Quality of Life and Management of Living Resources

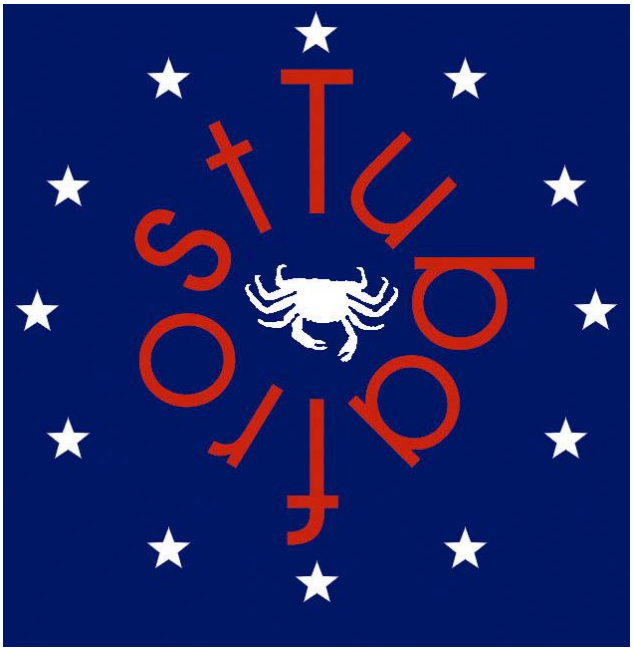
European Human Frozen Tumour Tissue Bank

TUBAFROST

QLRI-CT-2002-01551

Deliverable D 2.2

Use of a basic prototype system at the participating institutions



TUBAFROST Work Package 2: Development of storage system(s) for frozen tissue (technical aspects). (Deliverable 2.2 February 2004)

This deliverable builds upon **the selection of state of the art suitable tools to build a practical system** for frozen tissue storage. More specifically this deliverable aims to evaluate the best practical procedure to store micro-biopsies for subsequent nucleic acids extraction.

Problems to consider

In Deliverable 3.2 the specifications for sample vessels are:
"Cryovials, cryomolds or other storage vessels (e.g. cryostraws) used for storing tissue for the TuBaFrost tissue bank must be:

- specifically designed for storing biological materials at temperatures as low as -190°C;
- stable when submitted to sudden low temperatures (snap freezing), when held at low temperatures for long periods of time (years) or when taken through several freeze-thaw cycles; and
- as leak proof as possible (applicable to cryovials) even at the lowest cryogenic temperatures."

The sample vessels used in the pathology departments and by TUBAFROST participants vary considerably in volume and shape. This lead to several problems:

- The volume of tissue stored in the sample vessels influences the tissue quality and eventually the quality of derived material (nucleic acids, proteins)
- This heterogeneity limits the development of automatized systems.

In many instance in oncology micro-samples are the only possibility to get access to tissue especially when organs are small, hardly accessible or when sequential samplings (for instance under treatment) are to be considered. Large sample vessels (for instance with a volume of 2ml or more) are inadequate to store these

micro-samples. Moreover, cytological samples are being largely considered as fine needle aspirations could bring enough material to study the transcriptome, proteome or even to characterize immune response.

In Deliverable 3.2 the specifications for bar codes are:

- "The use of barcodes is recommended as it improves the accuracy of sample identification and can facilitate sample management and distribution. (…) the label and its adhesive must be able to a) withstand a wide range of temperatures (the minimum being approximately -196°C boiling point of liquid nitrogen) b) withstand an archival life of many years at extremely low temperatures and c) be self adhesive on many different materials;
- it is essential that the label remains firmly affixed and legible".

However, it is difficult to fulfil these specifications without standardization of sample vessels and usually the 2ml cryovials does not allow secure adhesion.

In Deliverable 3.2 the specifications for storage of cryovials in liquid nitrogen begin by the following diagnosis:
"There is currently no screw top cryogenic vial on the market today that can claim to be leak proof in liquid nitrogen so the following recommendations are particularly important[4,6]. If liquid nitrogen is trapped inside a container that is sealed, then expansion on warming above -196°C may cause an explosion, giving rise to danger from contamination by the vessel's contents as well as injury from fragments of the vessel itself." Indeed, these "explosions" of the cryovials when warming up are a daily experience in most of the pathology departments.

