HLT02 METVES

EMRP JRP – HLT 02 MetVes

Protocols to collect and prepare human blood, urine, and saliva for measurement of microvesicles

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I. Introduction

Human blood contains fluid and cells, proteins, lipoproteins, and microvesicles (MV) of different cellular origins [1]. Also urine and saliva contain not only MV but also cells and other "particulate matter" [2-4]. Because of the complexity of the body fluids, the collection and handling of body fluids affects MV measurements. Especially in the case of blood, pre-analytical variables such as collection, handling, and storage of MV-containing blood fluid (plasma) may strongly affect the numbers of MV [5]. Therefore, in clinical studies, not only standardisation of analytical but also of pre-analytical variables is important. One of the goals in HLT02 METVES is to develop standard protocols for preparation, isolation, freezing, and storage of MV-containing samples. As part of task 1.4. of METVES, protocols to collect and prepare human plasma, urine, and saliva for measurement of MV have been developed and/or optimised.

II. General requirements

(see Figure 1 for general equipment and disposables)

1. When feasible, all body fluids should be collected from overnight fasting donors; please be aware that collection of body fluids may require (written) informed consent and/or permission from the local medical ethical committee; furthermore, all body fluids or fractions thereof, also when collected from healthy donors, should be considered as potentially infectious biological materials. The latter may have consequences for use of personal protection items, such as gloves, waste, etc. Please be also aware that blood may only be collected by specially trained personnel. Finally, be aware of the fact that most collected body fluids may be used for study materials, but not for comparative clinical studies. Also providing names and other details of donors on labels should be circumvented. The detailed description is based on procedures performed on our laboratory (Laboratory of Experimental Clinical Chemistry, Academic Medical Centre of the University of Amsterdam, Amsterdam, Netherlands), but also similar alternatives can be used.
2. 15-ml and 50-ml polypropylene centrifuge tubes (ref. nr. 188271 and 227261; Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands)
3. Micropipets (Transferpette® Brand; Wertheim, Germany)
4. 2-200 µl disposable Eppendorf pipet tips (ref. nr. 0030000870; Sigma-Aldrich, Zwijndrecht, The Netherlands)
5. 1 ml disposable pipet tips (ref. nr. 740290; Greiner, Bio-One B.V.)
6. 1.5 ml polypropylene tubes (ref. nr. 72.607; Sarstedt B.V., Etten-Leur, The Netherlands)
7. Screw caps for 1.5-ml tubes (ref. nr. 65.716; Sarstedt B.V.)
8. Disposable plastic Pasteur pipets (Ref. nr. 612-1681; VWR International B.V., Amsterdam, The Netherlands)
9. Tube racks for 1.5, 15, and 50-ml tubes
10. Label (ref. nr. 062379; Brady, Egelsbach, Germany)
11. Soft nitrile gloves (ref. nr. 102451, Medeco B.V., Oud-Beijerland, The Netherlands)
12. Shaved ice and ice container
13. Tabletop liquid N_2 container (Dilvac Dewar flask; Essex, United Kingdom)
14. Waterproof cryo-gloves (Tempshield, Maine, USA)
15. Safety google
16. Long plastic tweezer
17. Liquid Nitrogen (N_2)
18. Tabletop centrifuge with a swing rotor (Rotina 46 RS; Hettich zentrifugen, Tuttlingen, Germany)
19. -80 °C Freezer

