Ref. Ares(2021)2478168 - 12/04/2021

REVERT

DELIVERABLE

D4.1

Report on SOPs for sample collection, processing and storage, including indicators to monitor quality of samples

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

DOCUMENT INFORMATION

AUTHORS			
Fay Betsou	LIH*	Massimo Gion	AULSS3
Monica Marchese	LIH	Aline S.C. Fabricio	AULSS3
Antonette Leon	AULSS3	Pablo Conesa Zamora	UCAM
Anette Gjörloff Wingren	UMU	Jenny Persson	UMU
Peter Winter Lukas Jost Klaus Hoffmeier Ricardo M. Figueiredo	GXP		
Contributors			
Silvia Riondino	SR	Stefania Nobili	UniFi
Stefano Rapi	UniFI	Enrico Mini	UniFI
Luca Messerini	UniFI	Marco Peluso	ISPRO
Lieke Mevis	LIH**	Camille Bellora	LIH

(*) As of November 2020, the new affiliation is LNS - Laboratoire National de Santé, Luxembourg

(**) As of the beginning of 2021, Lieke Mevis has left LIH

Deliverable lead partner	LIH
Contributing partner(s)	AULSS3
Work Package	Tools for biomarker translation to clinical practice: standardization and quality management
Deliverable type	Report
Contractual delivery date	30.06.2020
Actual delivery date	12.04.2021
Dissemination level	Public
Version	2.4

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

STATEMENT OF ORIGINALITY

This deliverable contains original unpublished work except where clearly indicated otherwise. Acknowledgement of previously published material and of the work of others has been made through appropriate citation, quotation or both.

DISCLAIMER

The content of this deliverable represents the views of the author only and is his/her sole responsibility; it cannot be considered to reflect the views of the European Commission and/or the Consumers, Health, Agriculture and Food Executive Agency (CHAFEA) or any other body of the European Union. The European Commission and the Agency do not accept any responsibility for use that may be made of the information it contains.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

TABLE OF CONTENTS

DOCUMENT INFORMATION 2
TABLE OF CONTENTS 4
LIST OF TABLES 7
LIST OF FIGURES 7
1 Introduction 8
1.1 Scope 8
1.2 Basic definitions 8
1.3 Standard precautions 8
2 Body fluids 10
2.1 Specimen collection 10
2.2 Specimen identification 10
2.3 Risk of hemolysis 10
2.4 Venipuncture 10
2.5 Blood drawing sequence 11
2.6 Serum – general laboratory tests 12
2.6.1 Blood transport, shipment and storage conditions 12
2.6.2 Blood processing, serum aliquoting and storage conditions 12
2.7 EDTA plasma– general laboratory tests 12
2.7.1 Blood transport, shipment and storage conditions 12
2.8 Serum – fragile analytes (i.e. inflammatory biomarkers) 13
2.8.1 Blood transport, shipment and storage conditions 13
2.8.2 Blood processing, serum aliquoting and storage conditions 13
2.9 Plasma from EDTA – fragile analytes (i.e. inflammatory biomarkers) 13
2.9.1 Blood transport, shipment and storage conditions 13
2.9.2 Blood processing, serum aliquoting and storage conditions 13
2.10 Plasma from CTAD – fragile analytes (i.e. inflammatory biomarkers) 14
2.10.1 Blood transport, shipment and storage conditions 14
2.10.2 Blood processing, serum aliquoting and storage conditions 14
2.11 Whole blood (general laboratory tests) 14
2.11.1 Complete blood count and HbA1c measurements 14
Whole blood shipment and storage conditions 14
2.11.2 aPTT, D-dimer and Fibrinogen measurements 14
Whole blood shipment and storage conditions 14

