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Position Paper

Standard operating procedure for the collection of fresh frozen tissue samples

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ABSTRACT

Studies using fresh-frozen tissue samples originating from different centres, as is often the case in EORTC related translational research, can show conflicting research results due to heterogeneity in the quality of samples and associated data from each centre. The development of infrastructure for the European Human Frozen Tumour Tissue Bank (TuBaFrost) anticipated this problem and Standard Operating Procedures (SOPs) have been developed to ensure samples collected are of consistent high quality and variation in research results is minimised. The SOPs drew on the best practice standard workflows and operating procedures employed by members of the TuBaFrost Consortium and key tissue bank initiatives worldwide. It was essential to provide workable solutions that reflect the variety in infrastructure and resources at the potential collecting centres and also the fact that it is not necessary to standardise every step of the collection and storage process in order to collect high quality tissue. Hence, the TuBaFrost SOPs detail the compulsory measures that must be implemented in order to become a TuBaFrost collecting centre and also make advisory recommendations regarding the less critical factors. Accordingly, the TuBaFrost SOPs are very flexible and to illustrate this the complete SOP for collecting, freezing and storing tissue at the Erasmus MC Tissue Bank is included. These TuBaFrost SOPs could equally be applicable to centres collecting samples for EORTC related translational research studies in order to standardise sample quality and produce reliable and reproducible research results.

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1. Introduction

Frozen tissue samples are used in a wide variety of experimental techniques and when sourced from a distributed network, for example through multi-centre studies or virtual tissue banks, there must be conformity in tissue collection and storage techniques to ensure results from these experiments are reproducible and comparable.¹ This Standard Operating Procedure (SOP) originates from SOPs developed for the collection and storage of human tumour and corresponding normal or unaffected tissue for the European Human Frozen Tumour Tissue Bank (TuBaFrost) www.tubafrost.org. Here, we document the series of actions required as a guide for implementing the SOP in an EORTC-related centre, this is based on the more detailed and descriptive version of the TuBaFrost SOPs published recently.² For trial related multi-centre translational research studies as performed at the EORTC the implementation of this SOP at participating collection centres sets the standard for avoiding intrinsic bias of samples. To further facilitate trial related translational research on frozen tissue samples, the EORTC pathobiology group can offer virtual tissue banking and a histology review system, both of which can be supported by virtual microscopy and tailored to the needs of a particular trial. A compensation of the costs may be required, depending on the type of trial, the amount of work involved and the level of adaptation to the basic system needed.

The TuBaFrost SOPs drew on the best practice standard workflows and operating procedures employed by members of the TuBaFrost Consortium and key tissue bank initiatives worldwide^{3–5} and have been developed through identification of all the actions that take place during the tissue collection and storage process, as depicted in Fig. 1. This chronologic series of actions must be taken into account in order to produce high quality frozen tissue samples and associated documentation. The impact of each action on tissue specimen quality was considered and either compulsory measures or advisory recommendations were described – in this way the implementation of unnecessary and costly measures is avoided. In addition to the general TuBaFrost SOP, a working description of the implementation and the resulting SOP is provided, as used at the Erasmus MC Tissue Bank.

2. General TuBaFrost SOP

2.1. Safety

Carry out all procedures 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.

2.2. Collection of tissue

2.2.1. Ward

1. Consent taken from patient (if applicable – according to the law in the collecting country).

2.2.2. Operating theatre

2. Deliver notification of tissue collection (and consent form if applicable) to surgeon or highlight on operating list.

2.2.3. Surgeon

Perform normal operative procedure, record time of excision of specimen.

3. Complete pathology form (if possible in advance).

4. Place specimen in labelled sterile pot/bag and put on ice.

2.2.4. Operating theatre staff

5. Notify TuBaFrost dedicated technician or transport service – send specimen immediately to pathology department fresh and unfixed.

2.2.5. Histopathology department

6. Notify pathologist and dedicated TuBaFrost research technician (if not already present).

7. Check paperwork and allocate pathology number to specimen as routine.

2.2.6. Pathologist

8. Macroscopically describe specimen as routine.

9. Using clean instruments and on a clean surface (sterile foil or clean dissection board) dissect the specimen – clean or change instruments between dissecting normal and tumour tissue.

