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Position Paper

European Organisation for Research and Treatment of Cancer (EORTC) Pathobiology Group standard operating procedure for the preparation of human tumour tissue extracts suited for the quantitative analysis of tissue-associated biomarkers

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ABSTRACT

With the new concept of 'individualized treatment and targeted therapies', tumour tissue-associated biomarkers have been given a new role in selection of cancer patients for treatment and in cancer patient management. Tumour biomarkers can give support to cancer patient stratification and risk assessment, treatment response identification, or to identifying those patients who are expected to respond to certain anticancer drugs. As the field of tumour-associated biomarkers has expanded rapidly over the last years, it has become increasingly apparent that a strong need exists to establish guidelines on how to easily disintegrate the tumour tissue for assessment of the presence of tumour tissue-associated biomarkers. Several mechanical tissue (cell) disruption techniques exist, ranging from bead mill homogenisation and freeze-fracturing through to blade or pestle-type homogenisation, to grinding and ultrasonics. Still, only a few directives have been given on how fresh-frozen tumour tissues should be processed for the extraction and determination of tumour biomarkers. The PathoBiology Group of the European Organisation for Research and Treatment of Cancer therefore has devised a standard operating procedure for the standardised preparation of human tumour tissue extracts which is designed for the quantitative analysis of tumour tissue-associated biomarkers. The easy to follow technical steps involved require 50–300 mg of deep-frozen cancer tissue placed into small size (1.2 ml) cryogenic tubes. These are placed into the shaking flask of a Mikro-Dismembrator S machine (bead mill) to pulverise the tumour tissue in the capped tubes in the deep-frozen state by use of a stainless steel ball, all within 30 s of exposure. RNA is isolated from the pulverised tissue following standard procedures. Proteins are

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extracted from the still frozen pulverised tissue by addition of Tris-buffered saline to obtain the cytosol fraction of the tumour or by the Tris buffer supplemented with the non-ionic detergent Triton X-100, and, after high-speed centrifugation, are found in the tissue supernatant. The resulting tissue cell debris sediment is a rich source of genomic DNA.

1. Introduction

Clinical features and histomorphological factors have been used traditionally to predict individual cancer patient outcome or direct cancer treatment.[1,2] With the new concept of 'individualized treatment and targeted therapies', tumour-associated biomarkers, present in the tumour tissue or released into the blood, have been given a new role in selection of cancer patients for treatment and in cancer patient management.[3,4] Tumour biomarkers can give support to cancer patient stratification and risk assessment, treatment response identification, or to identifying those patients who are expected to respond to certain anticancer drugs.[5–7] Owing to high-throughput technologies, gene-, protein-, and tissue-arrays, we are now faced with various 'cancer gene signatures' rather than with single tumour markers.[8–20]

This scenario of multiple biomarkers requires novel methods for the simultaneous assessment of tumour tissue biomarkers; quality assessment and quality assurance is also an important issue to consider as well as state-of-the-art statistics to evaluate and validate the tumour biomarkers in question.[21–36] Since there is the need for a methodical development process to bring a tumour biomarker from the bench to the bedside, well designed and controlled biomarker studies have to be conducted to allow validation of the prognostic or predictive ability of the tumour biomarkers. Only then, a new tumour biomarker can be recommended for clinical routine application.[35]

Considering that a tumour tissue-associated biomarker can be DNA-, mRNA-, or protein-based, the recently reported advances in gene and protein array technology are having a considerable impact in this field. Gene- and protein-expression profiling can help distinguish between patients at high risk and those at low risk for developing distant metastases, and so identify patients who will benefit from chemotoxic or endocrine therapy, or immunotherapy. Despite major technological inventions and improvements, a number of important issues still remain regarding the clinical utility of gene and protein expression profiling, including differences in study design, patient selection, array technology or methods of analysis, but also concerning the choice, source, and attributes of the piece of tumour tissue to be assessed.[4]

One should be aware that assay results can be heterogeneous, depending on the nature and composition of the tumour tissue specimen, the way of tissue processing, biomarker extraction procedure, and design and specificity of the biomarker assay.[35] Also, tumour tissue-associated biomarkers may occur in different molecular forms and such variant molecular forms may be present in different areas of the section of tumour tissue being investigated.[37,38] Importantly, if the biomarker extraction methodology is uncontrolled and not standardised, the information based on the biomarkers may also be incorrect and of no use for clinical decision-making.[39,40] The EORTC PathoBiology Group therefore takes notice of the fact that it is highly necessary to establish a standardised criterion (standard operating procedure) for the disintegration of tumour tissue in its frozen state and its extraction procedure to allow the quantitative assessment of tumour tissue-associated biomarkers.