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

Whole blood processing, plasma aliquoting and storage conditions 14
2.12 Buffy coat from EDTA 15
2.12.1 Blood processing (EDTA) 15
2.12.2 Buffy coat processing and storage conditions 15
3 Other Body fluids 16
3.1 Ascites and pleural fluid 16
3.1.1 Liquid biopsy for cfDNA purification: shipment and storage conditions 16
3.1.2 Liquid biopsy processing, supernatant and pellet storage conditions 16
3.2 Urine 16
3.2.1 Urine shipment and storage conditions 16
3.2.2 Urine processing, supernatant and pellet aliquot storage conditions 16
3.3 Stool 17
3.3.1 Stool collection 17
3.3.2 Stool shipment and storage conditions (for genetic or metagenomics analysis) 17
3.3.3 Stool processing and aliquoting 17
4 Tissues (Pathology) 18
4.1 Tissue collection 18
4.2 Preparation of flash frozen samples 18
4.3 Preparation of formalin-fixed paraffin-embedded samples (FFPE) 19
5 Tumor organoids 21
5.1 Tissue preparation for organoids culture 21
6 Next generation sequencing (NGS) 22
6.1 Sample preparation and shipment 22
6.1.1 Fresh-frozen tissue 22
6.1.2 Blood (plasma and buffy coat) 22
6.1.3 Cell culture material and organoids 23
6.1.4 FFPE material 23
6.2 Sequencing of nucleic acid 23
6.2.1 mRNAs: massive analysis of cDNA ends (MACE-Seq) 23
6.2.2 Small RNAs: sRNA-Seq 24
6.2.3 Genomic DNA: Exome-Seq 24
6.2.4 Genomic DNA: Methyl-Seq 24
6.3 Bioinformatics 24
6.3.1 Bioinformatics processing and statistical analysis of MACE-Seq data 24
6.3.2 Bioinformatics processing and statistical analysis of sRNA-Seq data 24

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

6.3.3 Bioinformatics processing and statistical analysis of Exome-Seq data 25
6.3.4 Bioinformatics processing and statistical analysis of Methyl-Seq data..... 25
7 Essential References..... 26
8 Revision History..... 29

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

LIST OF TABLES

Table 1: Revised, simplified recommended order of draw for vacuum tubes or syringe and needle, based on United States National Committee Clinical Laboratory Standards consensus (2003)..... 11

LIST OF FIGURES

Figure 1: Diagnostic and research sample preparation 18

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

1 Introduction

1.1 Scope

Many variables, including anticoagulant volume and concentration, type of tube additive, duration and temperature of specimen storage, and surface of containers used for specimen collection and storage, may affect the reliability and accuracy of laboratory tests.

This document concerns the standard operating procedures (SOPs) for the collection, transport, processing and storage of specimens for laboratory tests, including general information appropriate for the majority of tests and specific information regarding laboratory tests that will be evaluated in the REVERT project.

1.2 Basic definitions

Specimen

The discrete portion of a body fluid or tissue taken for examination, study, or analysis of one or more qualities or characteristics, to determine the character of the whole.

Sample

One or more parts taken from a system and intended to provide information on the system, often to serve as a basis for decision on the system or its production (ISO 15189)10; NOTE: A sample is prepared from the patient specimen and used to obtain information by means of a specific laboratory test.

Room temperature (RT)

The temperature prevailing in a working area. Controlled room temperature is a temperature maintained thermostatically, encompassing the usual and customary working environment of approximately 18°C - 25°C. NOTE: labeling can indicate storage at 'controlled room temperature' or 'up to 25 °C'.

1.3 Standard precautions

Considering that it is often impossible to know which specimens might be infectious, all patient and laboratory specimens are treated as infectious and handled according to 'Standard Precautions'.

Standard precautions are guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of all infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the US Centers for Disease Control and Prevention.

