction from tumor or normal tissue is important to obtain precise and intact protein

expression profiles for the proteomic analysis (1–3, 12). To prevent the degradation and dephosphorylation of the protein, it is essential to preserve the analytical quality during extraction. It is also necessary to maximize the reproducibility and to minimize waste. Containment of the sample is important to avoid the risk of contamination to and from each sample. Either the ionic detergent SDS or the chaotrope, urea, provides efficient ways to solubilize water-insoluble protein subunits.

2 Materials

2.1 Tissue Sample Preparation

1. Surgical materials: The use of surgical specimens must be approved by the institution's ethics committee, and written informed consent obtained from all of the patients.
2. Multi-beads shocker (Yasui Kikai, Osaka, Japan).
3. High speed refrigerated microcentrifuge MX-100 (TOMY Digital Biology, Tokyo, Japan).

3 Methods

3.1 Protocol for Sampling and Storage

1. Weigh the frozen clinical tissue samples.
2. Wash these samples twice with PBS at room temperature (see Note 1).
3. Cut the tissues into appropriately small pieces (see Note 2).
4. Place the cut pieces in tubes (see Note 3).
5. Store tissues in liquid nitrogen or -80°C until they are used.

3.2 Protocol for Protein Extraction

1. Transfer the frozen sample to a new special collection tube that is provided with the Multi-beads shocker (Fig. 5a) (see Note 4).
2. Crush the frozen tissue to powder with the Multi-beads shocker after cooling with liquid nitrogen at $250\times g$ for 20 s (Fig. 5b) (see Note 5).
3. Add urea-lysis buffer (6 mol/L urea, 2 mol/L thiourea, 3 % CHAPS, and 1 % Triton X-100) to the liquid nitrogen pools (Fig. 5c, d) (see Note 6).
4. Incubate on ice for 30 min (Fig. 5e) (see Note 7).
5. Centrifuge at $17,400\times g$ for 30 min at 4°C (Fig. 5f, g) (see Note 8).
6. Collect the supernatant, and place it in a new collection tube (Fig. 5h).
7. Determine the concentration of the total proteins (see Note 9).

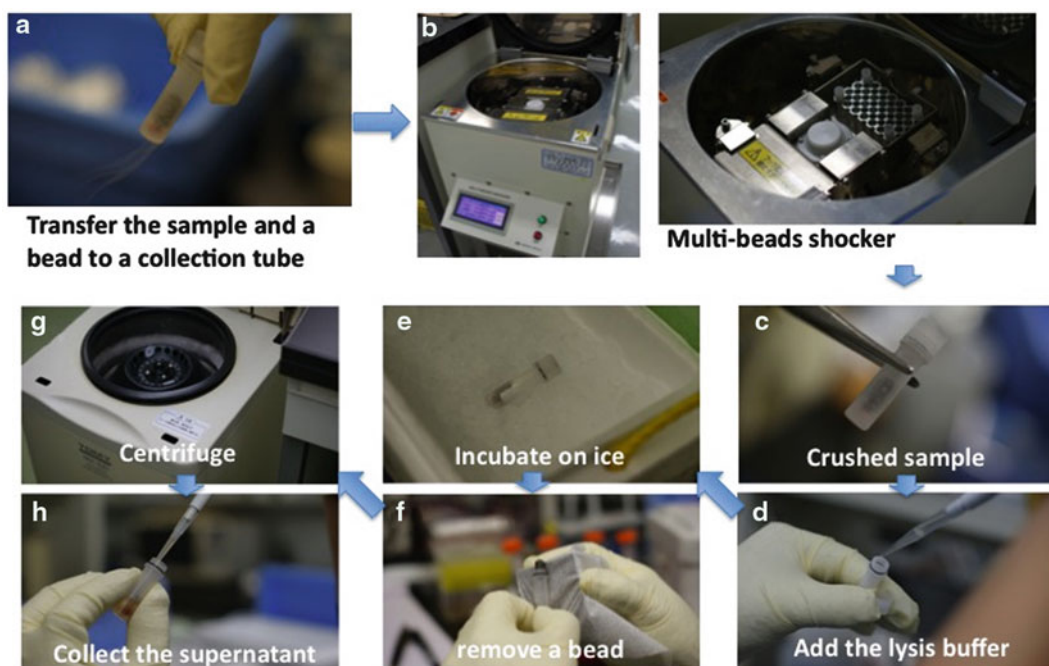


Fig. 5 Protocol for protein extraction. **(a)** The frozen sample is transferred with a bead to a new special collection tube that is provided with the Multi-beads shocker under cooling with liquid nitrogen. **(b)** The frozen tissue is crushed to powder with the Multi-beads shocker under cooling with liquid nitrogen at $250\times g$ for 20 s. **(c)** Crushed sample. **(d)** The urea lysis buffer is added to the liquid nitrogen pools. **(e)** Incubate on ice for 30 min. **(f)** The bead is removed from the tube. **(g)** The tubes are centrifuged at $17,400\times g$ for 30 min at 4°C . **(h)** The supernatant is collected and placed in a new collection tube

4 Notes

1. The purpose of such washing is to remove any excess serum from the harvested samples by this procedure.
2. Usually, the sample should be about the size of a grain of rice (5 mm in diameter).
3. We recommend the use of tubes designed for the storage of biological materials, human or animal cells, at temperatures as low as -190°C (e.g., Cryotubes, Cryogenics). Label the details of the samples on these tubes. Make a list and data sheet for the samples to organize them during both storage and analysis.
4. Be careful to prevent any potential contamination. Place an optimal amount of frozen tissue in each tube.
5. During crushing, be careful not to dissolve the frozen sample. Be careful to avoid potential contamination caused by the cracking of the tubes.

6. The amount is adjusted according to the size of the sample (50–500 μL). Be careful to avoid contamination.
7. Mix gently two or three times during this incubation. Wait until the sample is completely dissolved, even if it takes more than 30 min. Be careful to avoid potential contamination.
8. Remove the bead from the tube before the procedure of centrifuge.
9. If the concentration is too high (over 50 $\mu\text{g}/\mu\text{L}$), dilute using urea-lysis buffer.

Acknowledgments

This work was supported by a grant from the Japan Society for the Promotion of Science (JSPS), a science Grant-in-Aid for Young Scientists B, No-22791405. The authors appreciate critical comments and support from Dr. Makoto Endo and Dr. Eisuke Kobayashi.

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Proteomics for Biomarker Discovery

Zhou, M.; Veenstra, T. (Eds.)

2013, XI, 320 p. 57 illus., 34 illus. in color., Hardcover

ISBN: 978-1-62703-359-6

A product of Humana Press