10. Take representative parts for routine diagnosis (for fixation and embedding) as priority and determine if there is sufficient material available for the tissue bank.

11. Supply research technician with sample(s) for cryostorage – representative parts of the lesion, normal tissue and pre-malignant conditions.

2.2.7. Technician

12. Prepare the sample for snap freezing on a clean surface and using clean instruments – change instruments in between preparing normal and tumour tissue. The recommended minimum size of tissue for snap freezing is approximately 0.5 cm³, this is consistent with other tissue bank initiatives and also the requirement for various studies,⁶ though the amount of tissue available will differ depending upon the sample site. Smaller fragments should still be snap frozen for the tissue bank. If there is sufficient material freeze duplicate samples – therefore there may be many samples per biopsy.

13. 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 for pre-cooling:

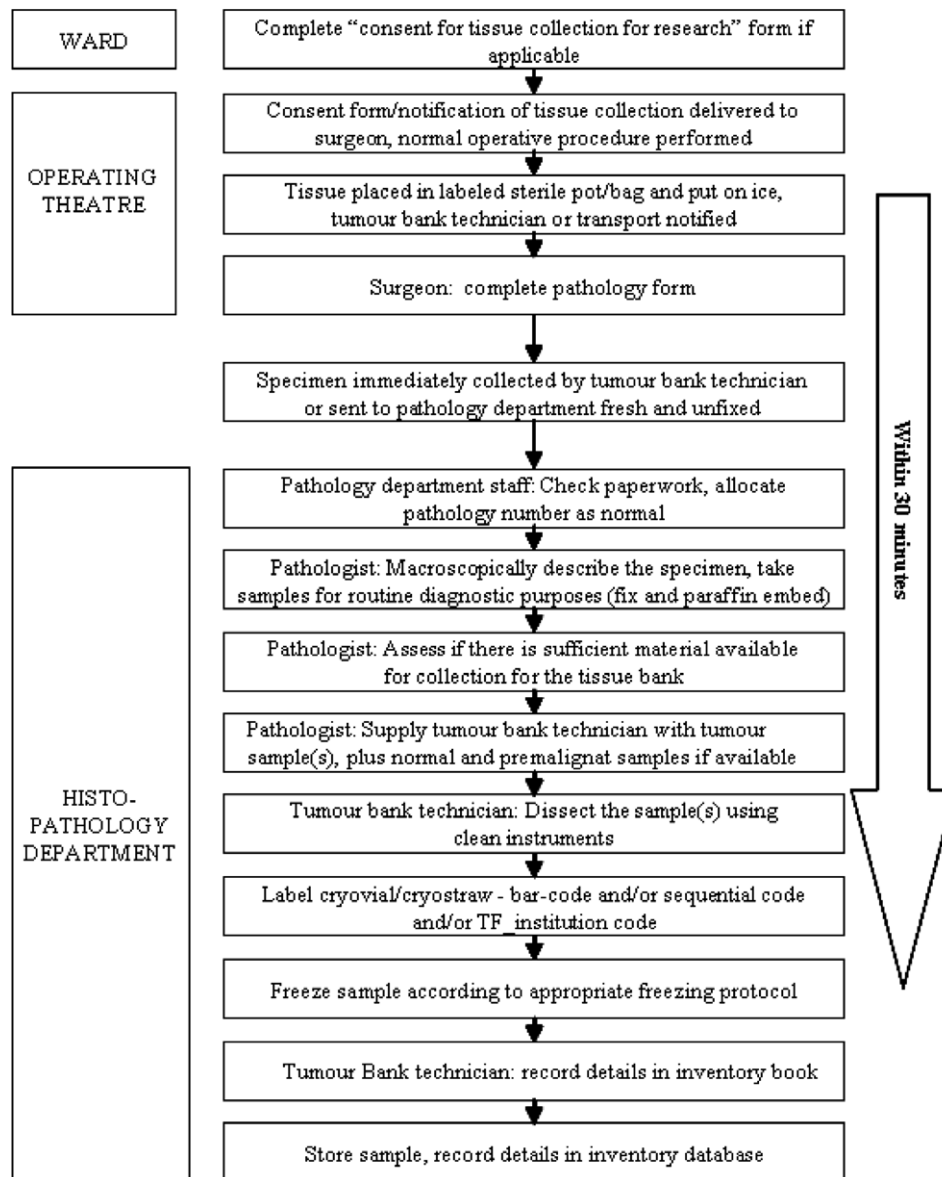


Fig. 1 – Work flow for collection of tissue.

- (A) Liquid nitrogen: suspend a vessel of isopentane in liquid nitrogen.
- (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.
14. Label cryovials, cryomolds or cryostraws with a bar-code and/or sequential code and/or TF_institution code (depending upon local laboratory practice). Use 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 bar-code is used a readable code must also be provided to make the sample identifier usable at institutes where there are no bar-code readers.
15. Record the local sequential code, pathology number, date, lag time from excision to freezing, the type of tissue (site and whether the sample is tumour/normal/premalignant) in the inventory book. If a bar-code system is in use this can be scanned into the Laboratory Management System and the above data recorded.
16. Tissue must be snap frozen within 30 min of excision from patient. Tissue subject to a delay of up to 2 h should still be collected and the delay noted within the local inventory database.
- Options for freezing
- (A) Freeze the sample directly in isopentane or embed the tissue sample in optimal cutting temperature (OCT) compound and freeze 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 s or less depending on size), but ensure the 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 s or less depending on size), but ensure the 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 s or less depending on size), but ensure the sample does not crack.