2. Factors to consider in tumour tissue disruption methods

Cells and tissues must be disrupted by mechanical force or lysed by hydrolytic enzymes or detergents to uncover cell-associated and tissue-linked molecules. However, several issues must be considered in selecting the best method for tumour cell/tissue disintegration and tumour biomarker extraction. For cultured tumour cells and small-size tumour tissue specimens (ca. 50–300 mg wet weight), often simple mechanical or chemical/enzymatical methods are satisfactorily used. Although enzymatic methods are easy to use, the disadvantages of the enzymatic methods are low reproducibility and that the enzyme must be removed or inactivated to permit quantitative detection and/or isolation of the desired tumour biomarker molecule.

Mechanical methods have the advantage of being applicable to small or large sample volumes, and several different apparatuses have been successfully applied to biological samples difficult to disrupt (Table 1). Disruption methods may vary considerably in the severity of the disruption process, the equipment and/or reagents needed, and in ease of use. The practical aspects of mechanical cell/tissue disruption, from bead (or ball) mill homogenisation and freeze-fracturing through to grinding, mechanical blade homogenisation and ultrasonics were discussed by Hopkins[41,42]; further basic information can be found on the internet.[43]

Laboratory-scale mechanical methods for cell disruption and tissue disintegration use glass, ceramic, tungsten, or stainless steel beads which are added to cells or tumour tissue in the native or frozen state and exposed to high level agitation. The simplest method is to place the beads and cells/tissue into a tube and then this suspension is mixed on a laboratory vortex mixer. At the more sophisticated level, bead-based methods use a closed flask (or vials) holding the cells/tissue and the beads while providing vigorous agitation with an electric motor. As the sample heats significantly owing to the extreme agitation, some form of cooling is necessary.

The Mikro-Dismembrator S (Sartorius AG, Goettingen, Germany) uses a shaking flask which is pre-cooled in liquid

Table 1 – Overview on apparatuses and technologies of tissue and cell disruption

Apparatus	Technology	Remarks
Bead mill homogenisers	0.1–2.5 mm ∅ glass or ceramic beads added to native cells or tissue in a test tube. Crushing action of the beads as they collide with the cells during vigorous agitation (vortexing)	Works successfully with tough to disrupt native cells or tissue. This handheld method can be slow and tedious
Shaking-type bead mills	Acts through a shaking device holding a pre-cooled shaking flask. Shaking flasks available which hold up to four cryogenic tubes into which deep-frozen tumour tissue and a grinding stainless steel ball of 9 mm ∅ are placed which during the shaking process will crush the deep-frozen piece of tissue into a fine but still frozen powder	Works best with small pieces of deep-frozen tissue (50–300 mg) which is pulverised in the cold state within 30 s. Fixed, paraffin-embedded tissue should not be processed this way
Rotor-type bead mills	Larger capacity bead mill cell disrupters agitate the beads using a rotor rather than by shaking. Equipped with efficient cooling jackets, larger sample volumes can be processed without overheating	Disrupts between 15 and 250 mL of cell suspension or tissue volume in 3–5 min
Rotor-stator homogenisers	The cell suspension or tissue is drawn into the apparatus by a rotor sited within a static tube (stator) and is then thrown centrifugally outward to exit through slots or holes on the tip of the stator. Tissue is rapidly reduced in size by a combination of turbulence and scissor-like mechanical shearing that occurs at high speed within the gap of the rotor and stator	The process is quite fast and, depending on the volume and toughness of the tissue, completed within 10–60 s
Blade homogenisers	A set of either bottom- or top-driven steel cutting blades rotate at high speed inside a glass, plastic, or stainless steel container. As aerosols are readily formed while blending, use a sealed blender container. Operate the blender in a well-ventilated hood. Blade homogenisers can process sample sizes from a few millilitres to several litres	Accessories for blenders include cooling jackets for temperature control and closed containers to minimise aerosol formation and entrapment of air
Freeze-fracturing	Tissue is frozen in liquid nitrogen and then ground with a common mortar and pestle at the same low temperature	The frozen tissue is fractured under the mortar because of its brittle nature while the ice crystals formed at this low temperature will act as an abrasive
Grinders	Cells or tissue are ground in a mortar or tube with fine sand, aluminium, or glass powder	The method is labour intensive and disruption efficiency is poor
Pestle and tube homogenisers	Pestle and tube homogeniser consist of test tubes made of glass, inert plastic, or stainless steel into which is inserted a tight-fitting pestle made of like material (clearance about 0.1–0.2 mm). The pestle is worked to the bottom of the tube, thereby tearing and fragmenting tissue as it is forced to pass between the sides of the pestle and the tube walls.	Tissues must be minced or chopped into small pieces (1–5 mm) with scissors or a single-edged razor blade prior to processing. The method is well suited for the processing of small pieces of tissue and of cryosections
Meat mincer and solids press	Tissue is mechanically pressed through holes in a metal sieve plate while rotating blades slowly sweep across the face of the plate, thereby cutting the meat into 0.3–0.5 mm fragments	Useful as a preliminary step to efficiently cut tissue before complete homogenisation using other physical methods

