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Adele Pakka

Optimising the isolation of white blood cell fractions

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Inflammatory bowel diseases such as Crohn's disease and Ulcerative colitis are common in the Western world. These diseases and their treatment are therefore also one of the interests of immunobiological research. Peripheral blood mononuclear cells (PBMCs) offer a tool to investigate these diseases.

Peripheral blood mononuclear cells have uniform, round nucleus. This group is formed by mainly lymphocytes and monocytes, which can be isolated from EDTA whole blood samples by using suitable gradient medium and density centrifugation.

The aim was to observe how the pre-analytical factors such as storage temperature and time effect on PBMC viability. This was done by counting viable cells at different time points and by preserving them in three different temperatures. Viability was detected by using trypan blue dye in two automated cell counters and Bürker's chamber. The results of automated cell counters were compared with the manual counting results.

Based on the results received room temperature seems to be the best option to store the whole blood samples and that the PBMC viability doesn't drastically drop overnight. However, due to the difficulties during the experiments made, the results of this thesis aren't reliable. There was also a notable variation in the cell counting results, which made the result comparison difficult.

Keywords	PBMC, cell counting, viability

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1 Introduction

Inflammatory bowel diseases (IBD), such as Crohn's disease and Ulcerative colitis, have increased during the past 25 years. This increase has widely been identified in the Western world. In Finland there are approximately 40 000 individuals under the diagnoses of inflammatory bowel diseases and each year over 2000 new patients are identified (IBD - tulehdukselliset suolistosairaudet). Although the aetiology of these diseases is partially unknown, some genetic and environmental factors have been recognised. For example, genetic factors can cause abnormalities in the mucous membrane of the bowel. These genetic factors can also cooperate with the environmental ones, which include diet, smoking and in its entirety, the western lifestyle. (Färkkilä 2014, Mustajoki 2017a, Mustajoki 2017b.)

IBD can be treated with biological medicinal products which are manufactured or extracted from biological sources. These sources are living systems, such as plants, animal cells and microorganisms. The main differences to synthetic drugs are bigger molecular size and complexity of the structure. Due to these differences the route of administration differs as well. Synthetic drugs can be taken through the mouth, but biological medication is often given as an injection or intravenous therapy. As a drug, biologicals are developed to be precise, meaning that they only effect on certain structures in the body. (Biologiset lääkkeet ja biosimilaarit 2017.)

Despite the efficiency and variety of medication, some patients lack proper response to the treatments. Identifying these patients is important in order to avoid unnecessary usage of drugs and the adverse effects following. Differences between the patients and responses to medication can be examined by analysing biopsies, stool and blood, from which the white blood cells (WBCs) are important. This thesis was executed in a research group of Päivi Saavalainen (IBD study) and concentrates on the isolation of peripheral blood mononuclear cells (PBMCs) as well as comparison of automated cell counters.

2 Components of blood

Blood consists of different components such as white blood cells, red blood cells (RBCs), platelets and plasma. These components transport oxygen, carbon dioxide, hormones, energy sources and metabolic waste. WBCs, RBCs and platelets form a group called blood cells, from which the white blood cells are the target of interest when it comes to immunological research. (Bjålie – Haug– Sand– Sjaastad– Toverud 2014: 316-326.)

2.1 Plasma and platelets

Approximately 92 % of the blood plasma is water which allows it to transport water-soluble substances. Other components in plasma are mostly proteins; albumins, fibrinogens and globulins, which transport the other water-insoluble substances and protect the human body against infections. (Yleistä verisoluista; Bjålie et al. 2014: 316-326.)

Platelets are small cells which don't have nucleus. These cells derive from megakaryocyte, each producing approximately 1000-5000 platelets. Platelets, also known as thrombocytes, participate in hemostasis by forming clots with the help of coagulation factors. (Bjålie et al. 2014: 325-328; Tietoa verestä; Verihiutale L. Trombosyytti, Blodplättar; Lassila – Porkka – Remes – Savolainen 2015: Trombosyyttien tuotanto.)

2.2 Red blood cells

Red blood cells transport oxygen and carbon dioxide. Just like platelets these cells also lack nucleus and are rather small (7-8 µm) compared to the white blood cells (Ek 2009: 49). RBCs are flat by shape which makes the oxygen – carbon dioxide swap easier, by increasing the surface area. Due to the fact that these cells don't have nucleus or any other cell organelles they don't consume oxygen themselves. A protein called hemoglobin is necessary in order to transport oxygen from lungs to tissues. (Bjålie et al. 2014: 317-318; Erytrosyytit eli punasolut.)

Red blood cells are formed in red bone marrow through multiple cell divisions. This process takes approximately 7 days. A normal production of red blood cells requires suffi-

cient nutrition concerning iron, proteins, vitamin B and folate. A hormone called erythropoietin regulates the RBC production. Red blood cells live approximately for 120 days. (Erytrosyytit eli punasolut; Bjålie et al. 2014: 318-321.)

2.3 White blood cells

White blood cells are responsible for immune response, in which each of them has their own task. Blood, for WBCs, is mainly just a passageway in order to get to the areas of infection. As a group WBCs are diverse and can be divided into two main groups based on the shape of the cell nucleus, appearance of granules and the place of formation. These two groups are polymorphonuclear leukocytes and mononuclear leukocytes as presented in figure 1. (Leukosyytit eli valkosolut; Bjålie et al. 2014: 322-323.)

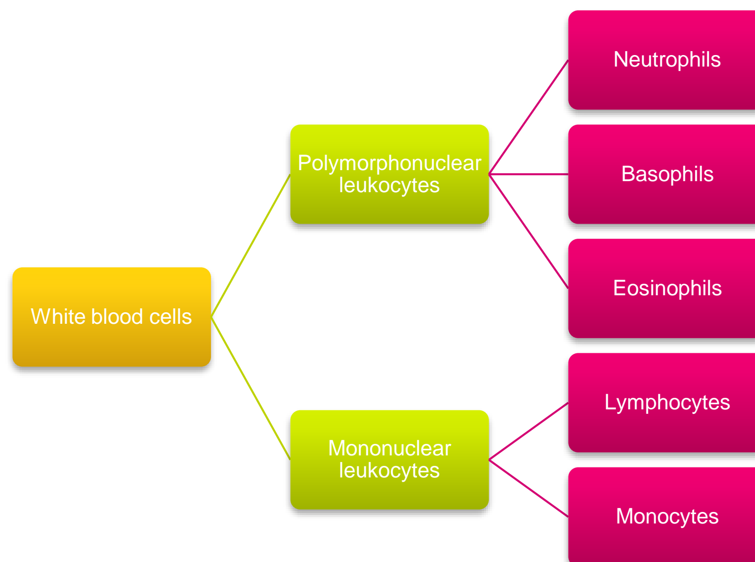

Figure 1. Division of white blood cells.