- (D) Introduce a carrot of tissue into a cryostraw, thermally seal each extremity and place in liquid nitrogen.

2.3. Storage of tissue

17. 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°C freezer or liquid nitrogen storage facility in liquid or vapour phase.
- (B) Place cryostraws in a designated visiotube within a goblet (removable liquid nitrogen storage elements) and place within the locked liquid nitrogen repository.

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

18. Check the alarm system of the storage repository. This should be a tri-phase alarm system with (a) local visual and acoustic alarms where the storage repository is located, (b) a distant acoustic and visual alarm in a central surveillance facility, and (c) a remote alarm capable of automatically dialling out pre-programmed telephone numbers.
19. Check the back-up system for the storage repository – either a back-up freezer running constantly or adequate supplies of liquid nitrogen.
20. Record storage details in the inventory book and check earlier data that was entered. At a minimum the information recorded will include: inventory number (local sequential code); location co-ordinates; pathology number; type of tissue (site and also whether the sample is tumour/normal/premalignant); lag time between excision and freezing; and date.
21. Transfer details to the computerised database system.
22. Update the database when samples are moved or depleted.

2.4. Quality assurance

23. In the first year of collection an institute will be subject to a 6-month quality review of 2% of newly collected sam-

ples. In the absence of issues encountered during the first year of collection, the quality review will be reduced in subsequent years to 1% of new cases received annually. The review will focus on records, equipment and frozen sections.

24. Stained H&E frozen tissue sections will be reviewed by a pathologist to confirm the diagnosis and to assess how representative the sample is.
25. RNA will be extracted and the quality checked using an agarose gel or a bioanalyser – the bioanalyser requires less material and provides integrity and concentration information.
26. Sample records will also be checked for accuracy and physical checks of the sample identification and location will also allow the durability of the sample vessels and the inventory containers to be assessed to ensure they have remained stable at low temperatures.

3. Erasmus MC tissue bank SOP

The numbers for each step of the Erasmus MC Tissue Bank SOP reflect the numbering of the TuBaFrost SOP and illustrate the flexibility when implementing the SOP.