When drawing and processing blood, the following standard precautions apply:

1. wear disposable gloves when collecting and processing specimens
2. wash hands thoroughly with disinfectant soap prior to leaving the work area
3. cover skin cuts or abrasions. If the phlebotomist accidentally sustains an injury from a contaminated needle stick, the wound will be thoroughly cleansed with soap and water. Local physicians should be notified to evaluate the need for analysis of the participant's serum for possible infections
4. store needles in a locked cabinet when the collection center is closed

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

5. use 0.1% sodium hypochlorite (household bleach) to clean up any spills of blood, plasma, or serum. Use this solution to clean up all laboratory work surfaces at the completion of work activities
6. dispose of all needles and tubing in puncture-resistant containers for safe disposal
7. do not perform any pipetting by mouth especially of any blood, serum, or plasma
8. avoid formation of potentially infectious aerosols by careful pipetting and centrifugation
9. place in biohazard bags for disposal all used vacutainer tubes and blood products

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

2 Body fluids

2.1 Specimen collection

If a blood sample is poorly collected, the results may be inaccurate and misleading to the clinician, and the patient may have to undergo the inconvenience of repeat testing. The three most common issues resulting from errors in collection are inaccurate labeling, hemolysis and contamination.

Prior blood collection, the patient/donor must have signed an informed consent if biomarkers, normally not included in the current clinical practice for disease management, are requested for research purposes.

2.2 Specimen identification

The patient and all patient specimens must be positively identified at the time of collection. Specimens should be labeled in the presence of the patient right after blood draw.

In case of clinical trial, at participant arrival, the tube ID must match the participant specimen ID. Each tube for laboratory testing must be identified with a firmly attached label bearing at least the following information:

- unique identification code
- date and time of collection
- specimen type if a secondary or aliquot tube is used (i.e., anticoagulant type vs serum)

If an encoded (barcode) label is used, the label must be attached according to the established institutional policy.

2.3 Risk of hemolysis

The phlebotomist must reduce the risk of hemolysis.
Factors that increase the risk of hemolysis, and therefore should be avoided, include:

1. use of a needle of too small a gauge (23 or under 23), or too large gauge for the vessel
2. drawing blood specimens from an intravenous or central line
3. underfilling a tube so that the ratio of anticoagulant to blood is greater than 1:9
4. mixing a tube too vigorously
5. failing to let alcohol or disinfectant dry from the skin surface

2.4 Venipuncture

For a successful venipuncture, the phlebotomist will:

1. anchor the vein by holding the patient's arm and placing a thumb below the venipuncture site
2. cleanse the venipuncture site with alcohol/disinfectant prep, using a circular motion from the center to the periphery and allow to dry

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

3. assemble the butterfly-vacutainer set
4. enter the vein swiftly at a 30 degree angle or less, and continue to introduce the needle along the vein at the easiest angle of entry
5. release the tourniquet before withdrawing the needle, once sufficient blood has been collected, and always before it has been in place for two minutes or more. Some guidelines suggest removing the tourniquet as soon as blood flow is established. Considering the influence that a tourniquet can have on inflammatory/angiogenic markers, NIH procedures recommend to leave the tourniquet on for no longer than two minutes. If a tourniquet must be applied for the preliminary vein selection, it should be released and reapplied after two minutes
6. withdraw gently the needle and apply gentle pressure to the site with a clean gauze or dry cotton-wool ball. Ask the patient to hold the gauze or cotton in place, with the arm extended and raised. Ask the patient NOT to bend the arm, because doing so causes a hematoma
7. invert gently tubes with additives for a thorough mixing. Erroneous test results may be obtained when the blood is not thoroughly mixed with the additive

2.5 Blood drawing sequence

A blood drawing sequence must be followed to avoid cross-contamination of additives between tubes. As colour coding and tube additives may vary, recommendations with local laboratories must be verified.

Table 1: Revised, simplified recommended order of draw for vacuum tubes or syringe and needle, based on United States National Committee Clinical Laboratory Standards consensus (2003).