2.3.1 Polymorphonuclear leukocytes

White blood cells, which have lobed nucleus and granules in cytoplasm are called polymorphonuclear leukocytes (PMNLs). PMNLs include three ''subgroups'' of cells, which are neutrophils, basophils and eosinophils. These cells are produced in bone marrow

and are short-lived, living approximately up to 4-5 days. (Hedman – Heikkinen – Huovinen – Järvinen – Meri – Vaara (edit.) 2011: Immuunijärjestelmän solut ja niiden kehitys; Bjålie et al. 2014: 324–323.)

Neutrophils are the most frequent type of leukocyte in bloodstream. These cells are capable of eliminating bacteria by using phagocytosis. During the infection the amount of neutrophils increase remarkably and they use blood as a route to get to the areas of infection. Granules are also meaningful for immune response by containing substances, which activate the immune system. These substances can, for example, activate the complement system and enhance the phagocytosis. (Neutrofiili; Hedman et al. 2011: Immuunijärjestelmän solut ja niiden kehitys; Bjålie et al. 2014: 324.)

Eosinophils are often connected to allergic reactions and parasitic infections (Hedman et al. 2011: Immuunijärjestelmän solut ja niiden kehitys). These cells are easy to recognize based on the red-orange coloring of the granules. Eosinophils excrete enzymes and proteins in order to damage cell membranes and eliminate parasites. (Bjålie et al. 2014: 324; Eosinofiili; Ek 2009: 32.)

Basophils have large purple colouring granules, which are released during allergic reactions and inflammation (Ek 2009: 33). These granules contain histamine, which is a neurotransmitter causing typical symptoms of allergy and inflammation (Hedman et al. 2011: Immuunijärjestelmän solut ja niiden kehitys). Alongside eosinophils, these cells are capable of phagocytosis, but don’t play an important role during bacterial infections (Bjålie et al. 2014: 324–325; Basofiili).

2.3.2 Mononuclear leukocytes

Peripheral blood mononuclear cells (PBMCs) is a term used to describe cells with uniform, round nucleus. This group mainly consist of lymphocytes whereas monocytes are a minority. PBMCs play an important role in the immune system, protecting the body from pathogens such as harmful viruses and bacteria. Common methods used in order to protect the body from these pathogens are phagocytosis and the production of antibodies. (Hedman et al. 2011: Immuunijärjestelmän solut ja niiden kehitys; Bjålie et al. 2014: 322–325; Pollak 2016.)

3 to 8 % of white blood cells are monocytes, cells which are large by size (15-25 µm) and capable of phagocytosis (Monosyytti). These cells have an oval/horseshoe shaped nucleus and azurophilic colouring granules (Ek 2009: 27; Farquhar, Marilyn G. – Ford Bainton, Dorothy – Nichols, Barbara A. 1971). Monocytes circulate in blood for 1-3 days before proceeding into tissues where they differentiate into macrophages. Other tasks, besides phagocytosis, are presenting antigens and cleansing, which includes elimination of other unwanted particles and damaged cells. (White blood cells; Bjålie et al. 2014: 325; Hedman et al. 2011: Immuunijärjestelmän solut ja niiden kehitys.)

Lymphocytes form 20-30 % of all white blood cells (Lymfosyytti). Main subpopulations are B (bone marrow derived) and T (thymus derived) lymphocytes and natural killers, which all have the same basic structure. These cells are important for acquired immunity by recognising antigens and producing antibodies. Lymphocytes come in various sizes between 7 – 30 µm. (Bjålie et al. 2014: 325; LeBien– Tedder 2008; Ek 2009: 18-26.)

B cells are responsible for humoral immunity. Humoral immune response is pervasive due to the production of antibodies, which spread to the entire body through blood. B cells express immunoglobulins A, D, E, G and M, which enables the adherence and identification of antigens. These cells require activation in order to transform into antibody producing plasma cells or memory B cells. This usually requires presence of antigens and T helper cells. The final differentiation is achieved through multiple cell divisions and selection of better antigen binding cells (Hedman et al. 2011: Lymfosyyttien aktivaatio). Memory B cells can be activated again by the same pathogens, which leads to generation of new plasma - and memory B cells, enabling rapid immune response. (Lymfosyytti; B cells – Function, activation, lineage and markers.)

T cell immune response is antigen specific and more localised compared to the humoral response. These cells can be divided into several subgroups, inter alia, cytotoxic-, helper- and regulatory cells. Differentiation takes place in lymphatic tissues and requires a presence of antigen presenting cells. Each group has a role in immune response. Cytotoxic T cells protect the body from intracellular pathogens. Helper T cells also protect the body from pathogens, but they also activate B cells into producing antibodies. Regulatory T cells suppress T cells responses, which prevents tissue injuries. (Hedman et al. 2011: Lymfosyyttien aktivaatio; Sytotoksiset T-solut.)

Natural killers eliminate cells, infected by viruses and parasites, by secreting cytotoxic substances. These cells are also capable of recognising and eliminating body's normal altered cells, like cancer cells. (Hedman et al. 2011: Immuunijärjestelmän solut ja niiden kehitys.)

3 PBMC isolation

Centrifugation is a method used to separate different components of whole blood based on the particle size and density. Centrifugal force causes particle sedimentation, in which denser and larger particles sediment faster and form a layer to the bottom of the centrifugation tube. (Lehtonen – Jaarinen – Jansson – Pohjakallio – Repo 2012: 44-45.) Density gradient centrifugation methods are based on different particle densities but also the density of the gradient medium in use (Ficoll-Paque PLUS, Frei: 7-14). This method can be used to isolate PBMCs from whole blood samples.

Density gradient centrifugation separates particles based on their buoyancy density and sedimentation rate. Denser particles, such as red blood cells, sink to the bottom whereas the blood plasma sediments on the top. PBMC fraction can be found right below the plasma layer, from which it can be separated and washed for further analyses (Figure 2). During the thesis process Ficoll-Paque medium and Leucosep tubes were used in order to separate PBMCs from whole blood samples. (Instruction Manual – Leucosep; Gradienttisentrifugointi; Boujtita – Collart – Hatton – Oceana.)

Figure 2. Leucosep tube after centrifugation. Top yellow layer is plasma and right below it PBMC fraction. Red blood cells and PMNLs sediment at the bottom.

3.1 Gradient medium

The gradient medium is used to separate the particles and choosing the right separation medium is crucial in order to reach the optimal separation. The medium should be chosen based on its application. For example, inorganic salts can be used as a medium to separate DNA or RNA, whereas polysaccharide mediums like Ficoll can be used for the isolation of mammalian cells. Besides medium's application it should also have certain properties such as adequate solubility and accessibility. The medium should also be inert with the components of the sample to retain the sample's biological activity. (Frei: 7-14.)

Ficoll is commonly used medium for PBMC isolation. This method works also for smaller volumes of blood enabling the isolation procedure to be done, for example, from children's samples as well. The PBMC fraction formed after the centrifugation consist of, to some extent, also from PMNLs, platelets and red blood cells (max. 10%). Platelets and red blood cells can be removed by washing procedure. (Ficoll-Paque PLUS, Boujtita et al.)

3.1.1 Other density gradient centrifugation methods

Histopaque is a method that can be used for PBMC and neutrophil isolation. Gradient medium, which contains polysucrose and sodium diatrizoate is adjusted to certain density. Medium with a density of 1.077 g/mL can be used for PBMC isolation, which combined with 1.119 g/mL medium enables the isolation of both PBMCs and PMNLs. (Histopaque – 1077; Histopaque – 1119.)