3.1. Safety

Tissues are handled in the pathology post-mortem suite, which is convenient for receiving fresh tissue samples from the pathologist and is also equipped with all the necessary measures required to handle potentially infectious material. Specifically

- Personal protective equipment (surgical overcoat and latex gloves) must be worn in this room.
- Safety goggles for eye protection, silencers for ear protection, laboratory coat and cryogenic gloves must be used when working with large amounts of liquid nitrogen. With small amounts safety goggles and a laboratory coat will suffice.
- The diagnostic process must not be in any way compromised by the tissue bank activities [3 and 4]. The pathologist is responsible for the diagnosis and therefore has the authority to decide if tissue is available for storage for experimental purposes.

3.2. Collection of tissue

3.2.1. Ward

1. No action is required in the ward, as the patient does not need to give consent for the secondary use of residual tissue in the Netherlands. If the patient objects to the use of their tissue for experimental use, they can 'opt out' by completing an objection form and filing this with the central records office of the hospital. Tissues can only be used if no objection is registered at the hospital of treatment.

3.2.2. Operating theatre

2. Operating schedule is made available to Tissue Resource Technicia.

3.2.3. Surgeon

3. Preferably, fill out the pathology form before starting the operation and apply the identification sticker to the form.
- Perform normal operative procedure.

3.2.4. Operating theatre staff

4. As soon as the operative procedure allows, place the sample in a sterile vessel, preferably on scraped ice. In addition, ensure the pathology form is finalised, apply the identification sticker and record the time of excision.

Send the specimen immediately to the pathology department, therefore the operating theatre staff are responsible for the transport of the specimen to the pathology department or for contacting the tissue bank staff through the hospital paging system. Direct office phone numbers will be used to establish contact if the paging system is ineffective. Details of the location where the surgical resection specimen will be placed for collection and transfer are then agreed. The surgical resection specimen together with the pathology form is then transported to the agreed location.

3.2.5. Histology department staff

7. Upon receipt of the fresh sample at the pathology tissue reception facility, check the fresh tissue and the pathology form and register the details in the Laboratory Management System (LMS). Label the transport vessel with the automatically generated pathology number (printed on stickers). Transfer the specimen and form to the pathology post-mortem suite.

3.2.6. Pathologist

- The pathologist, together with the assisting technician, assess as soon as possible if there might be tissue available for the tissue bank.
6. If there appears to be residual tissue available to freeze, contact the tissue bank technician (if not already present) via the hospital paging system or direct office phone numbers.
8. Macroscopically describe specimen as routine.
9. Use clean instruments and work on a clean surface (sterile foil or clean dissection board).
- Dissect the specimen.
 - Clean or change instruments between dissecting normal and tumour tissue.
10. Take representative parts for routine diagnosis (for fixation and embedding) as priority and decide if there is sufficient material available for the tissue bank.
11. Supply tissue bank research technician with sample(s) for cryostorage – representative parts of the lesion covering

the variety of states encountered, e.g. tumour, normal and pre-malignant tissue.

- Available tissue samples are collected for freezing on a dedicated disposable wax plate on which the location of tumour, premalignant and normal, in case they are available, is clearly marked with a waterproof marker together with the pathology number routinely allocated to this case.
- In the ideal case, where sufficient tissue is available, a maximum of four samples of about 1 cm³ of every available state (if present, tumour/premalignant/normal) will be frozen. This may only be done if the diagnostic process will not be disturbed.
- The wax plate and the corresponding Pathology form are then transferred to the Tissue resource technician who is responsible for verification of facts on transfer of the material.

3.2.7. Tissue resource technician (research technician dedicated to the tissue bank)

- Upon receiving the tissues together with the form, check whether the correct patient material has been collected and that it corresponds with the pathology number on the form.
12. Prepare the sample for snap freezing on a clean surface and use clean instruments.
- Clean instruments in between preparing different states, e.g. normal and tumour tissue.
 - The size of tissue for snap freezing will differ depending upon the sample size and the available amount of tissue (maximum of four per state, about 1 cm³).
16. (B) The received tissues are bisected in such a way that the resulting pieces can be frozen with the shared cut plane orientated upwards on cork and a piece of filter paper wetted with physiologic salt solution.
- To enable orientated freezing, a piece of cork is cut to support the size of the tissue to be frozen. On top of the cork a piece of filter paper is placed of a similar size. The filter paper is wetted with physiologic salt solution (0.9% NaCl). The tissue is positioned on top with the shared cut plane upwards.
 - The other half of the tissue is placed with the shared plane facing downwards in a cassette, it is then fixed and embedded in paraffin.

