EuroPMP

PROTOCOLS FOR LABORATORY PREPARATION OF BLOOD, CELLS, AND TISSUES

FOR USE IN PSEUDOMYXOMA PERITONEI RESEARCH IN EUROPE

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PROCEDURE FOR PREPARING PLASMA FROM EDTA BLOOD

** Procedure originally shared by the Flatmark lab, Oslo University Hospital, Norway*

1. This procedure requires at least 2, 20 ml blood samples in EDTA tubes.
2. Centrifuge the samples at 820 *g* for 10 minutes, as soon as possible after collection and absolutely within 1 hour. Use a swing-out rotor without a brake.
3. Pipette the plasma from the spun EDTA tube as soon as possible and transfer it to new 2 ml tubes. Be careful not to disturb the buffy coat layer in the EDTA tube; only pipette to about 0.5 cm above this layer to avoid drawing it up into the pipette with the plasma.
4. Centrifuge the 2 ml plasma samples at 10,000 *g* for 10 minutes to get rid of any remaining cells and debris.
5. Pipette the plasma from the smaller sample tubes into a single larger tube to mix them together and then distribute the plasma into 1 ml aliquots and freeze at -80˚C.
6. *This step is to preserve cells for extraction of germline DNA later.* With <u>one</u> of the original EDTA tube(s), pipette 1 ml of the upper portion of the tube (normal blood including the buffycoat) remaining after the first centrifugation. The remainder of the tube and the remaining material from the other EDTA tube should be discarded.
7. All sample tubes should be labeled appropriately and frozen at -80˚C.

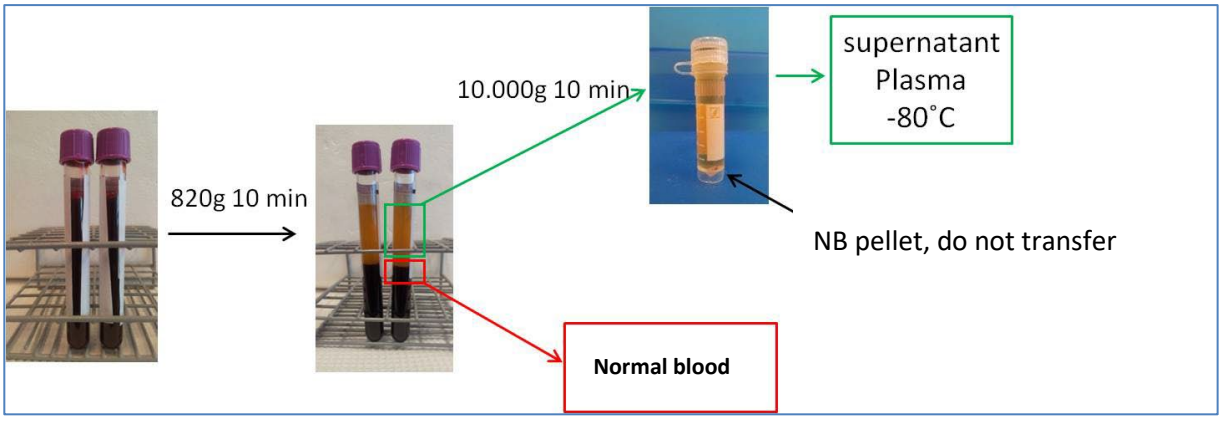

Figure 1: Modified Figure from Heidi Namløs

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PROTOCOL FOR HANDLING BIOBANKED TISSUE FROM FREEZER UNTIL DNA/RNA

PREPARING FROZEN SECTIONS FROM TISSUE SAMPLES

1. Prepare and pre-label three (3) cryotubes with, for example, “Sample_ID” and letter A, B or C, before starting. Prepare 2 slides and have Tissue-Tek OCT ready for use.
2. Divide the tissue into 3 parts (these will become samples A, B, and C) in a petri dish set on a block of dry ice to keep the samples frozen (see Figure 2).