When adding the solutions, a proper technique is required. Denser solution is added first, after this Histopaque 1.077 g/mL and the blood sample are added dropwise. After centrifugation the PBMC layer is formed below plasma, just like when using Ficoll. PMNLs will form a layer on top of the red blood cells. (Frei: 14-16.)

3.2 Leucosep

Leucosep tubes have a porous barrier, which consists of high-grade polyethylene. Although the isolation of PBMCs could be done just by using the gradient medium, the barrier does make the isolation process faster and easier. For example, the sample material can't be mixed with the Ficoll medium, which has been centrifuged below the barrier. After centrifugation, the PBMCs form a layer on top of the barrier which makes the collection easier and prevents contamination with the red blood cells and PMNLs. (Instruction manual – Leucosep.)

4 Quality in laboratory testing

Laboratory tests are utilized to evaluate the condition and the treatment of the patient. Therefore, it is essential to minimise the errors occurring during the laboratory process to avoid unnecessary expenses and delays in the treatment. This process includes three phases, which are pre-analytical, analytical and post-analytical. Although all these are important when it comes to quality, the main concentration was directed on pre-analytical and analytical phases. All three phases and their contents are described in figure 3. (Matikainen – Miettinen – Wasström 2010: 9-13.)

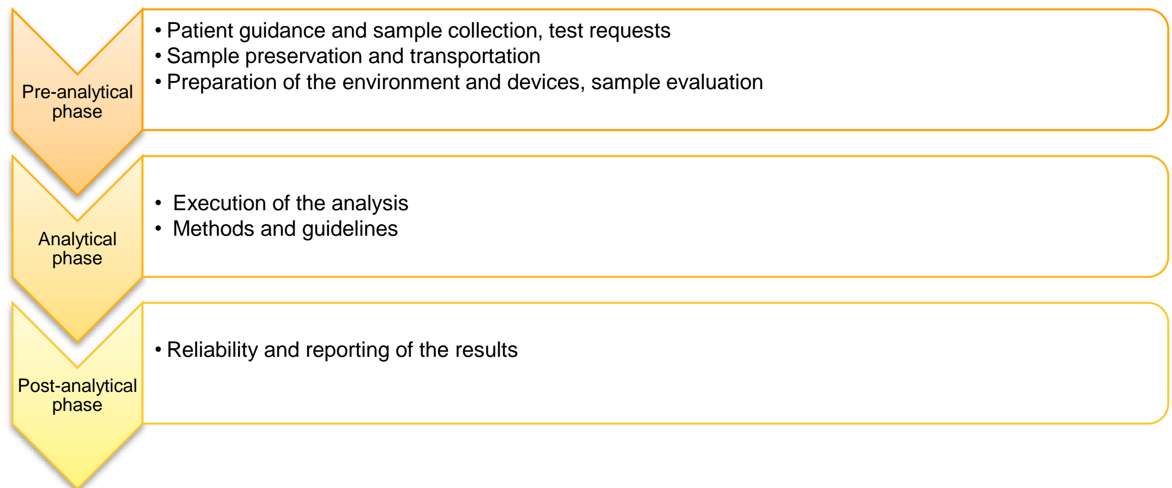

Figure 3. The three phases of laboratory process presented in vertical order.

4.1 Pre-analytical phase

The pre-analytical factors are patient guidance, sample collection, sample preservation and transportation. The errors which emerge from these components account for 50-70% of all aberrations, making it the most high-risk stage in the entire laboratory process. (Hoitotyön Tutkimussäätiö; Plebani 2012.)

4.1.1 Sample collection

Although home sample collection (urine and stool samples) is considered to be more sensitive for errors, laboratory sampling has its' own challenges as well. Representative sample is sufficient, uncontaminated and it fulfils the requirements for further analyses.

Some aberrations that emerge during the sample collection are sample contamination, long usage of tourniquet, incorrect volume and heterogeneity. Mixing the collection tubes reduces clotting and hemolysis, which can interrupt the laboratory analyses. Contamination can be prevented by aseptic working methods. Long usage of tourniquet causes hemolysis but also leads to high concentrations of proteins, cells and lipids. (Kurec 2016; Plebani 2012.)

During the thesis process all samples were taken in standardized conditions. Tourniquet wasn't used during the blood collection. EDTA anticoagulant tubes were mixed after sampling to reach homogeneity and to avoid clots.

4.1.2 Sample preservation and transportation

It is important to notice that the sample, even after collection, is active when it comes to chemical reactions. Depending on the sample material it might require certain temperature conditions or protection from sunlight. Inappropriate conditions in preservation or transportation have an impact on sample quality and eligibility for laboratory analyses. (Kurec 2016; Matikainen et al. 42; Plebani 2012.)

In order to have the same starting point for all the samples analyzed for this thesis, the transportation phase was excluded. This was to avoid the variable conditions and delays occurring during the transportation.

4.2 Analytical phase

The analytical phase consists of specimen testing. Some causes for analytical errors are malfunctioning equipment, incorrect calibration, user related factors and improper methods (Hemminki – Hiltunen – Hägg – Linko – Järvenpää – Kärhä – Saarinen – Simonen 2011: 38-42; Asibey – Ephraim – Laing – Sadique – Sakyi 2015). It should be notified, that no result is completely accurate. Consistent results are considered reliable, which can be achieved by defining and reducing the probability of possible errors. In laboratory, this can be achieved by internal and external quality assessment. (Hemminki et al. 2011:63.)

During the thesis process we examined whether the red blood cells (RBCs) have an effect on the results received from the automated cell counters. RBC contamination can induce errors in measured PBMC concentrations. (Gandhi – Hei – Kuksin – Laverty – Li-Ying Chan – Nejad – Qiu 2012.)

4.3 Post-analytical phase

Post-analytical phase consists of assessment of the reliability, laboratory reporting and follow-up planning (Matikainen et al. 2010: 12). Missing or delayed laboratory reporting, wrong interpretation of the results and improper follow-up planning are errors that occur during the post-analytical phase (Hawkins 2011).

5 Aim

The aim of this thesis was to answer following research questions:

1) How pre-analytical factors (time and temperature) effect on PBMC viability
2) Which automated cell counter is more reliable when assessing cell viability
3) Does RBC contamination effect on cell viability

In order to assess the effect of pre-analytical factors the samples were held in different storage temperature for 3 hours. The temperatures used were +4, +37 and room temperature. As a reference one sample was isolated immediately after the collection.

Results comparison was executed for two automated cell counters (Countess II FL and TC 20 Automated cell counter). Manual counting with Bürker chamber was used as a reference. In addition, the effect of possible RBC contamination was assessed by comparing the results between washed and not washed samples (Gandhi et al. 2012).

6 Methods

6.1 Blood collection and processing

Peripheral blood samples were collected in EDTA anticoagulant collection tubes without tourniquet. After collection, samples were mixed in sterile container to ensure homogeneity and subsequently divided into same sized portions. Blood samples were collected from the same person each day. Mixing was done every time, except when the effect of tourniquet and time (24 hours) were examined.