(*continued on next page*)

Table 1 – *continued*

Apparatus	Technology	Remarks
Ultrasonic disintegrators	Ultrasonic disintegrators disrupt cells and tissue by generating intense sonic pressure waves in liquid media which cause the formation of microbubbles that grow and collapse violently. The implosion generates a shock wave with sufficient energy to break cells and tissue. As ultrasonic disintegrators generate considerable heat during processing, the sample should be kept ice cold if at all possible.	Tough tissues should either be macerated first in a tissue press or grinder, or else pulverised in liquid nitrogen. Be aware that free radicals can be generated during sonication and that these can react with most biomolecules
The 'cell bomb'	In this process, cells are placed under high pressure of an inert gas (usually nitrogen) and then the pressure is rapidly released. The rapid pressure drop causes the dissolved gas to be released as bubbles that ultimately lyse the cell	Well suited to subcellular fractionation studies of single cells. Not suited to larger pieces of tissue
High-shear mechanical methods	Valve type processors disrupt cells by forcing the media with the cells through a narrow valve under high pressure. As the fluid flows past the valve, high shear forces in the fluid pull the cells apart	Due to the high energies involved, sample cooling is generally required, especially for samples requiring multiple passes through the system. Best for cells, not for tissue

This table briefly describes various common apparatuses and technologies applied to disintegrate tumour tissues and cells.
Some of the apparatuses are designed for small sample volumes up to a few millilitres, others for very large volumes of several litres. Basic data for this table were assembled from Hopkins.[41] For more details see Hopkins[41,42] and http://www.processingtalk.com/news/mir/mir100.html.

nitrogen and into which four disposable cryogenic tubes of 1.2 ml each are placed, thereby allowing simultaneous, individual processing of deep-frozen samples but preventing sample cross contamination. The deep-frozen tissue is fractured by a moving stainless steel ball of 9 mm in diameter resulting in a still frozen but fine tissue powder. Glass beads are not recommended in this set-up as they may adsorb significant amounts of charged biomolecules, such as nucleic acids and proteins. As the time of agitation is short (less than 30 s) sample heating is not an issue.

It is rare that a tissue disruption process produces a directly usable biomaterial; often subsequent (bio)chemical treatment of the disintegrated tissue is necessary. This is different for the tissue powder generated in the Mikro-Dismembrator S. After pulverisation of the frozen tissue block by use of the dismembrator machine, addition of physiological buffer (e.g. Tris-buffered saline, TBS) to the tissue powder, followed by ultracentrifugation, yields the so-called cytosol fraction. A large fraction of soluble cell/extracellular matrix proteins is contained in this cytosol fraction. Alternatively, addition of TBS, supplemented with the non-ionic detergent Triton X-100, to the tissue powder, will free additional proteins and other cell components from cell membranes, intracellular organelles, and the extracellular matrix, respectively, in addition to the already released cytosol proteins.

