

---

## Selected protocols

---

Most of the following protocol guidelines are derived from the methods and protocols publicly available and were adapted or replaced according to comments of experienced bio-resource experts in the field. However, it must also be noted that these methods have been developed with a specific end use of specimens in mind (e.g. proteomics), and thus may not be suitable in all cases. Therefore these protocols are only given as indications.

### Processing of blood specimens

If serum and plasma are being collected, it is important to prioritize the separation of them so they can be frozen as soon as possible. This is critical for time-sensitive samples for protein studies, for example. For processing of blood specimens we recommend to use the following protocols.

#### *Filter papers*

Always handle filter papers wearing gloves and only by the upper corner, marked out for labelling. Do not allow the card to come into contact with any unclean surface (e.g. bench, base of hood). Use EDTA/ACD tubes to produce filter papers.

1. Mix anticoagulated blood containing vacutainers by inversion before starting.
2. Wipe top of vacutainers with 70% ethanol before opening the lid.
3. Make 2 filter papers by placing 40µl of blood in the circle using a micropipette.
4. Air dry the filter papers thoroughly in the back of the Class II Biological Safety Cabinet.
5. Store filter papers in a paper envelope (not plastic) at room temperature.

#### *Blood pellets (white cells)*

Blood pellets can be used for the isolation of DNA (from EDTA / ACD tubes)

1. Transfer blood from the original tube to a labelled 50ml tube.
2. Fill tube with Tris-EDTA buffer (formula) and mix vigorously. Place on ice for 5 to 10 minutes.
3. Spin at 1200xg for 10 minutes.
4. Carefully pour off supernatant into a beaker containing chlorine bleach. Briefly vortex the pellet and add 50ml Tris-EDTA buffer. Shake vigorously.
5. If division of the sample is necessary, at this point pour 25ml of the sample into another falcon tube.
6. Spin both tubes at 1200xg for 10 minutes.
7. Repeat washing if red cells persist.
8. Carefully pour off supernatant.
9. Using a swirling motion, remove the pellet with a pipette and transfer to labelled cryovial.
10. Store in -80°C or liquid nitrogen until further use.

As an alternative, red cells can be lysed by using an ammonium-containing lysis buffer.

## *Plasma*

Plasma collected in EDTA and ACD tubes, can be used for bioassays, plasma DNA isolation, proteomic analysis, and biomarker discovery.

1. Spin vacutainer (about 9ml) at 815xg for 10 minutes at 4°C to separate plasma from blood cells.
2. After wiping each tube with 70% alcohol, remove about 3ml plasma. Tube can be retained for white blood cell extraction.
3. Transfer to a labelled 15ml tube and centrifuge at 2500xg for 10 minutes at 4°C.
4. Aliquot plasma into 1ml labelled cryovials (3 to 4 aliquots).
5. Place in liquid nitrogen Dewar to snap freeze.
6. Store at -80°C or in liquid nitrogen.

The purpose of double spinning the plasma is to remove all cellular contaminants so that the plasma is suitable for plasma DNA analysis. It is extremely important, therefore, not to disturb the buffy coat after the first spin, and any pellet after the second spin.

## *Platelet-poor plasma*

Platelet-poor plasma can be used for the isolation of plasma DNA (from EDTA tubes)

1. Spin blood at 3200xg for 12 minutes at room temperature.
2. Pipette off plasma using a plastic pasteur pipette. Transfer into tube.
3. Spin plasma at 2000xg for 10 minutes at 4°C.
4. Aliquot into 1ml aliquots in labelled cryovials.
5. Store at -80°C.

## *Serum*

The blood is collected into tubes without addition of anticoagulants. Then two phases are distinguishable, a solid phase containing fibrin and cells, and a fluid phase containing the serum.

This process should be completed after 30 minutes at room temperature, after which the process described below starts.

1. Spin blood at 1500xg for 10 minutes at room temperature.
2. Aliquot 1ml portions of supernatant into labelled cryovials.
3. Place into liquid nitrogen Dewar or dry ice to snap freeze.
4. Transfer to -80°C freezer or liquid nitrogen.

## *White Blood Cells*

White blood cells collected in EDTA and ACD tubes can be used for DNA extraction and the creation of cell lines.