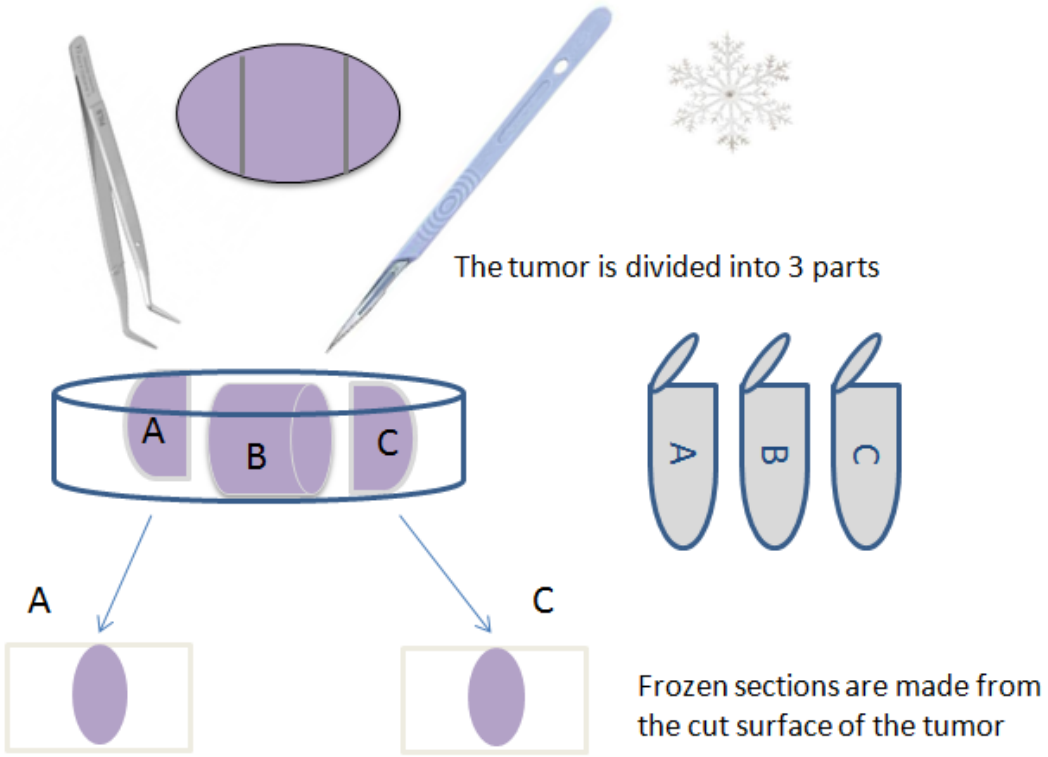

Figure 2: Cutting of the tumor sample

3. Sample B should be placed directly into the pre-marked tube labelled “B” and stored on dry ice or in a long-term -80°C freezer.
4. Using a cryostat, make 2 frozen sections from samples A and C.
5. The cryosections should be placed on slides.
6. The remaining parts of samples A and C should be covered in “Tissue-Tek OCT” (glycol/resin used to set tissues prior to cryostat), put into their pre-

marked cryotubes, and stored on dry ice or in the -80°C freezer.

7. The two slides should then be hematoxylin and eosin stained. Tumor content should be determined by a pathologist using the two slices; the average is taken to reflect the entire sample.

ALIQUOTING TISSUE

8. Before starting, prepare four pre-marked cryotubes with, for example, “Sample_ID” and “Tumormix 1 to Tumormix 4”.
9. A piece of paper towel (or similar, the point is not to contaminate the dry ice between tumor samples) is placed on top of the dry ice. Samples A and C are placed into the petri dish on top of the towel.
10. Remove all the Tissue-Tek OCT from around the tissue sample. Add sample B to the dish with the cleaned samples A and C (see Figure 3).