The use of acidic buffers to extract biomarker molecules from tumour tissues is not recommended as low pH buffers favour the activity of cysteine-type proteases cathepsin B and L with enzymatic activity optima between pH 3 and 5. At this pH, cathepsins B and L efficiently degrade extracellular matrix proteins but as important, target and degrade/activate other proteins as well. A prime example is the enzymatically inactive proform of the urokinase-type plasminogen activator, pro-uPA, which at low pH is activated by the proteolytic action of cathepsins B and L.[44,45] Depending on the type of biomarker analysis, protease inhibitor cocktails can be added to the tissue extracts to block endogenous proteolytic activity.

Tumour tissue powder is not only a source for protein-based biomarkers but also a rich source of RNA and DNA. RNA can be extracted from such a powder by standard RNA extraction protocols (see below). DNA can be extracted from the tissue powder as well, or alternatively, from the tissue debris sediment collected after high speed centrifugation of the cytosol fraction or the detergent fraction.[46]

3. Standard operating procedure for the preparation of human tumour tissue extracts suited for the quantitative analysis of tumour tissue-associated biomarkers

The described standard operating procedure is suited for the gentle but rapid disrupture of tumour tissue in its deep-frozen state and the subsequent extraction of RNA-, DNA-, and protein-based biomarkers from the generated tissue powder, using the Mikro-Dismembrator S machine equipped with a new, four cryogenic tubes holding shaking flask. It is very necessary to cool both the tissue specimen and the shaking flask in liquid nitrogen before you start the short-term disintegration procedure. As a result, a still frozen tissue powder is generated. If the shaking flask and the sample are not cold

enough, the disintegration procedure will fail and the result will be an amorphous mass of partly disintegrated tissue.

The shaking flask bead mill technology is already in use and has been applied by several centres for the extraction of RNA, DNA,[46–56] or biomarker proteins from various sources of human and animal tissues.[57–68] In the following, guidelines are given on how to store tumour tissue (see Fig. 1) and how to process it for the extraction of RNA, DNA, or protein.

3.1. *Freezing and long-term storage of tumour tissue specimens*

3.1.1. *Materials needed for freezing and storage of tumour tissue specimens*

1. Fresh tumour tissue specimen.
2. 1.2 ml cryogenic tube (#5012, Nalgene, Neerijse, Belgium, Fig. 2a).
3. Liquid nitrogen.
4. Liquid nitrogen tank with storage system (Fig. 2b–d).
5. Forceps, scalpel.
6. Latex or plastic gloves.
7. Protective eye wear and safety gloves to handle liquid nitrogen containing devices (Fig. 4).

In general, tissue specimens should be snap-frozen in liquid nitrogen and then stored in liquid nitrogen containing refrigerator tanks. Alternatively, if long term storage in liquid nitrogen is not feasible, the snap-frozen tissue specimens can be stored in −80 °C freezers. If transport of the deep-frozen tissue specimens is necessary, this can be done in special liquid nitrogen transport vessels, such as those of the Voyageur series (Air Liquide, Paris, France; http://fi.vwr.com/app/catalog/ Catalog?parent_class_id=9&parent_class_cd=59803). The Voyageur range of vessels is specially designed for transport

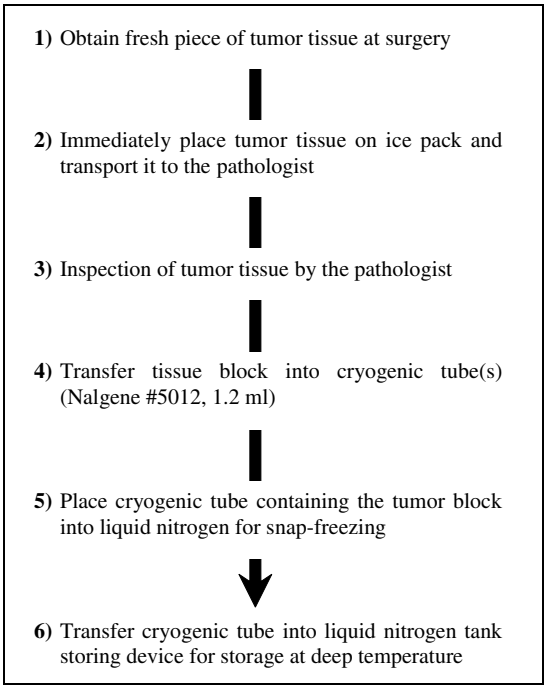

Fig. 1 – Surgery, inspection of the tumour tissue section by the pathologist and snap-freezing, and storage in liquid nitrogen.

