Clinical sample collection for biomarker profiling
We strongly recommend contacting the ProMiFa staff before starting any study.

This guideline is meant to set an identifiable standard for the collection of fresh samples and/or frozen tissues, to ensure an acceptable level of quality in these samples and to enhance the reproducibility and comparability of test results derived from these specimens.

1. When collecting samples from patients, care should be taken to keep a consistent protocol and to minimize biological variation due to activities, such as eating.
2. For any body fluid, the colour should be noted. If haemolysis has occurred, the fluid will be pink or red, and these samples should be avoided.
3. Collect at least 30 samples per disease state or dose.
4. Collect as much sample as possible (although only small amounts are needed for the profiling, bigger amounts are needed for further identification of the biomarker).
5. Samples should have been handled identically and care should have been taken to minimize the number of thaw/freeze cycles.
6. If a pen is used to label a cryovial or other receptacle that will be stored in freezing conditions, ensure that the pen is waterproof/solvent-proof and can withstand long-term freezing conditions.

1. Plasma sampling requirements for proteomic analysis

1. Venipuncture blood is collected into a sterile tube containing EDTA as an anti-coagulant (EDTA monovettes BD No 366456).
2. After blood collection, the tube must be immediately placed in an ice bath (2-4°C). This is to prevent protein degradation which may occur between sample collection and storage, due to the presence of proteases.
3. In the lab, the samples must be centrifuged immediately at 2000 x g for 10 min at 2-6°C. After centrifugation, the supernatant, while kept on ice, is distributed into 0.5 ml aliquots in polypropylene cryovials Falcon (2 ml) and immediately placed on dry ice. All aliquoting process is completed within 60 min of specimen collection.
4. Samples are stored at -80°C until analysis.
5. Transport of samples from one site to another has to be done on dry ice.

2. Serum sampling requirements for proteomic analysis

1. Venipuncture blood is collected into a sterile tube (vacutainer BD No 367957).
2. After blood collection, the blood sample is allowed to clot for 30 min at room temperature. The tube must then be placed in an ice bath (2-4°C).
3. In the lab, the samples must be centrifuged immediately at 2000 x g for 10 min at 4°C. After centrifugation, the supernatant is added of protease inhibitors, aliquoted in 2 ml polypropylene cryovials Falcon (6 x 0.5 ml; 3 ml total) and immediately placed on dry ice.
4. Samples are stored at -80°C until analysis.
5. Transport of samples from one site to another has to be done on dry ice.

3. Urine sampling requirements for proteomic analysis

1. Urine (24h, first morning or random urine collection has to be defined according to the type of study; the midstream is always recommended) is collected into a sterile container and placed in an ice bath (2-4°C).
2. In the lab, the samples must be centrifuged immediately at 3000 x g for 5 min at 4°C. After centrifugation, the supernatant is transferred to new sterile polypropylene tubes (Falcon tube, 50 ml), where it is aliquoted (25 ml/each) and immediately placed on dry ice.
3. Samples are stored at -80°C until analysis.
4. Transport of samples from one site to another has to be done on dry ice.
4. Tissue sampling requirements for proteomic analysis

1. Time points for collection will be protocol-specific.
2. Samples have to be snap-frozen in liquid nitrogen using 2 ml cryovials Falcon.
3. Specimens should be numbered as they are removed from the patient (e.g. the first core often contains more tumor than subsequent cores).
4. Fresh tissue samples should be frozen as soon as possible. If they cannot be frozen immediately, they need to be frozen within 30 minutes.
5. The number of cores depends on the size of the specimen.
6. In the preoperative research setting, we recommend 2-4 fresh/frozen cores be taken for research at each of the following time points:
   - Baseline
   - On treatment
   - End of therapy/at surgery

   Cores can be taken at the time of diagnosis or after diagnosis. As regards size, cores of 3 mm or less are acceptable.
7. During surgery, fresh/frozen tissue may be prepared either in the operating room, taking cores and immediately freezing them after the removal from the patient, or transferring the surgical specimen to the pathology department in a container without fixatives.
8. Preoperative and surgical specimens should be frozen using the same freezing method.
9. Once snap-frozen, the samples should be immediately transferred either to liquid nitrogen, or to a -80°C freezer until analysis. If a local site has only a -20°C freezer, the specimen may be stored at -20°C until shipping, for no longer than 1 week.