Blood samples were held in different storage temperatures for three hours. These temperatures were +4, room temperature and +37. As a reference one or two EDTA samples were isolated immediately.

PBMC isolation was done by following the sample handling guide (appendix 1). To eliminate RBCs, additional wash with water and 2 x phosphate-buffered saline (PBS) was executed. The additional wash was done for one sample per storage temperature.

6.2 Cell counting

Cell viability was determined by using trypan blue dye. Results of two automated cell counters, Countess II FL and TC 20 Automated cell counter were compared with the manual counting results.

6.2.1 Trypan blue

Trypan blue solution is a cell stain, commonly used to assess cell viability. This stain is negatively charged, and in order to dye the cells it needs to interact with the internal region of the cells. Viable cells, which have intact and selective cell membrane do not take up the blue stain. Viable cells can be detected under a microscope as bright and undyed (figure 4.). (Ngo-Camus – Puhar – Ramarao – Tran 2011.)

Non-viable (dead) cells have damaged cell membranes, which enables the trypan blue solution to enter these cells. However, when exposed to trypan blue for significant amount of time, viable cells might blend with the dye or get damaged. This exposure induces errors in viability assessment. (TC20 Automated Cell Counter 2011: 5, 12; BIO-RAD support.)

6.2.2 Automated cell counters

Both Countess II FL and TC20 use trypan blue dye to determine the cell viability. Samples were diluted 1:1 with trypan blue solution. Both of the automated counters use their own disposable slides with two chambers marked with A and B. Volume of 10 microliters

was added to the chamber. As a method automated cell counting is fast, taking approximately 10-30 seconds. (TC20 Automated Cell Counter 2011: 13; Countess II Automated Cell Counters: 2.)

Results are numeral and presented visually. Both automated cell counters offer total and live cell quantity and percentage, which are counted per milliliter. Countess II FL also shows the dead cell count. Cell diameter range varies: Countess II FL has the range of 7-60 µm, whereas TC20 has 6-50 µm. This means that cells in these size ranges can be identified for viability. Both of the automated counters can count cell concentration up to 1 x 10^7. (TC20 Automated Cell Counter 2011: 4; Countess II Automated Cell Counters: 8.)

6.2.3 Manual counting

Isolated PBMC fraction was diluted 1:1 with trypan blue for manual cell counting. For this purpose, Bürker chamber was used and 3 x A square was counted from each sample. A-square has the surface of 1/25 square millimeters and is defined by three lines each side. Live cells that were on top of the lines on one vertical side and horizontal side were also included to the count.

Figure 4. Trypan blue dyed cells in Bürker chamber. Live cells can be seen bright and undyed.

Following formula was utilized to count the total amount of cells per mL:

$$\text{Cells/mL} = \frac{2{,}5 \cdot 10^5 \cdot \text{dilution factor (2)} \cdot \text{cells counted}}{3}$$

Manual counting is sensitive for errors related to the counting, but sometimes assessing the viability of individual cells can be challenging. This is because trypan blue has the ability to blend into the surfaces of viable cells.

7 Results

Cell viability in overnight or 24-hour samples was one area of interest. Beforehand it was already known that cell viability stays on acceptable level when it comes heparinized samples. Received results suggest that the cell viability remains on moderate level despite the usage of EDTA anticoagulant tubes. These samples were not mixed before the isolation.

Table 1. Results of longer storage samples and usage of tourniquet. * = sample taken elsewhere and kept in room temperature overnight. No RBC elimination

	Manual counting	Countess II FL	Biorad TC20
Tourniquet	4,67 x 10^6	2,25 x 10^6, 83%	2,49 x 10^6, 89%
No tourniquet	3,5 x 10^6	1,92 x 10^6, 76%	2,05 x 10^6, 90%
Immediately isolated	3,00 x 10^6	1,67 x 10^6 83%	2,00 x 10^6, 74%
24 hour sample	6,83 x 10^6	2,84 x 10^6, 73%	4,65 x 10^6, 62%
Overnight sample*	5,00 x 10^6	8,50 x 10^5, 81 %	4,17 x 10^5, 73 %

Results of manual counting and both automated cells counters. Table 2 shows the amount of living cells and table 3 total and living cells (only the automated counters). Numbers 1, 2 and 3 indicate different days for measurements (day 1, day 2, and day 3). Difference to the reference shows the % difference between the automated counting

results and manual counting. Letters B and C refer to the automated cell counters B = BIORAD TC 20 and C = Countess II FL. Wash refers to the RBC elimination.

Table 2. Living cells.

Living cells / mL

1. *Measurement*
2. *Measurement*
3. *Measurement*

Temperature and time	Manual counting (reference)	Countess	Biorad TC20	% difference to reference C= Countess B= Biorad
Room temperature Immediately isolated washed	**1.** 9,17 x 10^6 **2.** 9,33 x 10^6 **3.** 1,67 x 10^5	**1**. 2,58 x 10^5 70 % **2**. 1,39 x 10^6 83 % **3**. 5,86 x 10^3 7 %	**1**. 2,28 x 10^5 98 % **2**. 1,62 x 10^6 78 % **3**. 2,55 x 10^5 16 %	**1**. C. 97,2% smaller B: 97,5 % smaller **2**. C: 85 % smaller B: 82,6 % smaller **3**. C: 96,5 % smaller B: Reference is 52,7 % smaller
Room temperature Immediately isolated Not washed	**1.** 1,12 x 10^7 **2.** 8,83 x 10^6 **3.** 5,5 x 10^6	**1.** 3,06 x 10^6 87% **2.** 3,29 x 10^6 83 % **3.** 1,66 x 10^6 92 %	**1.** 8,89 x 10^5 69 % **2.** 5,01 x 10^6 84 % **3.** 1,84 x 10^6 69 %	**1**. C: 72,7% smaller B: 92,1 % smaller **2.** C: 62,7 % smaller B: 43,3 % smaller **3**. C: 69,8 % smaller B: 66,5 % smaller
+4, 3 hours Washed	**1.** 8,5 x 10^6 **2.** 6,83 x 10^6 **3.** 2,17 x 10^6	**1.** 8,56 x 10^5 81% **2.** 1,80 x 10^6 90 % **3.** 1,64 x 10^5 82 %	**1.** 1,30 x 10^6: 59% **2.** 2,42 x 10^6 93 % **3**. 5,53 x 10^5 17 %	**1.** C: 89,9 % smaller B: 84,7 % smaller **2.** C:73,6 % smaller B: 64,6 % smaller **3.** C: 92,4 % smaller B: 74.5 % smaller