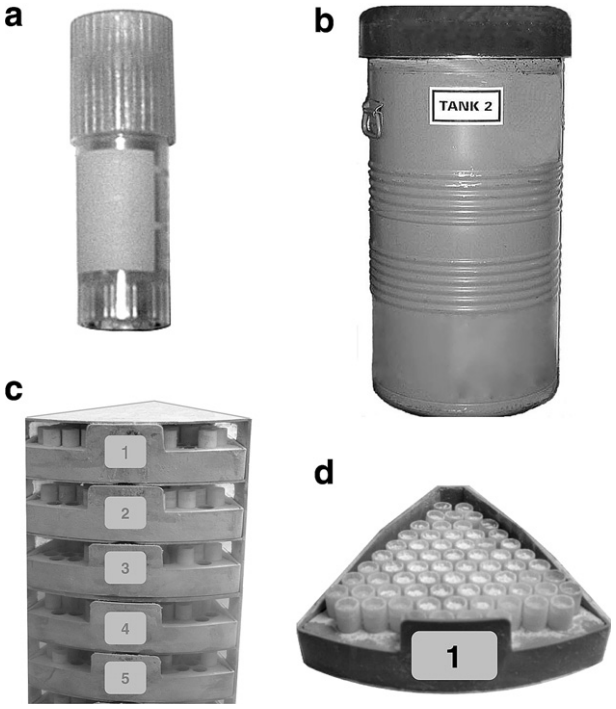

Fig. 2 – (a) 1.2 ml Nalgene cryogenic tube plus cap. The tumour tissue specimen is placed into this tube, closed, and then snap-frozen in liquid nitrogen. (b–d) Storage of the cryogenic tubes in holders and drawers (c,d) placed into a liquid nitrogen tank (b).

Fig. 3 – (a) A picture of a dewar, characterised by a narrow mouth, is depicted which is a holding tank for liquid nitrogen. From that device, liquid nitrogen is poured into some other device, e.g. a transportable refrigerator shown in (b). The shaking flask (Fig. 6e) is precooled in such a refrigerator for about 5 min and then transferred immediately to the shaking device (holder) (Fig. 6b) of the Mikro-Dismembrator S (Fig. 6a).

Fig. 4 – Personal protective equipment must be worn by all persons who dispense or handle liquid nitrogen or very cold objects such as cryogenic gloves (left) and protective eye wear (right).

of diagnostic and infectious substances products. A porous substance inside the vessel absorbs liquid nitrogen and preserves the samples in vapour phase. The products are safely transported as the liquid nitrogen is contained and will not be spilt if the vessel is knocked over. These vessels comply with international regulations applicable to transport of hazardous goods. Alternatively, deep-frozen tissue specimens can be transported in dry ice containing boxes.

Shipped biological specimens are regulated by governmental and non-governmental, consensus developmental organisations. IATA regulations are commonly encountered since they regulate transport by air of frozen materials packed in dry ice containing boxes or liquid nitrogen containing vessels. The University of Newhampshire has developed a special guide describing regulations concerning the transport of frozen biological material. The guidelines are available for download at http://www.unh.edu/ehs/shipping/UNH-Shipping-Biological-Materials.pdf.

Cryogenic tube Nalgene #5012 meets the United States of America Dangerous Goods Regulations for the transport of infectious and diagnostic specimens. These tubes should not be autoclaved. Use appropriate safety procedures as outlined in the Nalgene Cryopreservation Manual (Lit. No. 10096) when handling and disposing of vials. Do not use cryogenic tubes for storage in the liquid phase of liquid nitrogen unless correctly sealed in Nunc CryoFlex™ tubing. Such use may cause entrapment of liquefied nitrogen inside the vial and lead to pressure build-up resulting in possible explosion or biohazard release.

Personal protective equipment must be issued to all persons who dispense or handle liquid nitrogen or very cold objects. Safety procedures for dispensing and handling liquid nitrogen or very cold objects are found under www.sf.med.va.gov/e3/image-library/research/forms/safety/SOP%20Liquid%20Nitrogen%2009-12-05.doc where it is explained that liquid nitrogen is a hazardous substance and that, if misused, it may cause frostbite, eye damage, or asphyxiation. Therefore, when handling liquid nitrogen or very cold objects a face shield, cryogenic gloves, and cryogenic apron should be worn when dispensing liquid nitrogen. Liquid nitrogen or any item that has been immersed in liquid nitrogen should not touch any part of your body. Liquid nitrogen should be stored and dispensed in an approved container, only. Keep in mind not to use tightly sealed containers as these will build up pressure as the liquid boils and may explode after a short time.