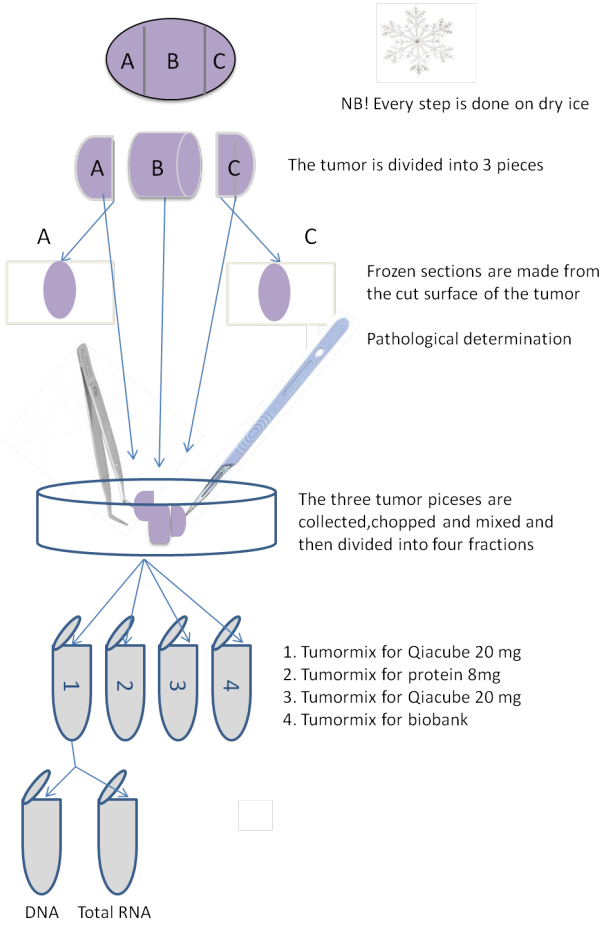

Figure 3: Procedure for determination of tumor content and alliquotting of the sample

11. While still frozen, the tissue should be quickly chopped into tiny pieces with a sterile scalpel and blended until the tissue mixture is as homogenous as possible.

12. Two aliquots of about 20 mg are put into tube 1 and tube 3 of the pre-labelled tubes (for use in the DNA/RNA extraction step below), and one sample of

about 8 mg (for protein analysis) is placed into tube 2. Any leftover sample should be placed into tube 4. All tubes are kept on dry ice to ensure they are completely frozen and then transferred into long-term storage at -80 ˚C.

TISSUE LYSIS AND HOMOGENIZATION (USING TISSUELYZER LT)

13. A 5 mm Stainless Steel Bead (http://www.qiagen.com) is washed in 1 ml Buffer RLT Plus (lysis buffer; http://www.qiagen.com) and transferred into the tube containing the tumor mix (labelled Qiacube) while still on dry ice.
14. Half the amount (175 µl) of lysis buffer mix, containing 342 µl Buffer RLT Plus (http://www.qiagen.com) and 7 µl Reducing agent DTT [2M] (https://no.vwr.com/app/Home), is added to the tumor mix on wet (normal) ice and then 0.9 µl Reagent DX (http://www.qiagen.com) is added to the same tube to prevent excessive foaming while the tumor mix is homogenized (30 Hz, 2 times for 4 min each time). The homogenization is done using a pre-cooled (4˚C) TissueLyzer LT Adapter on a TissueLyzer LT (http://www.qiagen.com).

Figure 4: Qiagen TissueLyser LT

HOMOGENIZATION WITH QIASHREDDER

15. The remaining lysis buffer mix (175 µl) is added, and the lysate is further homogenized by centrifugation (13200 rpm for 3 min) through a QIAshredder column (http://www.qiagen.com) at room temperature. The lysate (supernatant, without the pellet that reduces the total yield) is transferred into a new tube, before extraction of DNA, RNA and miRNA (see next step).

EXTRACTION OF DNA, RNA, AND MIRNA

16. DNA and RNA >200 base pairs are extracted from the same lysate as used above for each patient using the AllPrep DNA/RNA/miRNA Universal Kit (http://www.qiagen.com), automated with the use of QIAcube (http://www.qiagen.com). The extraction is performed following the custom protocol “AllPrep DNA/RNA/miRNA Universal Kit” for 60 µl input. Both the DNA and RNA fractions are immediately stored on wet ice, before long-term storage at -25˚C and -80˚C, respectively. For some samples both the AllPrep DNA spin column and the RNeasy spin column have been eluted one extra time with 30 µl EB-buffer and RNAse-/DNAse free water, respectively to increase the yield.