Temperature And time	Manual counting (reference)	Countess	Biorad TC 20	% difference to refe-rence
+4, 3 hours Not washed	**1.** 8,5 x 10^6 **2.** 6,83 x 10^6 **3.** 3,33 x 10^6	**1.** 3,07 x 10^6 75 % **2.** 3,89 x 10^6 87 % **3.** 7,51 x 10^5 82 %	**1.** 6,16 x 10^6: 75% **2.** 3,53 x 10^6 85 % **3.** 4,50 x 10^5 36 %	**1.** C: 63, 9 % smaller B: 27,5 % smaller **2.** C: 43 % smaller B: 48,3 % smaller **3.** C: 77,4 % smaller B: 86,5 % smaller
+37, 3 hours Washed	**1.** 4,0 x 10^6 **2.** 1,67 x 10^5 **3.** 2,00 x 10^6	**1.** 7,39 x 10^5 78 % **2.** 0,00 0 % **3.** 3,34 x 10^5 86 %	**1.** 1,02 x 10^6 62 % **2.** 1,08 x 10^4 12 % **3.** 5,31 x 10^5: 36 %	**1.** C: 81,5 % smaller B: 74,5 % smaller **2.** C: 100 % smaller B: 93,5 % smaller **3.** C: 83,3 % smaller B: 73,5 % smaller
+37, 3 hours Not washed	**1.** 9,5 x 10 ^6 **2.** 2,67 x 10^6 **3.** 7,33 x 10^6	**1.** 2,77 x 10^6 65 % **2.** 5,98 x 10^5 77 % **3.** 1,08 x 10^6 79 %	**1.** 5,50 x 10^6 81 % **2.** 6,88 x 10^5 80 % **3.** 9,43 x 10^5 63 %	**1.** C: 70,8 % smaller B: 42,1 % smaller **2.** C: 77,6 % smaller B: 74,2 % smaller **3.**C: 85,3 % smaller B: 87,1 % smaller
Room tempera-ture 3 hours Washed	**1.** 6,17 x 10^6 **2.** 5,00 x 10^5 **3.** 1,67 x 10^5	**1.** 7,57 x 10^5 73 % **2.** 1,17 x 10^4 22 % **3.** 0,00 0 %	**1.** 5,69 x 10^5: 38 % **2.** 2,71 x 10^4 31 % **3.** 2,76 x 10^5: 15 %	**1.**C: 87,7 % smaller B: 90,8 % smaller **2.** C: 97,6 % smaller B: 94,6 % smaller **3.** C: 100 % smaller B: reference is 65,3 % smaller
Room tempera-ture 3 hours Not washed	**1.** 1,00 x 10^7 **2.** 2,83 x 10^6 **3.** 9,67 x 10^6	**1.** 2,31 x 10^6 74 % **2.** 3,40 x 10^5 73 % **3.** 3,89 x 10^6 79 %	**1.** 4,26 x 10^6 76 % **2.** 7,05 x 10^5 76 % **3.** 4,17 x 10^6 74 %	**1.** C: 76,9 % smaller B: 57,4 % smaller **2.** C: 88 % smaller B: 75,1 % smaller **3.** C: 59,8 % smaller B:56,9 % smaller

Table 3. Total cells and live cells from both automated cell counters.

Temperature and time	*Countess*	*Biorad (TC20)*
	T: Cells in total *L: Live cells*	
Room temperature Immediately isolated Washed	**1.** T: 3,69 x 10^5, L: 2,58 x 10^5 70 % **2.** T: 1,67 x 10^6 L: 1,39 x 10^6 83 % **3.** T: 8,80 x 10^4 L: 5,86 x 10^3 7 %	**1.** T: 2,33 x 10^5 L: 2,28 x 10^5 98 % **2.** T: 2,08 x 10^6 L: 1,62 x 10^6 78 % **3.** T: 1,61 x 10^6 L: 2,55 x 10^5 16 %
Room temperature Immediately isolated Not washed	**1.** T: 3,54 x 10^6, L: 3,06 x 10^6 87 % **2.** T: 3,95 x 10^6 L: 3,29 x 10^6 83 % **3.** T: 1,80 x 10^6 L: 1,66 x 10^6 92 %	**1.** T: 1,28 x 10^6 L: 8,89 x 10^5 69% **2.** T: 5,93 x 10^6 L: 5,01 x 10^6 84 % **3.** T: 2,66 x 10^6 L: 1,84 x 10^6 69 %
+ 4, 3 hours Washed	**1.** T: 1,06 x 10^6 L: 8,56 x 10^5 81% **2.** T: 2,01 x 10^6 L: 1,80 x 10^6 90 % **3.** T: 1,99 x 10^5 L: 1,64 x 10^5 82 %	**1.** T: 2,18 x 10^6 L: 1,30 x 10^6 59 % **2.** T: 2,60 x 10^6 L: 2,42 x 10^6 93 % **3.** T: 3,22 x 10^6 L: 5,53 x 10^5 17 %

Temperature and time	Countess	Biorad
+4, 3 hours Not washed	**1.** T: 4,09 x 10^6 L: 3,07 x 10^6 75% **2.** T: 4,47 x 10^6 L: 3,89 x 10^6 87 % **3.** T: 9,15 x 10^5 L: 7,51 x 10^5 82 %	**1.** T: 8,26 x 10^6 L: 6,16 x 10^6 75 % **2.** T: 4,17 x 10^6 L: 3,53 x 10^6 85 % **3.** T: 1,24 x 10^6 L: 4,50 x 10^5 36 %
+ 37, 3 hours Washed	**1.** T: 9,44 x 10^5 L: 7,39 x 10^5 78 % **2.** T: 6,45 x 10^4 L: 0,00, 0 % **3.** T: 3,87 x 10^5 L: 3,34 x 10^5 86 %	**1.** T: 1,66 x 10^6 L: 1,02 x 10^6 62 % **2.** T: 9,21 x 10^4 L: 1,08 x 10^4 12 % **3.** T: 1,50 x 10^6 L: 5,31 x 10^5 36 %
+ 37, 3 hours Not washed	**1.** T: 4,25 x 10^6 L: 2,77 x 10^6 65 % **2.** T: 7,74 x 10^5 L: 5,98 x 10^5 77 % **3.** T: 1,38 x 10^6 L: 1,08 x 10^6 79 %	**1.** T: 6,77 x 10^6 L: 5,50 x 10^6 81 % **2.** T: 8,62 x 10^5 L: 6,88 x 10^5 80 % **3.** T: 1,50 x 10^6 L: 9,43 x 10^5 63 %
Room temperature 3 hours Washed	**1.** T: 1,03 x 10^6 L: 7,57 x 10^5 73 % **2.** T: 5,28 x 10^4 L: 1,17 x 10^4 22 % **3.** T: 5,86 x 10^4 L: 0,00 0%	**1.** T: 1,48 x 10^6 L: 5,69 x 10^5 38 % **2.** T: 8,67 x 10^4 L: 2,71 x 10^4 31 % **3.** T: 1,81 x 10^6 L: 2,76 x 10^5 15 %

Temperature and time	Countess	Biorad
Room temperature 3 hours Not washed	**1.** T: 3,13 x 10^6 L: 2,31 x 10^6 74 % **2.** T: 4,69 x 10^5 L: 3,40 x 10^5 73 % ***3.*** T: 4,94 x 10^6 L: 3,89 x 10^6 79 %	**1.** T: 5,63 x 10^6 L: 4,26 x 10^6 76 % **2.** T: 9,27 x 10^5 L: 7,05 x 10^5 76 % ***3.*** T: 5,66 x 10^6 L: 4,17 x 10^6 74 %

7.1 Interpretation and reliability

These results suggest that the room temperature is on average the best storage temperature for PBMCs. However, these results aren't considered reliable because of small sample size and the variation between results. Because of this variation no conclusions were made about the reliability of automated cell counters or about the best storage temperature.