The term 'refrigerator' is generally applied to those devices for which the principal purpose is to provide cooling of samples, that is, long term storage in a cryogenic environment. Since samples are constantly being added to or withdrawn from the chamber, a refrigerator generally has a wide mouth [e.g. larger than 5 in. (12.7 cm)] (Figs. 2b and 3b). The term 'dewar' is generally applied to those devices for which the principal purpose is to serve as a reservoir or holding tank for liquid nitrogen, which is destined to be poured into some other volume or device that is to be cooled. Because it is not meant to store samples and is rather meant to be used as a pouring device, the units are characterised by a narrow mouth, typically about 2 in. (5 cm) in diameter (Fig. 3a). (Text modified from http://www.2spi.com/catalog/instruments/cryogenic-refrigerators.shtml).

3.2. *Pulverisation of deep-frozen tumour tissue specimens using the Mikro-Dismembrator S bead mill machine*

3.2.1. *Materials needed for the pulverisation of deep-frozen tumour tissue specimens*

1. Deep-frozen tumour tissue specimen (50–300 mg).
2. Microscale, spatula, forceps, scalpel, pipettes plus tips.
3. Mikro-Dismembrator S machine (Sartorius AG, Goettingen, Germany) (Fig. 6), consisting of a bead mill with shaking flask made of Teflon (pulverisation device) holding up to four Nalgene #5012 cryogenic tubes.
4. 9 mm ∅ grinding stainless steel ball.
5. Cryogenic tube (1.2 ml, #5012, Nalgene, Neerijse, Belgium).

With the Mikro-Dismembrator S from Sartorius, Goettingen, Germany, even very difficult materials can be reliably disintegrated (see Fig. 5). The Mikro-Dismembrator S comes with a maximum shaking frequency of 3000 per minute and is

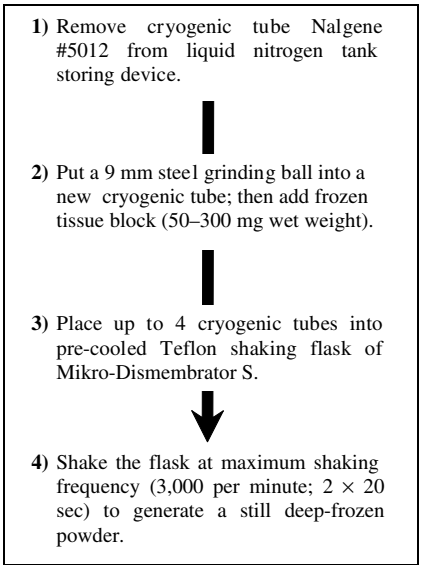

Fig. 5 – Disintegration of the deep-frozen tumour tissue sample by use of the Mikro-Dismembrator S apparatus equipped with the four cryogenic tubes accommodating shaking device.

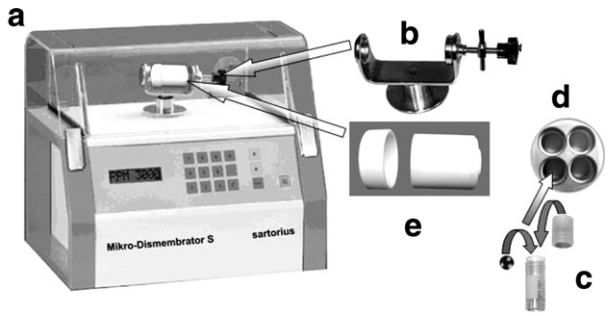

Fig. 6 – Mikro-Dismembrator S to be used for rapid pulverisation of deep-frozen tumour tissue. (a) The Mikro-Dismembrator S machine (bead or ball mill) acts through a shaking device (b) holding a pre-cooled Teflon shaking flask (pulverisation chamber) (e) into which up to four 1.2 ml cryogenic tubes (Nalgene #5012) (c,d) are placed. To the deep-frozen tumour tissue a grinding stainless steel ball is added which during the shaking process (maximum shaking frequency of 3000 per min, 30 s) will crush the deep-frozen piece of tissue into a fine but still frozen powder. Different from the conventional Mikro-Dismembrator II which is designed to process one tissue sample at a time, the Mikro-Dismembrator S is constructed for the simultaneous but independent processing of up to four tissue samples.

therefore well suited for fast homogenisation of biological samples deep-frozen in liquid nitrogen, including human tumour tissue of various origin. Owing to the high efficiency of shaking of the frozen tissue sample in the presence of a grinding steel ball, the deep-frozen tumour tissue sample is rapidly disintegrated allowing subsequent gentle and fast isolation of proteins but also of DNA and RNA (see www.sartorius.com).