1. Transfer the remaining blood from the plasma spin to a labelled 50ml tube containing 10ml RPMI 1640.
2. After alcohol swabbing the lid of this tube, aliquot 3ml Ficoll into each of two clearly labelled 15ml tubes.
3. Carefully layer 9ml diluted blood onto each tube of Ficoll. Treat gently, do not mix, but spin as soon as possible.
4. Spin at 450xg for 30 minutes. Note: when centrifuging, do not use brake.
5. Remove most of the top layer (RPMI 1640) using a 1ml Eppendorf tip and discard  $\approx$  3-4ml into waste container containing chlorine bleach.
6. Collect white blood cells with the same Eppendorf tip using a swirling motion to 'vacuum up' white blood

cells. Do not take too much Ficoll (third layer), as it is toxic to the cells. Place the white blood cells in a labelled 15ml tube containing 10ml RPMI.

7. Spin at 450xg for 10 minutes.
8. Pour off the supernatant into a waste container containing chlorine bleach. Add 3ml of cold freezing mix (10% DMSO, 20% FCS, RPMI 1640) and resuspend.
9. Dispense the white blood cells into 3 x 1ml labelled cryovials that have been sitting on ice.
10. Place on ice. Place vials in a rate-limiting freezer as to cryopreserve cells in conditions that maintain cell viability. This should be done as soon as possible as DMSO is toxic at room temperature.
11. Transfer on a weekly basis to liquid nitrogen tanks.

Instead of a separation based on Ficoll, a Percoll separation can be used alternatively.

### *Buffy coat cells*

The buffy coat is a thin, greyish-white layer of white blood cells (leukocytes and lymphocytes) and platelets covering the top of the packed red blood cells after 450xg centrifugation (from EDTA/ACD containing blood tubes).

1. After having spun the blood, take buffy coat off with about 100µl of plasma using a disposable sterile Pasteur pipette: be careful not to lift red cells.
2. Lyse remaining red cells by addition of red cell lysis buffer at room temperature.
3. Spin tube at 450xg for 10 minutes at room temperature.
4. Resuspend the pellet.
5. Aliquot as appropriate into labelled cryovials.
6. Place in liquid nitrogen to snap freeze.
7. Store in liquid nitrogen.

### *Whole blood*

To be prepared from EDTA tubes. The anti-coagulated blood can be snap frozen as it is. In case the blood cells are needed intact, DMSO is needed to keep them alive while freezing.

1. Dispense 50µl DMSO into two 1ml sterile cryovials.
2. Invert EDTA tube twice then add 450µl of blood to each cryovial.
3. Invert cryovial to mix the whole blood with the DMSO. Note: DMSO is cytotoxic at room temperature; therefore as soon as it is mixed with blood, it should be placed in a controlled-rate freezer.
4. Transfer to -80°C after at least 4 hours.

## **Processing of solid tissue specimens**

Careful and well-documented processing of tissue specimens is crucial to the overall usefulness of the repository as a resource for scientific research. This protocol for collecting and freezing tissue samples was developed within TuBaFrost, a European project aimed at producing a European virtual frozen tumour bank (TUBAFROST, 2003). We recommend using this protocol which contains choices and recommendations for preserving solid tissue.

### *Snap-freezing*

#### *Safety*

All procedures should be carried out in accordance with the local codes of practice. Working with liquid nitrogen and isopentane is hazardous—all procedures must comply with local safety rules specific to these chemicals. All tissue must be handled as if potentially infectious.

## **Collection of tissue**

### **Ward**

Consent must be obtained from patient before surgery (if applicable, according to the law in the collecting country)

### **Operating theatre**

Deliver notification of tissue collection (and consent form if needed) to surgeon or flag up on operating list.

### **Surgeon**

1. Complete pathology form (if possible in advance).
2. Perform operative procedure, record time of excision of specimen.
3. Place specimen in labelled sterile pot/bag and put on ice.

### **Operating theatre staff**

Send fresh tissue specimen immediately to pathology department.

### **Histology Department**

1. Notify pathologist and tissue bank research technician (if not already present).
2. Check paperwork and allocate pathology number to specimen as routine.

### **Pathologist**

1. Macroscopically describe specimen as usual.
2. Using clean instruments and on a clean surface (sterile foil or clean dissection board), dissect the tissue specimen. Clean or change instruments between dissecting normal and tumour tissue.
3. Take representative parts of tissue for routine diagnosis (for fixation and embedding) as priority and decide if there is sufficient material available for the tissue bank.
4. Supply research technician with tissue sample(s) for cryostorage; representative parts of the lesion, normal tissue and pre-malignant conditions.

### **Technician**

1. Prepare the tissue sample for snap-freezing on a clean surface and using clean instruments—change instruments between preparing normal and tumour tissue. The minimum size of tissue for snap freezing is approximately 0.5cm<sup>3</sup>, though the amount of tissue available will differ depending upon the sample site. Smaller fragments should still be snap-frozen and stored in the tissue bank. If there is sufficient material, freeze duplicate samples.