It should be noted that the differences between the results are caused by various aberrations and time period between the measurements, since those were not completed on consecutive days. Therefore, possible changes in donator's health and physical condition have an effect on the results as well.

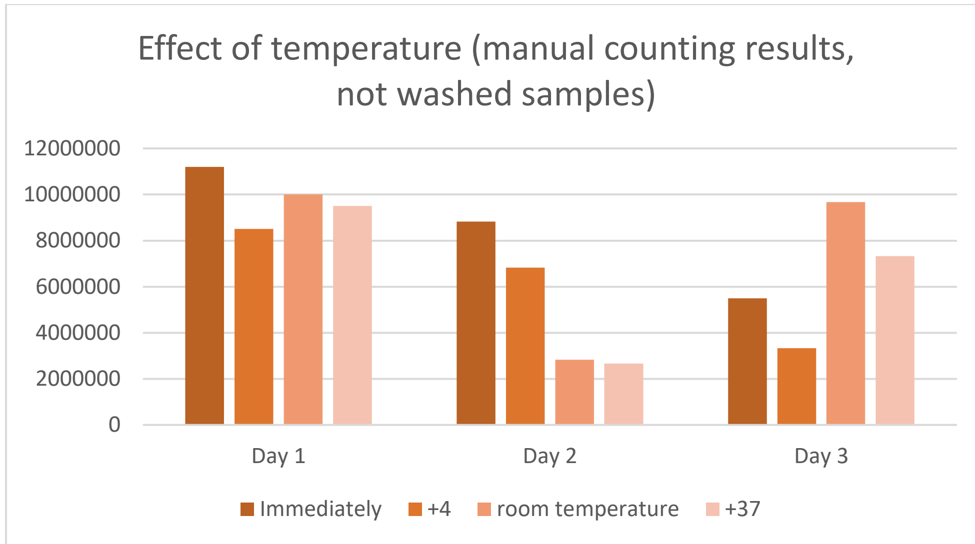

Figure 5. Effect of temperature on PBMC viability.

The additional wash with water was done for one sample per each temperature. There was no remarkable difference in the viability % between the not washed and washed samples. In some cases, the washing reduced the viability, which might have been because of the low number of RBCs and damaging effect of the water on WBCs. These findings were conflicted with practice-based observations before the thesis process. Figures 5 and 6 are visual presentation of the results.

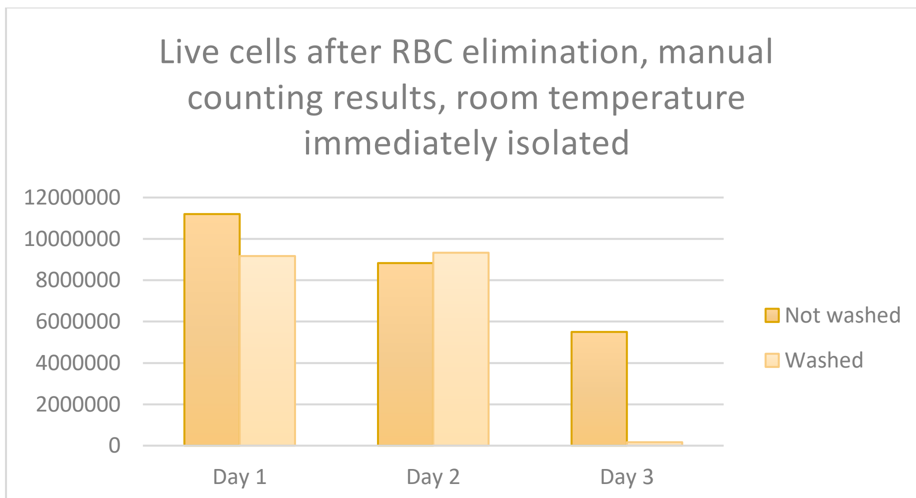

Figure 6. Live cells after RBC elimination.

7.2 Errors

Day 1 the samples were handled in a bit different way. Otherwise the protocol for the isolation was the same except after the first regular wash with PBS we divided the sample in two parts. This might have caused an error to the first results and to increase the reliability it was decided to process the samples with the same protocol (appendix 1).

Manual cell counting is sensitive for errors. This was due to my inexperience in manual counting but also because not all the cells were clearly blue or clearly bright which might cause errors. Also, during the sample processing, it was already noticed that some of the leucosep tubes did not have clear PBMC enriched layer, so the cells were unevenly divided already in the beginning of the isolation. This might have been caused by the reactivity of the cells, leading to white blood cell agglutination.

8 Research ethics and professional development

8.1 Ethics

Research ethics provides tools for professionally executed and reliable research. Some important aspects are reliability of results and information, sincerity, professional development, repeatability and valuation of dignity (Kankkunen – Vehviläinen-Julkunen 2015: 211-212). Before the execution thesis contract was made with the research group. Blood samples were collected from volunteers, preserving their anonymity. All the material was handled confidentially. (Kankkunen et al. 2015: 219.)

Literature and internet sources were evaluated for their reliability. This was done by comparing the information received with other sources and by avoiding the usage of secondary sources. Methods and results were described accurately and fabrication of the results was evaded. Therefore, the results presented in this thesis are authentic. Plagiarism was avoided by using turnitin program. (Kankkunen et al. 2015: 211-225; Moilanen – Ojasalo – Ritalahti 2015: 31-32.)

8.2 Professional development

During this project I gained deeper knowledge of cell biology and immunology, which are important sectors in laboratory work and research. Some important areas of development are work planning, information retrieval and execution of a research, which also includes interaction with the research group. All these skills are useful in working life, learning and future studies.

9 Discussion

Aim of this thesis was to optimize the PBMC isolation and compare the results of automated cell counters. This research was unable to answer given research questions, due to the small sample size and other variants occurring during the process. Comparison of automated cell counters might have been clearer if the execution time was significantly longer. Manual counting is sensitive for errors caused by differences between individuals and unclear cases.

In this thesis the PBMC viability was observed in a few points of time. It might be beneficial to assess the viability with more time points, but this would have also required longer execution time.

Results were sent to the manufacturers as presented in chapter 7 (Results), due to the remarkable variation between the results. These automated cell counters both base the viability assessment on trypan blue dye. However, there might be other differences between these counters, which remained unknown during the process.

Altogether working in a multi-professional research group has given me a good base to develop in a field of biomedical laboratory sciences. Feedback received from the research group and school has taught me a lot about the process of executing and writing a thesis. My internship included also a lot of practical work in a laboratory, which also helped me to preserve the connection to a clinical laboratory work.