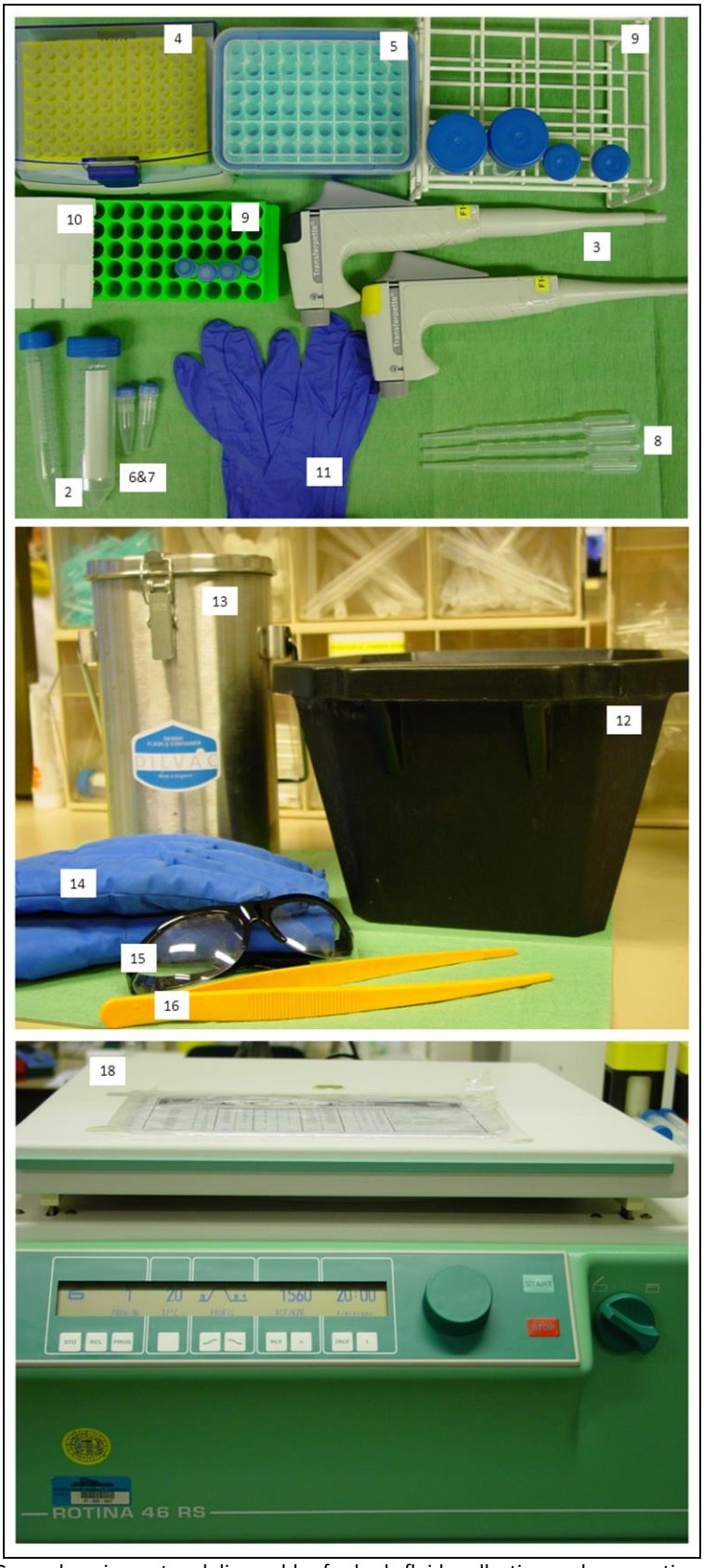

Figure 1. General equipment and disposables for body fluids collection and preparation

III. Blood

A. Materials

1. 21-G BD EclipseTM blood collection needle (ref. nr. 368607; Becton Dickinson, CA, USA)
2. BD vacutainer holder (ref. nr. 364815, Becton Dickinson)
3. 9NC (0.109 M) citrated plastic BD Vacutainer tubes (2.7 mL; ref. nr. 363048; Becton Dickinson)
4. Tourniquet (ref. nr. 840050, Greiner Bio-One B.V.)
5. Chlorhexidine 0.5% m/v in alcohol 70% v/v (Orphi Farma B.V.; Lage Zwaluwe, The Netherlands)
6. Adhesive bandages (ref. nr. N1170B; Nexcare, Wroclaw, Poland)
7. Needle disposal unit (Frontier medical group; Blackwood, United Kingdom)

B. Blood collection

(see online training material HLT02 MetVes D5.2.1)

1. Take at least two plastic tubes containing citrate (0.109 M)
2. Apply tourniquet on the upper arm and locate the vein
3. Disinfect the location for the venipuncture by using a cotton wetted with Chlorhexidine 0.5% m/v in alcohol 70% v/v
4. Insert the needle to the vein
5. Connect the first tube to the holder
6. Unconstraint the tourniquet as soon as the blood is released into the tube
7. Fill the tube with blood (2.7 ml)
8. Disconnect the tube from the holder and discard this tube as blood in this tube insufficiently reflects circulating (venous) blood
9. Connect the second tube to the holder
10. Fill the tube with blood (2.7 ml)
11. Disconnect the tube from the holder and immediately mix the blood with the citrate anticoagulant by inverting the tube gently for at least 5 times
12. Place the blood tube upright on the tube rack
13. Remove the needle and dispose of needles immediately upon removal from the donor's arm
14. Place a piece of a dry cotton on the puncture site, hold it in place until the bleeding has stopped, and apply an adhesive bandage
15. Label the blood collection tube with the ID number of the donor, the date and time of collection
16. Transport the tube containing blood immediately to the laboratory for plasma isolation (see HLT02 MetVes task 1.2. D1.2.5)