Therefore, the selection of the best suitable tools to build a practical system for frozen tissue storage should handle these problems.

Qualitative and quantitative evaluation of extracted RNAs from micro-samples

Objective

To evaluate the possibility to obtain 3 μg of RNA in 90% of the cases from a sample taken with 18 Gauges and 14 Gauges needles with a 28S/18S *ratio* above 1.6. The RNA quality was evaluated using Agilent micro-chip.

Material

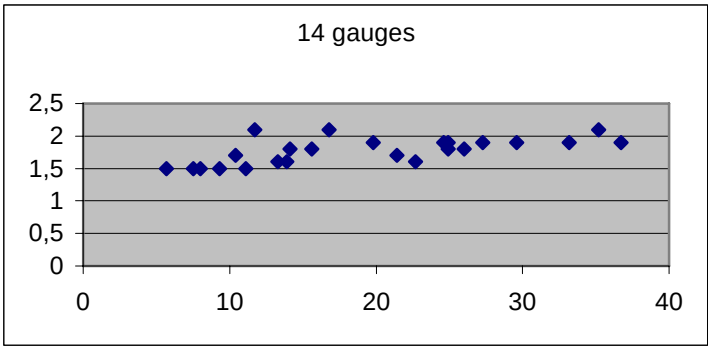
In 20 patients with breast carcinoma for whom a diagnostic fine needle biopsy have been performed, additional samples were obtained either with a 18G or 14G needle. In total, 46 samples were obtained for the study, distributed among breast (n=20), lever (n=9) and lymph node (n=17). For each site half of the samples were extracted with a 18G needle, the other half with a 14G needle. All the patients have signed a specific Patient Information Sheet/Informed Consent and the protocol was approved by the Institutional Review Board.

Methods

The sample was apposed against a glass slide for cytological evaluation, and then immediately put in a 2ml cryovial (Nunc cryovial) and the cryovial was directly immersed in liquid nitrogen. The samples were stored at least 7 days (range 7-25 days) in the standard nitrogen inventory container of the department. The samples extracted with a 14G needle were mechanically transformed in powder and then put in the lysis buffer (Qiagen) to follow the standard Qiagen procedure for double RNA/DNA extraction. The samples that were extracted with a 18G needle were directly added to the lysis buffer.

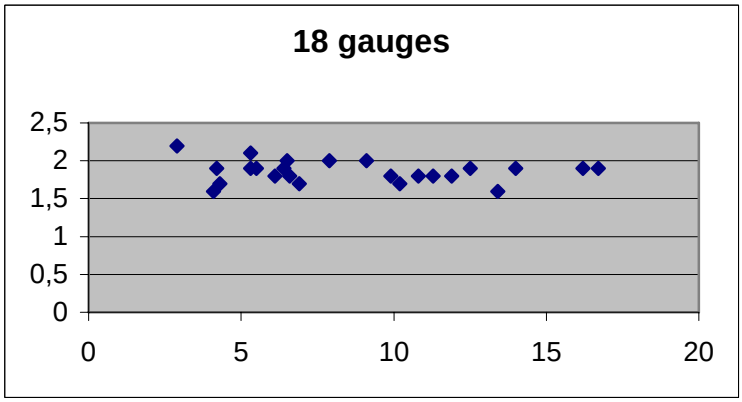
14G needles :

Average quantity in μg (range): 19.3 (5.7 – 36.7)
Average quality (range): 1,8 (1,5 – 2,1)
100% of the samples were above the target value (no technician-dependant effect)



18G needles :

Average quantity in μg (range) = 8,7 (2,9 – 16,7)
Average quality (range) = 1,9 (1,6 – 2,2)
100% of the samples were above the target value but in breast samples, 3 profiles were partly degraded.



Results are therefore excellent in terms of RNA retrieval with both 18G and 14G needle aspiration. However it is extremely difficult to remove the sample from the 2ml cryovials that appear not appropriate for these samples.