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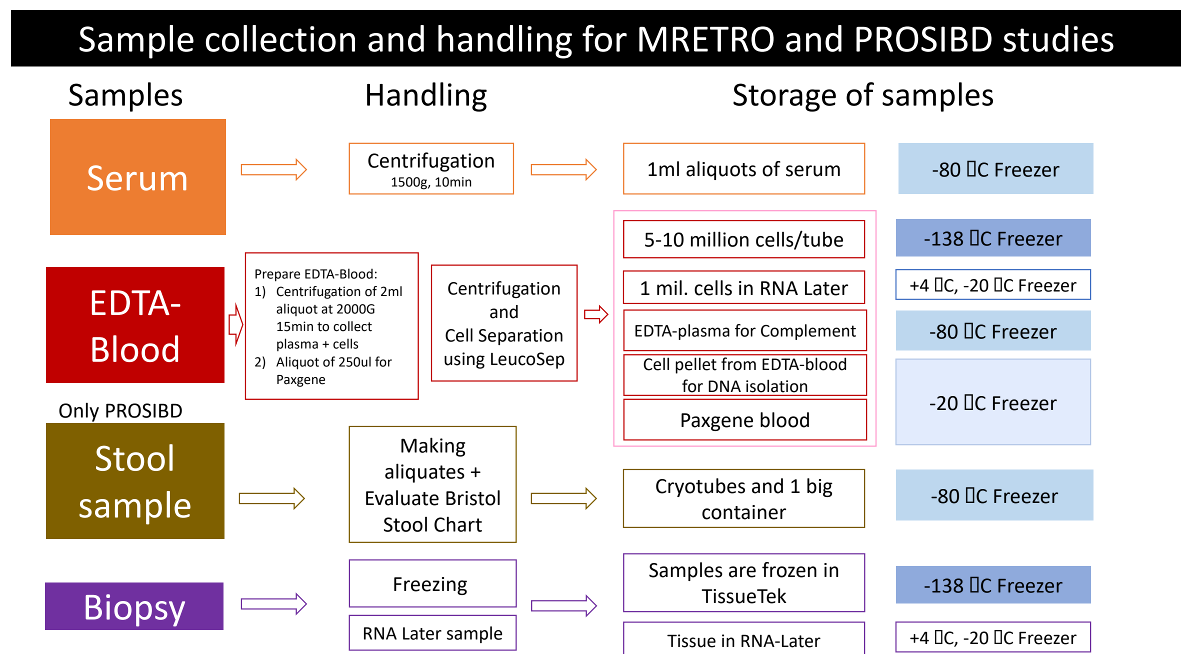
IBD sample processing

IBD-potilaiden näytteiden käsittely (23.1.2018 Eija Nissilä/Hanne Ahola)

Projektit MRETRO ja PROSIBD



Näytteiden kirjaaminen

Tiedot tutkimuspotilaista pidetään päiväkirjassa lukkojen takana. Se löytyy toiseksi ylimmästä lukollisesta laatikosta RNA-pöydän viereisestä työpisteestä. Avain on laatikossa hyllyllä. BM1:ssä kirja laukkukaapissa. Avain Hannella ja sovitusti toisaalla. Päiväkirjaan kirjataan:

Tutkimuskoodi, joka on RET tai PRO -sarjaa tutkimuksesta riippuen, tutkimuspotilaan nimi, henkilötunnus, pvm, mitä näytteitä on saatu ja miten ne on jaettu putkiin, sekä mihin laatikoihin ne on pakstettu. Pirkko antaa koodit PRO-näytteille, kirjaaja RET-näytteille. Samaan kohtaan liimataan tarra potilaan veriputkesta tai ylimääräisestä mukana tulleesta tarrasta, jossa on näytteenoton ajanhetki ja muita tietoja. Kirjataan myös RETRO/PROSIBD-excel-tiedostoon, mitä näytteitä on otettu, tiedot näytteistä ja soluista

sekä solujen pakastuspaikat. Kirjataan ylös päiväkirjaan myös mikäli jotain poikkeavaa on tapahtunut näytteiden käsittelyssä tai säilytyksessä.

Tarrojen printtaus

Tarrat printataan Kroko-ohjelmalla. Valitse ryhmän kansiosta H304/cd_group_ne/Kroko 36C pohjat/Yliopisto_epparitarrat kansiosta tarrapohjat seuraavasti:
Soluja varten Cryo-pakasteputkiin pidempi tarra: 2_rows_cryo
Muita putkia (seerumi, EDTA-plasma, RNA-later, Paxgene, uloste) varten: 2_rows_short
Näissä käytetään leveämpää (½) kasettia.
Korkkitarrat (RNA-later, Paxgene): Korkkitarra ja tähän käytetään *kapeampaa* (¼) kasettia.
MRETRO tai PRO-excelistä voidaan kopioida printattavat tiedot (koodi, näyte) 2-sarakkeissiin ruutuihin "stickers" -välilehdeltä. Klikkaamalla keskelläolevaa tarraruutua printtaus aktivoituu ja valitse printattava määrä. Jos teet samasta potilaasta useisiin näytteisiin tarrat, on helpompi tehdä koko paketti valmiiksi excelin puolella ja kopioida suoraan Krokoon, jossa valitaan sitten 1 kpl (esim 7 kpl serum, 1 kpl PaxGene jne).

Näyte-exceleissä on valmiit tarvittavat tarrasetit molemmille näytesarjoille stickers-välilehdellä. Lisää edessä olevaan ruutuun vain näytenumero ja kopioi koko setti Krokoon short-tiedostoon.

Seerumi

Laita veriputket jääkaappiin odottamaan, jos et heti fuugaa!
Sentrifugoi seerumiputket ja yksi EDTA plasmaa varten sopivan kokoisissa fuugin putkiadaptereissa 2000 g 10 minuuttia. Vie seerumiputket jääkaappiin ja aloita plasmanäytteistä! Katso EDTA-veri.

Fuugauksen jälkeen seerumi pipetoidaan 1ml:n eriin Cryoputkiin. Jos viimeinen erä on alle 500 µl, ei laiteta uuteen putkeen vaan voit laittaa edelliseen putkeen. Tallenna max 8 putkea / potilas. Näytteet pakastetaan -70°C pakkaseen MRETRO tai PROSIBD laatikkoon (ota laatikon numero ylös päiväkirjaan). Merkkaa kerätyt näytteet kirjaan.

EDTA-veri

Laita laminaarin suurempi teho päälle vähintään 20 min ennen työn aloittamista ja uv-valo päälle.

Ota heti aluksi 1) 2ml:n näyte EDTA-verta eppariin/putkeen komplementtimäärityksiä ja DNA:n eristystä varten, jos vain 2 EDTA-putkea. Fuugaa verinäyte 2000 G 10min ja ota a) plasma 2 putkeen ja pakasta -70°C heti. 2) Ota 250 μl:aa EDTA-verta putkeen, jossa 690ul PaxGene-liuosta, jos potilaasta ei tullut Paxgene-putkea. Paxgene -20°C pakkaseen. Näytteitä voi tulla myös valmiissa Paxgene-putkessa sekä 3 EDTA-putkessa. Tällöin Paxgeneputket pakastetaan -20°C pakkaseen. Loput EDTA-veret käytetään solujen eristykseen.