C. Plasma isolation

(see online training material HLT02 MetVes D5.2.1)

1. Centrifuge the blood within 15 minutes after blood collection at 1,560 x g for 20 minutes at 20 °C
2. Collect approximately 55% of plasma carefully using Pasteur pipet (see Figure 2 and note); only the upper part of the plasma should be collected, otherwise leukocytes and platelets contamination may occur

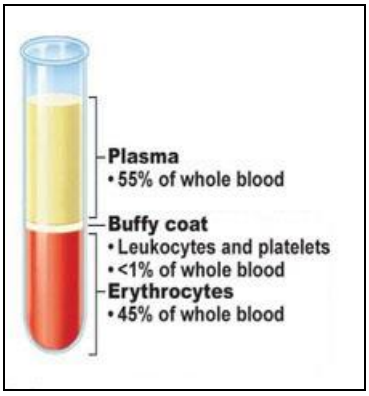

Figure 2. Isolation and collection of plasma

3. Transfer plasma into a 15-ml centrifuge tube
4. Centrifuge plasma at 1,560 x g for 20 minutes at 20 °C
5. Collect and transfer the supernatant to a 15-ml centrifuge tube
6. Mix plasma with pipet and make 250 µl aliquots of plasma in 1.5-ml tubes
7. Label the plasma tubes with the ID number of the donor, type of sample, and the date of isolation
8. Snap freeze the tubes containing plasma into the liquid N_2 and leave them for 15 minutes
9. Store plasma at -80 °C freezer for further measurement (see HLT02 MetVes task 1.2. D1.2.5)

Note: Discard hemolytic (red) plasma isolated at this centrifugation step to exclude artefacts due to erythrocyte lysis. Furthermore, platelets are thought to be one of the major sources of MV within human blood. Because platelets are easily activated, this should be circumvented. The risk of platelet activation is reduced by using plastics rather than glass, and by performing procedures at 20 °C, since especially at 4 °C platelets become activated.

IV. Urine

A. Materials

1. Plastic urine container (ref. nr. 75.562.105, Sarstedt B.V.)

Figure 3. Plastic urine container

2. 70% alcohol ketonatus (Orphi Farma B.V.)

B. Urine collection

1. Take a plastic urine container
2. Discard the first 10 ml urine produced
3. Collect 100 ml urine into the plastic container and close it
4. Clean when necessary the outside of the plastic container using 70% alcohol ketonatus
5. Label the container with the ID number of the donor, the date and time of collection
6. Place the urine on ice in the ice container and immediately transport it to the laboratory for isolation

C. Cell-depleted urine

1. Divide urine into two of 50-ml centrifuge tubes
2. Centrifuge urine at 300 x g for 10 minutes at 4 °C
3. Transfer each supernatant to a 50-ml centrifuge tube without disturbing the cell pellet
4. Centrifuge the supernatant at 1,560 x g for 20 minutes at 4 °C
5. Collect and transfer each supernatant to a 50-ml centrifuge tube
6. Mix urine with pipet and make 250 µl aliquots of urine in the 1.5-ml tubes
7. Label the tubes containing urine with the ID number of the donor, type of sample, and the date of preparation
8. Snap freeze the tubes containing urine into the liquid N_2 and leave them for 15 minutes
9. Store urine at -80 °C freezer for further measurement (see HLT02 MetVes task 1.3. D1.3.3)

V. Saliva

A. Materials

1. Plastic saliva container (ref. nr. 75.9922.721, Sarstedt B.V.)

Figure 4. Plastic saliva container

2. 70% alcohol ketonatus (Orphi Farma B.V.)
3. 0.9% sodium chloride solution (ref. nr. W7124, Baxter Healthcare, Zurich, Switzerland)

B. Saliva collection

1. Take a plastic saliva container
2. Rinse the mouth couple of times with water before saliva collection
3. Collect the saliva into the plastic container
4. Place the container on ice during the saliva collection
5. Clean when necessary the outside of the plastic container using 70% alcohol ketonatus
6. Label the container with the ID number of the donor, the date and time of collection
7. Place the saliva on ice in the ice container for immediate transport to the laboratory for isolation

C. Cell-depleted saliva

1. Divide saliva into two of 15-ml centrifuge tubes (see note)
2. Centrifuge saliva at 300 x g for 10 minutes at 4 °C
3. Collect and transfer each supernatant in a 15-ml centrifuge tube without disturbing the cell pellet
4. Centrifuge the supernatant at 1,560 x g for 20 minutes at 4 °C
5. Transfer and collect each supernatant in a 15-ml centrifuge tube
6. Mix saliva with pipet and make 250 µl aliquots of saliva in the 1.5-ml tubes
7. Label the tubes containing the saliva with the ID number of the donor, type of sample, and the date of preparation
8. Snap freeze the tubes containing urine into the liquid N_2 and leave them for 15 minutes
9. Store saliva at -80 °C freezer for further measurement (see HLT02 MetVes task 1.3. D1.3.3)

Note: Dilute a thick saliva 2-fold with 0.9% sodium chloride solution before centrifugation.