DNA/RNA CONCENTRATION AND SAMPLE QUALITY

17. It is important to measure both the concentration and the quality of the DNA and RNA. The quality (Abs 260/280 and 260/230 ratios) of DNA/RNA is measured using NanoDrop (http://www.nanodrop.com) and the concentration is further measured with Qubit (http://www.ThermoFischer.com). The total RNA integrity is measured using BioAnalyzer 2100 (http://www.home.agilent.com).

SEPARATION AND STORAGE OF PBMC AND PLASMA SAMPLES FROM ANTICOAGULANT-TREATED BLOOD

** Procedure originally shared by the MLC LAB, IOV, PADOVA*

Peripheral blood components are separated using Ficoll-Paque PLUS density gradient centrifugation as follows:

Figure 5: Example of 3 ml Ficoll + 6 ml blood

1. Anticoagulant-treated blood can be diluted 1:2 with isotonic medium such as RPMI medium or sterile PBS. Separation also works with undiluted blood samples. Pipette half of the total volume of blood into a 14 ml polystyrene round-bottom centrifuge tube.

2. Stratify the blood on the Ficoll very slowly and very carefully, avoiding the mixing with the Ficoll. Close the tube with the cap carefully.

Figure 6: Post-centrifuge layers

3. Centrifuge at 460 *g* for 30 min at 18 °C, with brakes off. Four (4) layers will appear:

   a) The upper is the separated plasma. This can be collected, divided into aliquots and stored at -20°C or -80°C accordingly to subsequent use.

   b) A ring of peripheral blood mononuclear cells (PBMC)

   c) The lower phase is Ficoll

   d) Pellet will contain erythrocytes and granulocytes.

4. Use a clean Pasteur pipette to transfer the PBMC to a clean tube.

5. Check the volume of the PBMC and add at least 5 times its volume of RPMI (or PBS). Centrifuge at 580 *g* for 30 min.

6. Remove the supernatant, being careful not to disturb/suction away the pellet at the bottom of the tube. Wash the pellet once again by adding more RPMI/PBS and centrifuging the tube at 350 *g* for 20 min.

7. Count PBMC (using microscope or automatic instrument as per your lab's protocols) and subdivide into pellets in sterile eppendorf tubes, centrifuge at 2900 *g* for 10 min, and store the dried pellets at -20°C or -80°C, depending on the subsequent processing step.

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PROTOCOL FOR PREPARING CFRNA FROM SERUM AND PLASMA

** Original procedure shared by Prof. Dr. Ulrike Stein https://www.mdc-berlin.de/stein*

SERUM

1. Following blood withdrawal with a 7.5-mL S-Monovette® (Sarstedt AG & Co., Nuembrecht, Germany), obtained blood samples were incubated at room temperature for at least 30 min to allow complete blood coagulation.
2. Within 1 hour post-blood drawing, serum was prepared by centrifugation for 8 min at 1800G at room temperature.
3. The obtained cell-free serum fraction should be immediately frozen at −80 °C until further processing. Unnecessary freeze–thaw cycles must be avoided. Samples should be blinded so that neither time of blood drawing nor any other information is disclosed during analysis.
4. Thaw samples on ice and immediately process after complete thawing.
5. For isolation of total RNA, the High Pure Viral RNA Kit (Roche Diagnostics, Mannheim, Germany) is used according to the manufacturer's instruction as described in [1-4]. Briefly, 350 µL serum is mixed with binding buffer, supplemented with Poly(A) carrier RNA (50 $\mu g \cdot mL^{-1}$). After RNA binding to the columns and washing steps, RNA is eluted in nuclease-free water.

PLASMA

6. Plasma was generated from cooled EDTA blood on the same day, within 7 hours post blood taking. The procedure for plasma separation is described in [3, 5].
7. 5 ml of cooled EDTA-treated blood is centrifuged at 1300 rpm for 10 min at 10°C.
8. The plasma supernatant is again centrifuged at 2500 rpm for 15 min at 4°C to remove all cell debris.
9. Samples were stored at −80°C as 400 µL aliquots.

** IMPORTANT: Investigators should be blinded as to the design, so that neither prognostic markers, nor the clinical course of the corresponding patient was disclosed during PCR-analysis.

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