Laimenna 2 putkea EDTA verta 1:1 PBS:llä niin, että huuhtelet EDTA-putken PBS:llä samalla määrällä kuin on verta yhteen 50 ml putkeen. Sekoita kääntelemällä pari kertaa. 2 putkesta tulee noin 40ml. Jaa tämä määrä verta kahteen LeucoSep-Falcon-putkeen, yhtä paljon molempiin. Pidä putket merkattuina, jos useampia näytteitä. Fuugaa 1000g 10min ILMAN JARRUA (= vajaa 20 min HI / BM1 n 45 min).

Fuugauksen jälkeen ime varovasti plasma pois steriilillä pasteurpipetillä, joka on imuletkussa kiinni. Varo imemästä soluja, joten jätä seerumia solukerroksen ylle riittävästi. Solufaasit kaadetaan yhteen uuteen 50ml:n putkeen. Lisätään 40ml PBS:ää solujen päälle ja fuugataan 250g ja 10min (nyt jarru päällä).

Fuugauksen jälkeen imetään imulla neste pois niin, että solut pysyvät putken pohjassa kallistamalla putkea. ”Ravista” solut irti toisista vetäen laminaarin ritiläpintaa vasten. Toista solujen pesu. Lisää 5ml:n pipetillä solumäärästä riippuen 5-10ml PBS falconiin ja suspensoi (sekoita) solut PBS:ään niin, että ne ovat tasaisena suspensiona. Tämä solususpensio laitetaan keltaisen 100μm:n Cellstrainerin läpi uuteen falcon-putkeen. Tarkista mittapipetillä solususpension tarkka volyymi solulaskentaa varten.

Soluista tehdään laimennos eppariin: 10μl trypanBlueta ja 10μl solususpensiota. Tämä sekoitetaan hyvin ja pipetoidaan 10 μl solulaskukammioon. Laitetaan kammio solulaskuriin ja kirjataan elävien solujen määrä ja elävien solujen %-osuus ylös päiväkirjaan.

Laske paljonko tarvitset solususpensiota µl:na, jotta saat 1miljoonaa solua RNA-later eppariputkea varten (yksi jaettuna solumäärä / ml. Muuta mikrolitroiksi (esim 0,07 = 70 µl). Fuugaa eppari 300g ja 3min epparifuugissa ja ota pipetoimasi nestemäärä pois. Lisää 1 miljoonan solun päälle 100µl RNAlater-liuosta. Lisää tarrat kanteen ja putkeen ja putki säilytetään eristysten ajan huoneen lämmössä ja sen jälkeen aluksi jääkaapissa.

Loput solut fuugataan 50ml:n Falconissa 330g, 5-10min. (Valmista 10% DMSO pakastusliuos: esim. 6,3ml FBS+700µl DMSO, jos aiot pakastaa 7 x 1 ml soluannosta, 15 ml putkeen). Jos soluja on ylenmäärin (yli 70miljoonaa), voidaan osa soluista ottaa pois jo ennen fuugausta. Fuugauksen jälkeen imetään supernatantti pois ja lisätään FBS+10%DMSO pakastusliuosta niin, että saadaan mukavia soluannoksia. Helpoin laskea koko elävien solumäärä ja jakaa se 7:llä tai pienemmällä, jos niukasti soluja. Jaa 1ml:n erät soluja cryoputkiin. Lisää putkiin pitkät tarrat ja laita pakastusastiaan ja jäädytä astia yön yli -20C tai -70°C pakasteessa riippuen boxista. Jäätyneet cryoputket siirretään -70°C pakasteesta –130 °C pakkaseen seuraavana päivänä. Perjantaina tehdyt solut maanantaina.

Ulostenäyte

Ulostenäytteet käsitellään vetokaapissa. Aluksi arvioi näytteen koostumus Bristol Stool Chartin luokituksen mukaan. Kirjaa tieto exceliin näytteen kohdalle. Jaetaan näytettä 4 cryoputkeen sekä yksi purkki pidetään sellaisenaan (jos niitä on). Siirrä uloste muovispatulalla kapealla päällä cryoputken sisälle. Ota isosta putkesta nimitiedot pois mustalla tussilla siltä osalta, jota tarra ei peitä. Pakastetaan näytteet -70°C pakasteessa. (Å2, Box2, PRO STOOL (II, tai <), Avaamaton purkki minigrippussiin, jossa lukee Hanne). Biomedicumissa oma minigrip ilman nimeä. Loput ulostejätteet kerätään minigrip-pussiin ja laitetaan roskiin. Sulje pussi hyvin hajuhaittojen takia.

Biopsianäyte

Hae K2 pakastehuoneesta hiilihappojäitä ja laita jäädytysmuotit kylmenemään niiden päälle. BM1:ssä kysy jäätä Citylabista, jos oma varastosi -70 °C kaapissa on tyhjä. Hätätilanteessa käytä -70 °C kylmäblokkia. Biopsianäyte tulee 2 eri putkessa, solumediassa

sekä RNA-laterissä. Labrassa oikealta ylhäältä (vesipisteen vierestä) löytyy biopsiatavarat boxista (Biopsy). BM1.ssä hannen alimman hyllyn vasen reuna. Kirjoitetaan näytteen nimi jäädytysmuottiin sopivalle kapealle paperilapulle lyijykynällä. Nostetaan pinseteillä kudospala ja näytteen nimilappu muottiin, joka on hiilihappojäiden päällä. Kumoa D-MEM kudospaloineen tiskikaapissa / pussissa olevalle petrimaljalle ja kallista sen verran, että kudospala jää "kuiville". Nyt saat siitä otteen ja voit siirtää sen kylmään jäähdytysmuottiin. Laita lyijykynällä kirjoitettu ID-paperi pystyyn toiseen reunaan, kuin missä biopsia on ja tiputa päälle TissueTek. Varo ilmakuplia! Tiputa varmistustippa jonnekin muualle ensin.   Valkoiseksi jäätynyt näyte laitetaan Biopsia-boxiin -70°C.
Biopsioista kirjataan päiväkirjaan mistä näytteistä laitettu jääblokki ja mitkä ovat RNAlaterissa. Ota RNAlater-putkista tarrat irti ja laita tilalle meidän omat, joissa sama kohdekoodi (esim 1a, 4b). Liimaa nämä tarrat ao näytteen kohdalle päiväkirjaan. Liimaa alkuperäiset tarrat työkirjaan erotellen RNA-later ja jääblokkinäytteet.

Lopuksi

Tarkista, että kaikki tiedot on kirjattu päiväkirjaan ja projektien excel-tiedostoihin. Yliviivaa putkista tarrat, joissa potilaan nimi ja henkilötunnus ovat näkyvillä. Kasaa mahdolliset ylimääräiset tarrat yhdeksi pinoksi ja suttaa mustalla tussilla kaikki identifikaatiotiedot päällimmäisestä tarrasta. Laita tarranippu roskikseen. Ylimääräisiä ei kannata tuoda hakupaikasta mukanaan, jos vain mahdollista jättää ne taakseen. Meille riittää yksi tarra jostain näytteestä.