3.3. *Extraction of pulverised deep-frozen tumour tissue specimens*

3.3.1. *Materials needed for the extraction of pulverised deep-frozen tumour tissue specimens*

1. Tris-buffered saline (TBS), pH 8.5, or 1% (w/w) non-ionic detergent Triton X-100 (Sigma–Aldrich, Munich, Germany) containing TBS as the tissue extraction buffer.
2. Rocking table to gently rotate the test tubes containing the tumour tissue suspended in TBS or 1% Triton X-100 containing TBS.
3. Refrigerator or cold room, ultracentrifuge plus rotor and ultracentrifuge tubes.
4. High-speed centrifuge plus rotor and appropriate centrifuge tubes.
5. 1.2 ml cryogenic tubes (#5012, Nalgene, Neerijse, Belgium).
6. Liquid nitrogen and liquid nitrogen tank with storing device.
7. Latex or plastic gloves.
8. Protective eye wear and safety gloves to handle liquid nitrogen containing devices.

With the rapid and gentle technique of tumour tissue disintegration in the frozen state, a still frozen tissue powder is generated which can be stored frozen or be immediately processed for tumour biomarker analysis (Fig. 7). This is particularly beneficial in proteome research. Comparable results are guaranteed, owing to the high reproducibility of the

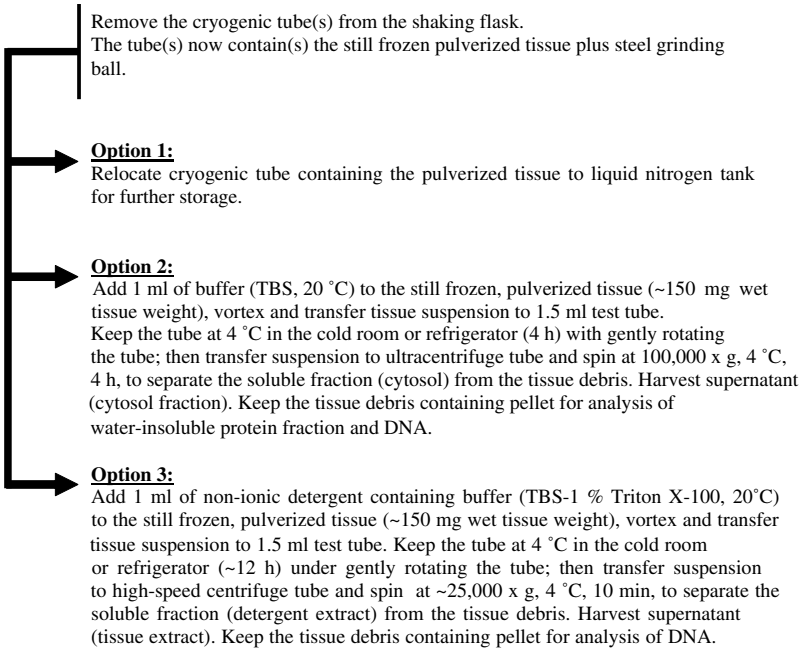

Fig. 7 – Preparation of the cytosol fraction and the detergent-released fraction from the pulverised deep-frozen tumour tissue. Proteins in the cytosol fraction and the detergent fraction are subjected to further analysis such as biomarker determination or proteomics.

disintegration process which is advantageous over manual processes of tissue disintegration.[41,42] Importantly, this disintegration process occurs when the tumour tissue is still deep-frozen; thus, thawing of the tissue is avoided. In addition, no foreign proteins, such as proteolytic enzymes often employed in other methods of tissue disintegration, are added before or during the grinding process. This allows state-of-the-art proteome analyses to be conducted on the proteins released, such as MALDI-TOF, SELDI, 2D-gels, and functional analyses.