A dewar barrel (Cryotech, diameter 20 cm, height 30 cm), stand and a home-made holder containing a glass tube resistant to high temperature variations (Schott AG, Germany) are located on the tissue bank preparation table. A home-made dipper is placed in the glass tube – see Fig. 2. Before use, the dewar barrel is filled with liquid nitrogen to a height where the holder containing the glass tube is submerged in the liquid nitrogen by about a third whilst being supported by the stand.

13. (A) Pre-cool the freezing medium, isopentane (2-methyl butane), to the point where opaque drops begin to appear in the isopentane and the solution becomes misty; this

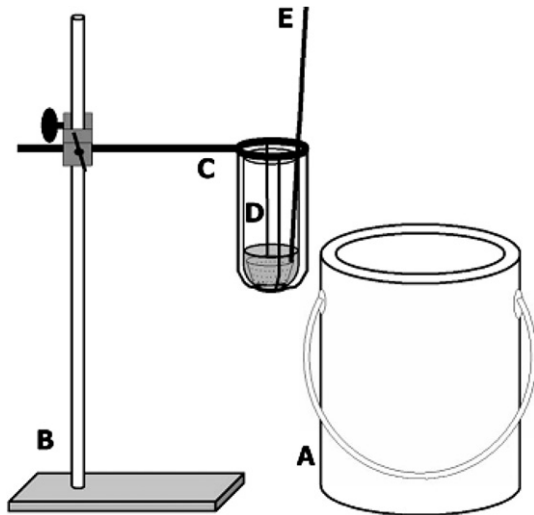


Fig. 2 – Snap freezing facility. Dewar barrel (A) filled with liquid nitrogen; stand (B); holder (C) (home made) metal construction to fully support the glass tube on a long arm that can be attached to a stand; glass tube (D) to withstand extreme temperature changes and filled with isopentane to fully submerge the dipper; dipper (E) (home made) the bottom consist of a metal grid and the cup has a large series of little holes to allow the fluid easily in and out, whereas the sample stays in the dipper.

means the isopentane is almost at its freezing point ($-160\text{ }^{\circ}\text{C}$), the optimal freezing point for tissue.

- At this point the holder with the glass tube containing the isopentane at freezing point is lifted and placed just above the liquid nitrogen, by bringing the holder on the stand to a higher position.
- The pre-numbered aluminium 3 ml cryo-vials (Sanbio) are taken from the drawer with pre-numbered cryo-vials that have been previously prepared (see Section 3.5).

Freeze directly in pre-cooled isopentane.

- The tissue orientated on cork is placed in the dipper and submerged in the pre-cooled isopentane. Do not remove the tissue from the isopentane until snap freezing is complete.
- Check whether the cryo-vial label corresponds with the snap frozen sample label.
- To pre-cool the vial, the lid is removed and the vial submerged with forceps directly in the liquid nitrogen until nitrogen boiling significantly decreases.
- The snap-frozen tissue is recovered from the dipper and placed in the pre-cooled cryo-vial, the lid is screwed on tight and the vial is stored in liquid nitrogen in the dewar barrel on the tissue bank preparation table until the end of the day.
- Embedding of the tissues is performed by embedding tissues of a format recognizable for Tissue Banking, yellow cassettes with a raster. The front surface of the cassette is annotated (using a charcoal pen fine writer) with the corresponding sequential number of the frozen sample.

- The tissue is placed in the embedding cassette with the shared cut face down.
- The cassette is placed in a formalin bath to await routine embedding and preparation of a H&E stained tissue slide.
- The pathology form is scanned to activate the pathology number in the Laboratory Management System and hence begins recording data. Choose ‘Storage’ from the menu and then ‘Tissue Bank’.