2. Pre-cool the freezing medium isopentane (2-methyl butane) to the moment when opaque drops begin to appear in the isopentane and the solution becomes misty; this will bring the isopentane towards its freezing point (-160°C), the optimal freezing point for the tissue. Options:

**A.** LN2: suspend a vessel of isopentane in LN2

**B.** Dry ice: add dry ice (cardice) to the isopentane until a slush is formed, or by suspending a vessel of isopentane in dry ice.

3. Label cryovials, cryomolds or cryostraws with a barcode and/or sequential code (depending upon local laboratory practice). Use a waterproof pen able to withstand long-term storage at low temperatures. The sequential code is the local inventory code and must not relate to the pathology number or other identifiers. If a barcode is used, readable recognition must also be included to make the sample identifier readable at institutions where there are no barcode readers.

4. Record the local sequential code, pathology number, date, lag time from excision to freezing, and the type of tissue (site and whether the sample is tumour/normal/premalignant) in the inventory book. If a barcode system is in use, this can be scanned into the Laboratory Information Management System and the above data recorded.

5. Freeze directly in isopentane. Do not remove the tissue from the isopentane until freezing is complete (only 5 seconds or less is needed depending on size) but ensure sample does not crack. Remove sample from isopentane and enclose in the labelled cryovial. Strive to snap freeze all tissue within 30 minutes of excision from patient. Tissue subject to a delay of up to 2 hours should still be collected and the delay noted within the local inventory database. Options for freezing:

**A.** Embed the tissue samples in optimal cutting temperature (OCT) compound and freeze in isopentane or freeze directly in isopentane. The isopentane used is either cooled by suspension in liquid nitrogen or through addition of dry ice. Do not remove the tissue from the isopentane until freezing is complete (5 seconds or less depending on size) but ensure sample does not crack. Remove sample from isopentane and enclose in the labelled cryovial.

**B.** Orientate the tissue on a piece of cork and an equally sized piece of Whatman paper soaked in physiologic salt solution. The isopentane used is either cooled by suspension in liquid nitrogen or through addition of dry ice. Do not remove the tissue from the isopentane until freezing is complete (5 seconds or less depending on size) but ensure sample does not crack. Remove sample from isopentane and enclose in the appropriate labelled storage vessel.

**C.** Embed samples in a cryosolidifiable medium in plastic cryomolds and immerse in the pre-cooled isopentane. The isopentane used is either cooled by suspension in liquid nitrogen or through addition of dry ice. Do not remove the tissue from the isopentane until freezing is complete (5 seconds or less depending on size) but ensure sample does not crack.

**D.** If the cryostraw system is used introduce a carrot of tissue into the straw, thermically seal each extremity and place in liquid nitrogen.

### *Storage of tissue*

Storage of tissue can be done according different protocols according the equipment available within the facility. Options for storage:

**A.** Transfer the snap-frozen sample from the isopentane to a pre-chilled storage container for transfer to either a locked  $-80^{\circ}\text{C}$  freezer or liquid nitrogen storage facility in liquid or vapour phase. For storage longer than 5 years, liquid nitrogen is recommended.

**B.** Place cryostraws in a designated visotube within a goblet (removable liquid nitrogen storage elements) and place within the locked liquid nitrogen repository.

B1. Store duplicate samples in a different storage facility if this is available.

B2. Check the back-up system for the storage repository—either a back-up freezer running constantly or adequate supplies of liquid nitrogen.

B3. Record storage details in the inventory book and check earlier data that were entered. At a minimum the information recorded will include: inventory number (local sequential code), location, pathology number, type of tissue (site and also whether the sample is tumour/unaffected (normal)/pre-malignant), lag time between excision and freezing, and date.

B4. Transfer details to the computerized database system.

B5. Update the database when samples are moved or depleted.

## **Formalin Fixation**

Formalin fixation is standard practice in most routine histopathology laboratories. The following guidelines address specific issues related to preservation of formalin-fixed specimens in BRCs.

1. Tissue specimens should not be bigger than 1.5 x 1 x 0.5cm.
2. Specimens will be fixed in fresh 10% neutral buffered formalin (NBF) Blue for a minimum of 4 and a maximum of 48 hours, after which time they will be embedded in paraffin following conventional techniques.
3. All reagents should be DNase- and RNase-free (e.g. prepared using DEPC water).
4. Fixation media such as Bouin's containing picric acid should be avoided, as this compound interferes with subsequent PCR analysis of extracted nucleic acids.
5. Alcohol fixation may be used as an alternative to formalin. For this, tissue is placed into 70% alcohol (diluted with DEPC water) for a minimum of 4 hours.