4. Determination of protein content in tumour tissue extracts by the BCA-microtiter plate format

In general, the concentration of the analytes in question in the cytosol fraction or detergent extract is expressed in ng (μg, mg) analyte per mg of protein. In the following, a microtiter plate-type photometric assay for the quantification of protein in tumour tissue extracts is described based on the Pierce BCA protein assay kit. This kit contains a detergent compatible formulation based on bicinchoninic acid (BCA) for the determination and quantification of protein. This method combines the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation using a unique reagent containing bicinchoninic acid. A purple colour will develop.

4.1. *Materials needed for the determination of protein content in tumour tissue extracts*

1. Tumour tissue extracts (cytosol fraction or Triton X-100 released detergent extract).
2. Control samples (tissue extracts with previously determined amount of protein; BSA control samples).
3. TBS, pH 8.5, plus 0.1% Triton X-100 and 0.05% Tween 20 (Sigma-Aldrich, Munich, Germany) for dilution of samples, controls, and standards.
4. Polystyrene 96-well microtiter plate (Nunc, Wiesbaden, Germany), pipettes with tips.
5. 96-well microtiter plate reader, filter set to 540 nm.
6. Computer for data storage and data analysing.
7. Latex or plastic gloves.
8. BCA protein assay reagent kit (#23225, Pierce, Rockford, IL, United States of America).
9. BCA protein assay reagent kit contains: BCA reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic assay, sodium tartrate, in 0.1 M sodium hydroxide). BCA reagent B (containing 4% cupric acid). Albumin standard ampules (containing 2 mg/ml of bovine serum albumin in 0.9% saline plus 0.05% sodium azide).

4.1.1. *Procedure*

1. Mix 20 ml reagent A with 400 μl reagent B to generate reagent mix AB. Add 200 μl of reagent mix AB to each well of a 96-well microtiter plate. Add 50 μl of standard (0–400 μg BSA/ml), control sample (Precipath U, #10171760; Roche, Mannheim), or test sample to each well.
2. If necessary, dilute samples with TBS, pH 8.5, plus 0.1% Triton X-100 and 0.05% Tween 20. Perform measurements in duplicate.
3. Cover plate with lid and incubate it overnight at room temperature. A purple colour will develop. Measure absorbance at 550 nm in a 96-well microtiter plate reader. Deduct the background average of the blanks from the standards and sample readings.
4. Construct standard curve by plotting the mean absorbance value calculated for each protein standard versus the corresponding protein concentration. Calculate the protein concentrations in the test samples by use of this standard curve. Multiply result by dilution factor (e.g. if diluted 1:20, multiply by 20).
5. Website www.piercenet.com lists compatible substances allowed in the BCA protein assay. Technical assistance is found at ta@piercenet.com.

5. Extraction of RNA and DNA from tumour tissue powder generated by use of the Mikro-Dismembrator S bead mill

Generate frozen tissue powder from ca. 100 mg tumour tissue by use of the Mikro-Dismembrator S machine (see above). For example, use the RNeasy Lipid Tissue Mini Kit (50) (Qiagen, Hilden, Germany, #74804) to extract RNA from this powder. The kit contains 50 RNeasy mini spin columns, collection tubes, QIAzol lysis reagent, and RNase-free reagents and buffers. Suspend tissue powder in 1 ml QIAzol lysis reagent for lysis, warm the tissue suspension to room temperature, then add 200 μl of chloroform. Shake vigorously for 2–3 min, and then spin at 12,000*g*, 15 min, 4 °C. Transfer the upper aqueous phase to a different test tube. Add 1 volume (usually 600 μl) of 70% ethanol and mix by vortexing. Apply sample to the RNeasy spin column for adsorption of RNA to the membrane. Use RNAse-free DNAse set (QIAGEN #79254) for convenient on-column DNase digestion during RNA purification according to the manufacturer's directions.

Extract genomic DNA from frozen tumour tissue powder or, alternatively, from tumour tissue debris pellet obtained after high-speed centrifugation of the tumour tissue cytosol or the detergent-extracted fraction (see above) using the QIAamp 96 DNA Blood Kit (Qiagen, #51161). This kit contains 4 QIAamp 96 Plates, QIAGEN protease, reagents, buffers, lysis blocks, tape pads and collection vessels to conduct the DNA extraction.[46]

Conflict of interest statement

None declared.

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