15. Record or scan:

- the local sequential code on the storage vial (see Section 3.5);
- the status from the appropriate bar code – tumour/normal/premalignant;
- the appropriate type of tissue (site or organ);
- the block sequential letter if one is prepared;
- any relevant information in the comments field, e.g. the lag time from excision to freezing.
- The data are later extracted through two dedicated queries and imported into the tissue bank database, where tracking of samples is initiated.

3.3. Storage of frozen tissue

17. At the end of the day the frozen tissues are transferred to the liquid nitrogen storage system – the 24 K container (Air products) with a capacity of 360 L, equipped with automated fill system, alarm system and a complete storage system. There are 15 large columns, of which each column contains 21 drawers and one drawer contains 49 cryo-vials. The small columns contain 21 drawers that contain only 16 cryo-vials. The aluminium cryo-vials (Sanbio) have a volume of 3 ml each. The alarm is a tri-phase alarm system with a local visual and acoustic alarm where the storage repository is located, a distant acoustic and visual alarm in a central surveillance facility and a remote alarm capable of automatically dialling out pre-programmed telephone numbers. A back-up container is constantly ready for use. The room is dedicated for liquid nitrogen storage and is equipped with an oxygen level alarm coupled to a ventilation system, in addition there is a liquid nitrogen tube system for the delivery of liquid nitrogen to the storage container fed from a large supply tank outside the facility.
- Identify the location of the column you need to retrieve, by consulting the diagram located next to the 24 K storage container depicting the position of the columns by number in the container.
 - Remove the lid from the storage container using cryogenic gloves.
 - Wait until the mist (which originates when air condenses through contact with the cold nitrogen vapour) is pumped away, so you can see which column you need.
 - Lift the appropriate column and remove the relevant drawer.

- Place the filled cryo-vials in the designated position in the drawer.
- Place the column back in the storage container.
- Remove any ice deposits that may damage the system or cause leakage and close the lid.

18. Check the alarm system of the storage repository.
19. Check the back-up system for the storage repository.
22. Update database when samples are moved, issued or depleted.

3.4. Quality assurance

23. Annually 1% of all new samples are randomly tested for:
 25. RNA quality.
 26. Accuracy of placement in the storage system.
24. Paraffin blocks and the derived H&E stained slides are of great value for the tissue bank. The H&E stained slides are used for recording digital histologic images, which are added to the database. These images can assist in the selection procedure when samples are requested by researchers. More importantly these slides are also used for quality control. Pathologists compare the given diagnosis of the patient with the histology on the tissue slide and confirm or reject the presence of the expected lesion. Upon rejection a note is made of the status and this is updated in the database. The blocks form a valuable resource, independent of the diagnostic archive, for Tissue Micro Arrays.

3.5. Preparations needed for receipt of fresh tissues

Equipment maintained at the Tissue Bank preparation table: clean instruments, forceps, disposable scalpels, embedding cassettes in Tissue Bank format, waterproof pen, charcoal pen fine writer, normal pen, 200 ml bottle containing isopentane, cork cut to 5 × 5 cm, filter paper cut to 5 × 5 cm, bottle with physiologic salt solution (0.9% NaCl)

14. Pre-number cryo-vials with bar code stickers (maintain 49 pre-numbered vials in reserve).
 - These vials are labelled with stickers (Brady BY217072) able to withstand extreme temperature changes and bearing a 2d bar code, including readable text, representing the storage location. In addition to the bar-code, the sample also bears a human-readable identification code to enable sample identification at institutes where bar-code readers are not employed. The stickers are printed with a Brady printer BP 1344 using Teklynx Codesoft version 7 software.

- The cryo-vials are labelled in advance to expedite the process.
21. A sequential code represents the position of the vial in the storage system. The first E characterises the storage system followed by the sequential number of the drawer or canister in the system followed by a hyphen and the position in the drawer, e.g. E514-24.
 20. The positions of the labelled cryo-vials in the drawers and the location of the drawers in the numbered columns are recorded in the database.
 2. Print out the operating schedule at the beginning of the day and mark those operations where tissue can be expected to be available for collection. Near the end of the day the received tissues are checked against the expected tissues and justification is sought for those operations for which tissue has not been received.

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