Alternatives to formalin can be desirable to use as a routine fixative, due to the chemical hazards of formalin. The effect on long-term storage using these alternative fixatives on the desired macromolecules is not always known, however, and should be empirically established.

## **RNA<sup>later</sup>**

This substance protects RNA in fresh specimens. It eliminates the need to immediately process or freeze samples.

### **Tissue**

Cut tissue to be less than 0.5cm in at least one dimension, then submerge tissue in 5 volumes of RNA<sup>later</sup> (e.g. a 0.5g sample requires about 2.5ml of RNA<sup>later</sup>).

### **Cells**

Resuspend pelleted cells in a small volume of PBS before adding 5–10 volumes of RNA<sup>later</sup>.

### **Storage**

RNA<sup>later</sup>-treated tissue and cell samples can be stored at 4°C for one month, at 25°C for one week or at -20°C for indefinite time. For RNA isolation, simply remove the tissue from RNA<sup>later</sup> and process.

## **Processing of urine and buccal cells**

**The following protocols for processing of urine and buccal cells contain recommended procedures.**

### **Urine**

1. Plastic or glass containers for collection of urine should be clean and dry, have a 50–3000ml capacity, a wide mouth and leak-proof cap, and should be clearly labelled.
2. When in transit, urine collections should be maintained on ice or refrigerated.
3. Urine should be aliquoted according to the volume necessary for analysis or storage.
4. Depending on the analyte to be measured, a preservative may be added during collection or before aliquoting.
5. Store urine at -80°C or lower in liquid nitrogen.

## *Buccal Cell*

1. A collection kit (containing mouthwash, 50ml plastic tube, plastic biohazard bottle, and courier packaging) is mailed or given to the participant, along with an instruction sheet. The participant is to brush the teeth as usual, rinse the mouth well twice with water, and then wait 2 hours. They should not eat or drink anything other than water during this time.
2. After 2 hours, 10ml of commercial mouthwash should be poured into the tube, and then 10ml tap water added. This diluted mouthwash should be placed into the mouth (without swallowing) and swished around vigorously for 30 seconds.
3. The mouthwash should then be spat back into the plastic tube, and the tube should be sealed tightly.
4. The sample should be sent back to BRC immediately for processing, or stored at 4°C until sent but should be sent within 24 hours.
5. On arrival at the laboratory, transfer mouthwash to 15ml conical test tubes.
6. Add 35ml Tris-EDTA to the mouthwash sample and spin at 450xg for 5 minutes.
7. Decant supernatant and discard.
8. Wash cells twice, each time with 45ml Tris-EDTA.
9. Resuspend cell pellet in 50µl Tris-EDTA and transfer to 2ml labelled cryovials.
10. Store sample at -80°C or in liquid nitrogen.

Note: Buccal cells can also be collected with other means such as brushes.

## *Saliva*

A research consortium at the University of California-Los Angeles was funded by the National Institute of Dental and Craniofacial Research (NIH grant UO1 DE 16275; PI: David T. Wong DMD, DMSc) to investigate the human saliva proteome. The protocol for saliva collection and processing is derived from their "Salivary Proteome Handbook Procedures and Protocols". (National Consortium for the human saliva proteome, 2007)

### *Collecting and Processing Saliva*

1. Saliva collection is recommended to be done in the morning (please aim for 10-11am if possible). Ask the subject to refrain from eating, drinking or oral hygiene procedures for at least 1 hour prior to the collection.
2. The subject should be given drinking water (bottled) and asked to rinse their mouth out well (without drinking the water).
3. Five minutes after this oral rinse, the subject should be asked to spit whole saliva (WS) into a 50ml sterile Falcon® tube.  
The subjects need to refrain from talking. It is better for subjects to drop down the head and let the saliva run naturally to the front of the mouth, hold for a while and spit into the tube provided. Subjects will spit into the collection tube about once a minute for up to 10 minutes. The goal for each whole saliva donation should be about 5ml. Require that the tube be placed on ice while collecting whole saliva. Remind the subjects not to cough up mucus as saliva is collected, not phlegm.
4. Submandibular saliva (SM) collection: use 2 x 2 inch cotton gauze to block the opening of each parotid duct; dry up the floor of the mouth and block the openings of the sublingual gland (both sides) and have the subject raise their tongue slightly to elevate the opening to the SM gland; begin to collect SM saliva by using a sterilized Wolf device. A sterilized and disposable yellow tip (for pipette P200) is connected into the device and changed between every collection. During the collection, at 2 minute intervals, a few grains of citric acid powder are swabbed with a moistened cotton applicator onto the lateral dorsum of the tongue to stimulate the secretion. Aim to collect at least 200µL SM.
5. Sublingual saliva (SL) collection: similar to the protocol described above for SM collection. The only difference is that the ductal orifices of the submandibular gland will be blocked off. Aim to collect >100µL

SL every time.