Evaluation of straws in replacement of 2ml cryovials for sample storage in liquid nitrogen

We evaluated two different type of high-security straws developed by Cryo-Bio Systems ™ for storage of biological samples in liquid nitrogen and nitrogen vapour. These straws have not been evaluated before for tissue storage as they were used only for liquids such as serum, plasma, buffy-coat and other blood fractions, cell suspension, bacterial or viral strains, gametes and embryos. These straws conform to ISO 9002 standards.

Two different straws have been evaluated: 0.5 and 1.0ml that were pre-barecode-printed.

Their specifications are as following:

<u>0.5 ml straws</u>:

Length: 133 (+/- 1) mm before sealing ; 130 (+/- 1) mm after sealing

Diameter: 2.5 mm (internal) and 0.30 mm wall thickness

<u>1.0 ml straws</u>:

Length: 133 (+/- 1) mm before sealing ; 130 (+/- 1) mm after sealing

Diameter: 3.7 mm (internal) and 0.35 mm wall thickness

The three-part closure system guaranteed the biosafety of the sample during filling and emptying procedures. Airtight heat seals on both ends of the straw prevent contamination of both the samples and the environment. The cotton part that will allow to push the sample out of the straw.

Barcode-based tamper-proof identification system.
The straw has to be opened to remove the identification jacket.

Sampling

Several systems were tested: semi-automatized devices (14G Trucut ™), 18G manual needles and 14G needles in several types of specimens received at the Department of Pathology of the Gustave-Roussy Institute. For the following types of specimens, between 3 and 17 samples have been made representing a total of 112 samples:

- Lever: free of lesion, colon adenocarcinoma metastasis, hepatocarcinoma
- Skin: squamous-cell carcinoma
- Lymph node: free of lesion, lymphoma
- Breast: free of lesion, adenocarcinom
- Ovary: free of lesion, serous adenocarcinoma, mucinous adenocarcinoma, serous borderline tumor
- Soft tissue part: leiomyosarcoma
- Colon: mucosa, adenocarcinoma
- Thyroid: papillary carcinoma
- Pancreas: normal tissue, adenocarcinoma.

Results

For exophytic tumors of more than 2 cm in diameter the 3 systems were perfectly usable. They allowed to take a carrot of 0.8-1.2 cm in length. However for flat tissue (mucosa or skin) and non-exophytic tumors (for instance ulcerated colon adenocarcinoma), a carrot of tissue could have been taken only using a bistoury as used classically.

Sample introduction in the straw

Whatever was the sampling system only the straws of 1.0ml measuring 3.7mm in diameter gave enough space to introduce the needle and to leave the carrot of tissue in the straw in pinching it. Several systems were developed to try improving the process but no one appeared to be usable in routine. However, manually it was always possible to introduce the carrot in the middle of the 1.0ml straw without modifying its specifications.

Sealing

After the sample have been introduced into the straw, each extremity is thermically sealed using a specific device developed by CBS ^{TM}.

Storage

Goblets are the removable storage elements placed in the liquid nitrogen container. Each goblet can hold sub compartments of different colors called visotubes. The straws are stored in a visiotube. The full size central visotube protects the straw seal when goblets are stored on top of each other. Pre-assembled goblets with 12 visotubes were used. Each visotube could hold 9 straws of 1.0 ml, thus 108 straws fit in one goblet. The color composition can be adapted to the organ.

Goblets are stored on top of each other within each canister and a metal strip is designed for lifting up the goblets.

Storage and tissue use

The straws were stored in liquid nitrogen in a round strorage repository. Thirty straws were randomly removed from the nitrogen and the carrot was removed out of the straw using a specific plastic baguette to push the cotton. Fifteen samples were weighted and used for histology control: after the whole freezing process, the tissue carrots weighted between 42 and 65mg. For all the specimens, after having been processed using a Cryostat at -20°C as for per-operative sections, the histology quality of the specimens was assessed by two observers and evaluated as “good” to “excellent”, apart for a lymphoma that was “poor”. The 15 other carrots were processed using the QiagenTM procedure for RNA retrieval (“midi” columns). The RNA quality and quantity was in the same range than for 14G needles stored in 2ml cryovials.

In conclusion, the developed process allows to fulfil all the requirements described in deliverable 3.2. It facilitates the standardization of the sample volume which is a key factor in tissue repository.