Don’ts

- Do not directly place the specimen in liquid nitrogen.
- Do not add serum to the specimen.
- Do not touch the biopsy without sterile gloves.
- Do not slow-freeze. Samples should be snap-frozen. Slow freezing promotes the formation of ice crystals. The slower a sample freezes, the larger the ice crystals. Older models of cryostats that require more than one minute to freeze a specimen should be avoided.
- Do not freeze a specimen directly on dry ice. If only dry ice is available, adding alcohol (e.g., ethanol) to the dry ice can make a slurry that will help to freeze the specimen more effectively. (The alcohol will increase the thermal conductivity of the dry ice.) This, however, is not a preferred snap-freezing method.
- Use of Whatmann paper in snap-freezing may increase chances for contamination.
- OCT (optimal cutting temperature), the generic name for the polyethylene glycol/sucrose-based freezing medium, can inhibit protein studies and should be avoided.
- High-throughput proteomics approaches have been only recently applied to formalin-fixed paraffin-embedded (FFPE) tissues. Fresh/frozen tissue offers a number of advantages to researchers over FFPE, such as protein measurements of certain antigens for which no suitable test in FFPE tissue is available.

Do’s

- Sterile equipment should be used, including for dissection and for snap-freezing.
- Instruments should be changed or cleaned between dissecting normal and disease tissue.
- When submitting a surgical specimen to pathology, document the research biopsies that have been taken in the operating room. This will help to avoid misinterpretation of margins and other perturbations of the specimen.
- Copies of any relevant pathology reports and material submission forms should be sent along with the specimens.
- Representative parts of the lesion and normal tissue should be provided.

General comments

1. Except for serum: Leaving any kind of sample at room temperature during processing (even if only for a few minutes) should be avoided. Leaving blood, serum or urine samples on ice bath (2-4°C) during processing should not exceed a total of 30 minutes.
2. If a previous core biopsy site is noted in the surgical specimen, cores should not be taken from near that site. Core biopsies alter the biology of tissue (e.g. genes involved in wound healing are very similar to those involved in cancer progression).

3. Prior to extracting biomolecules from fresh/frozen tissue, core samples should be histologically evaluated in order to reveal the presence of necrotic or inflammatory material.

4. Whether or not to perform laser-capture microdissection, or its equivalent, on fresh tissue for proteomics depends on the intended analysis. For examining global expression of proteins (i.e., in the stroma and epithelium), laser-capture microdissection should not be performed. If only information on the protein spectrum in the epithelium or stroma is sought, then laser-capture microdissection should be performed.

5. Details on storage

1. For proteomics, -80°C is preferred, although samples (except for tissues) can be stored at -20°C for up to 2 weeks without major problems. It is important to note that proteins are fragile macromolecules and are prone to degradation if not handled and stored correctly.

2. Samples should NEVER be thawed (especially when transferred between sites, or from -20°C to -80°C). Number of thawing needs to be recorded!

3. Samples should not be kept at -80°C AND THEN transferred and kept at -20°C.

6. Some pre-analytical variables that need to be considered

   **Patient information**
   1. Gender
   2. Age
   3. Diet
   4. Genetics
   5. Medical background
   6. Health background
   7. Special conditions: pregnancy, post/pre-menopausal, medications
   8. Social history: alcohol intake, smoking status
   9. Nature of sample: Fasting or random

   **Veniipuncture**
   1. Needle gauge
   2. Details of blood collection set

   **Phlebotomy**
   1. Tourniquet technique
   2. Patient position: seated/standing/lying
   3. Tube order - first versus last
   4. Blood source: venipuncture or from existing line

   **Collection device**
   1. Tube or bag
   2. If tube, glass or plastic (It has to be defined and consistent throughout the study)
   3. Gel or non-gel separators
   4. Other tube additives
   5. Manufacturer and device information

   **Blood derivative and processing**
   1. Sample type: plasma versus serum
   2. If plasma, nature of anticoagulant: EDTA
   3. If serum, clotting procedure used, and/or type of clot activator
   4. Details of processing: time, protocol, etc.
   5. Separation of blood from cells
   6. Centrifugation, speed and duration
   7. Aliquoting before analysis and handling/storage of those aliquots
   8. Length of time before analysis

   **Storage**
   1. Frozen before analysis: snap-frozen, e.g., dry ice-ethanol, or liquid nitrogen
2. Elapsed time and temperature prior to freezing
3. Short or long-term storage
4. Storage temperature
5. Expiration dating
6. Storage materials: glass *versus* plastic. If plastic, type of plastic
Number of freeze/thaw cycles