VI. Commentary

Single versus double centrifugation

Blood should be processed immediately after collection to obtain plasma. The centrifugation conditions, speed, and time to obtain plasma vary widely among studies [5]. Although the first centrifugation will remove most cells, in most studies 1,500–2,500 x g for 15–20 minutes, this is usually insufficient for plasma, because usually 1-5% of small platelets will remain present [5, 6]. These residual platelets may become activated when plasma is centrifuged, or during freeze-thaw procedures, which may lead to production of platelet MV. Therefore, a second centrifugation step has to be applied to remove residual platelets to circumvent the risk of artefacts [7]. To be noted, the applied centrifugation may also deplete larger vesicles such as “apoptotic bodies”, since these vesicles are approximately at the size range of platelets (1-5 µm) [8, 9]. Therefore, other centrifugation protocol may be more suitable for inclusion of apoptotic bodies [8]. Finally, it should also be mentioned that centrifugation of platelets, even at 1,500-2,000 x g may result in platelet activation. To which extent addition of anticoagulants or platelet inhibitors is useful, remains to be investigated.

For body fluids, the goal of the first centrifugation steps is the removal of cells in order to prepare MV-containing fluid. Subjection of MV-containing fluids to higher g forces, for example to pellet MV or to prepare MV-depleted fluid, should be performed with caution. For example, in the case of plasma, which contains mainly MV originating from platelets and erythrocytes, we observed that MV from platelet easily aggregate whereas MV from erythroyctes will not (see HLT02 MetVes task 1.1 D1.1.7). The consequence of such treatments can be artefacts and losses of certain subpopulations. Furthermore, not all MV will be pelleted at the same speed of centrifugation because sedimentation also depends on the density or ‘‘cargo’’ of a vesicle and the distance a vesicle travels [10].

Freezing and storage

Smaller or larger volume of aliquots can be prepared depending on the volume needed for measurements. Aliquots are snap frozen in liquid N_2 (see HLT02 MetVes task 1.1. D1.1.8). Small volume of aliquots require less time for snap freezing in liquid N_2 and thawing than the large volume of aliquots. On the other hand, preparation of small volume of aliquots may be more time consuming and these aliquots require bigger storage capacity.

The impact of storage delay between 1 week and 1 year has been evaluated in another study [7]. In these conditions, no major change in MV counts, thrombin generation or clotting time was observed after 12 months at -80 °C. However, we observed that the concentration of MV in stored plasma samples changes already after 6 months of storage (see HLT02 MetVes task 1.2. D1.2.5). Thus, storage time may affect the outcome, and care needs to be taken that this variable will not affect outcome or comparison of results between laboratories. Whatever storage conditions are used, matched control samples should be analyzed for comparison. Also be aware that especially in clinical studies, samples are thawed mostly only once. Therefore, it is recommended to freeze MV-containing body fluids in aliquots.

Before measurement, frozen body fluids containing MV should be thawed on melting ice. If salt crystals are formed in urine after thawing, warm the urine for a minute at 37 °C prior to measurement. Repeated freeze/thaw cycles should be avoided as these processes will impact the measurement of MV concentration and initiate the exposure of phosphatidylserine on the surface of MV especially MV derived from blood (see task 1.1 D1.1.8, task 1.2 D1.2.5, and task 1.3 D1.3.3).

VII. References

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VIII. Appendix

Table of collection and preparation procedures of microvesicles from body fluids

Body fluids	Blood	Urine	Saliva
Collection	21-G needle Plastic tube containing 0.109 M citrate No tourniquet during withdrawal Discard the first tube containing 2.7 ml of blood	Discard the first 10 ml of urine	Rinse the mouth couple of times with water
Cell depletion	1,560 x g for 20 minutes at 20 °C (2x)	300 x g for 10 minutes followed by 1,560 x g for 20 minutes at 4 °C	300 x g for 10 minutes followed by 1,560 x g for 20 minutes at 4 °C
Freezing	Snap freezing in liquid N_2	Snap freezing in liquid N_2	Snap freezing in liquid N_2
Storage	-80 °C	-80 °C	-80 °C