6. Parotid saliva (PR) collection: use a parotid cup to collect PR. Parotid cups may be placed bilaterally if the clinical investigator so chooses. This will allow for the simultaneous collection from each parotid gland. The citric acid stimulation will be performed as described above. Aim to collect >1mL PR. The first 0.1ml of parotid saliva collected will be discarded to insure that fresh parotid saliva is obtained.

NOTE: COLLECTED SAMPLES ARE TO BE KEPT ON ICE AT ALL TIMES PRIOR TO PROCESSING.

7. Sample processing using proteinase inhibitors: To each 100µl saliva, add:

- a) 0.2µL proteinase inhibitor cocktail from standard stock solution (Sigma, cat# P8340), invert gently.
- b) 0.3µL Na3OV4 (Sigma, cat# S6508) from standard stock of 400mM, invert gently.

8. Centrifuge specimens at 2600xg for 15 minutes at 4°C; (if you note that incomplete separation has occurred, increase the spin time to 20 minutes).

Remove the supernatants from the samples and mark them as plus “super” which stands for the supernatant phase of the saliva. Taking care not to disturb the pellet and keeping the pellet as is in the original tubes, mark the original tubes with “pellet”.

9. Freeze samples at -80°C.

## Processing of cervical cells

In a Pap smear test, a sample of cells is taken from the uterine cervix using a spatula or brush, smeared onto a slide, and examined under a microscope for abnormal cells (precancer or cancer). This protocol is a selected protocol from diverse collection procedures.

Note the following:

1. It is best not to take a smear from women who are actively menstruating or have symptoms of an acute infection. Slight bleeding is acceptable.
2. Pregnancy is not an ideal time for a Pap smear, because it can give misleading results.

Taking the smear with a wooden spatula or a brush

1. Insert the long tip of the spatula into the cervical os, and rotate it through a full circle (360 degrees). If the cervix broom brush is used, the tip of the brush is placed within the cervical os and then rotated gently for three 360° circles.

Taking a Pap smear

2. Smear both sides of the spatula (or the contents of the brush) onto the glass slide with one or two careful swipes. If you see any abnormalities outside the area sampled, take a separate specimen and smear it on another slide.
3. Immediately fix each slide. Either use spray fixative, at a right angle to, and a distance of 20cm from, the slide, or immerse the slide in a container of 95% ethanol for at least 5 minutes. If the slide is not fixed immediately, the cells will dry and become misshapen; it will then not be possible to read the slide accurately in the laboratory.
4. Gently close and remove the speculum.
5. Place all used instruments in decontamination solution.

After taking the smear

6. Label the frosted edge of each slide carefully
7. On the patient record, note and illustrate any features you have noted: visibility of the transformation zone, inflammation, ulcers or other lesions, or abnormal discharge. Note whether other samples were taken, for example Pap smear of other areas and, if the woman has been referred elsewhere, to whom and when.

## Processing of hair and nails

These protocols are recommended for collecting hair or nails specimen.

### *Hair*

Head hair may be collected as follows:

1. Along an imaginary line drawn across the middle of the back of the head from the centre of one ear to the centre of the other, gather a lock of hair at least the thickness of a pencil, and tie it together near the root end (near the scalp) using a small string or a rubber band.
2. Cut the hair as close to the scalp as possible without cutting the scalp.
3. Maintain the horizontal position of the hairs in the bundle by wrapping the cut section in aluminium foil or plastic wrap.
4. Indicate the root-end and tip-end by marking the foil or plastic wrap with a permanent marker or with a paper label. **Do not use tape on the hair itself.**
5. Place the specimen in a clean, dry, labelled paper envelope for shipment to the laboratory. Note whether bleaches, hair dye or medications (e.g. selenium or minoxidil) were used.

Please note that hair from other sources (pubic, axillary, beard, moustache, chest, etc.) may also be analyzed if head hair is not available (NMS labs).

### *Nails*

A clean pair of nail clippers is to be used. To clean thoroughly, nail clippers are rubbed with alcohol swabs. Nails should be clean of all polish, dirt and debris. Nail clippings from each finger or toe should be collected and packaged separately in plastic bottles. Each bottle should be labelled with the mass of the nail collected and its source, e.g., right index finger (NMS labs and Expertox).