Order of use	Type of tube
1	Blood culture bottle
2	Non-additive tube
3	Sodium citrate
4	Clot activator (silica)
5	Serum separator
6	Heparin
7	Heparin and gel separator
8	EDTA
9	ACT
10	CTAD
11	Oxalate/fluoride

EDTA, ethylenediaminetetraacetic acid; ACD, acid-citrate-dextrose; CTAD, trisodium citrate, theophylline, adenosine, dipyridamol.
Source: Table adapted from World Health Organization. WHO Guidelines on Drawing Blood: Best Practices in Phlebotomy. World Health Organization press 2010; Geneva.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

2.6 Serum – general laboratory tests

2.6.1 Blood transport, shipment and storage conditions

1. Gently mix the blood tube by inversion 5-6 times
2. Transport tubes, in a vertical position, in transport box at RT within 15 minutes from blood draw
3. Allow >30 minutes and ≤45 minutes between collection and centrifugation start
4. Immediately aliquot and store at -80°C

Non-metabolomic studies

If temporary storage and shipment at RT

- < 4h between collection and centrifugation start
- < 6h between collection and storage of processed sample at -80°C

Metabolomic studies

If temporary storage and shipment at 2-8°C

- < 4h between collection and centrifugation start

2.6.2 Blood processing, serum aliquoting and storage conditions

1. Allow the blood to clot for minimum 30-45minutes at RT
2. Centrifuge at 2000g, 10 minutes, soft brake, RT
3. Transfer the serum into a conical tube and homogenize it
4. Aliquot the serum up to 12x220µl in 0.5ml Matrix cryotubes and store aliquots at -80^{o}C

2.7 EDTA plasma– general laboratory tests

2.7.1 Blood transport, shipment and storage conditions

Gently invert the blood tube by inversion 3 times.

Non-metabolomic studies

If temporary storage and shipment at RT:

- < 6h between collection and centrifugation start
- < 8h between collection and storage of processed sample at -80°C

If temporary storage and shipment at 2-8°C:

- < 24h between collection and centrifugation start
- < 28h between collection and storage of processed sample at -80°C

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

Metabolomic studies

If temporary storage and shipment at RT:

- < 3h between collection and centrifugation start
- < 5h between collection and storage of processed sample at -80°C

If temporary storage and shipment at 2-8°C:

- < 24h between collection and centrifugation start
- < 28h between collection and storage of processed sample at -80°C

2.8 Serum – fragile analytes (i.e. inflammatory biomarkers)

2.8.1 Blood transport, shipment and storage conditions

1. Gently mix the blood tube by inversion 5-6 times
2. Transport to the laboratory, in a vertical position, in transport box at RT within 15 minutes from blood draw
3. Allow >30 minutes and ≤ 45 minutes between collection and centrifugation start
4. Immediately aliquot and store at -80°C

2.8.2 Blood processing, serum aliquoting and storage conditions

1. Allow the blood to clot for minimum 30-45 minutes at RT
2. Centrifuge at 2500g, 15 minutes, no brake, at +4°C
3. Transfer the serum into a conical tube and homogenize it
4. Aliquot the serum up to 12x220µl in 0.5ml Matrix cryotubes and store aliquots at -80°C

2.9 Plasma from EDTA – fragile analytes (i.e. inflammatory biomarkers)

2.9.1 Blood transport, shipment and storage conditions

1. Gently mix the EDTA blood tube by inversion 8-10 times
2. Transport to the laboratory, in a vertical position, in transport box at RT within 15 minutes from blood draw
3. Allow only ≤15 minutes between collection and centrifugation start
4. Immediately aliquot and store at -80°C

2.9.2 Blood processing, serum aliquoting and storage conditions

1. Centrifuge within 15 minutes from blood draw
2. Centrifuge at 2500g, 15 minutes, no brake, at +4°C
3. Transfer the plasma into a conical tube and homogenize it
4. Aliquot the plasma up to 12x220µl in 0.5ml Matrix cryotubes and store aliquots at -80°C

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

2.10 Plasma from CTAD – fragile analytes (i.e. inflammatory biomarkers)

2.10.1 Blood transport, shipment and storage conditions

1. Gently mix the CTAD blood tube by inversion 4-5 times
2. Transport to the laboratory, in a vertical position, in transport box at RT within 15 minutes from blood draw
3. Allow only ≤15 minutes between collection and centrifugation start
4. Immediately aliquot and store at -80°C

2.10.2 Blood processing, serum aliquoting and storage conditions

1. Centrifuge within 15 minutes from blood draw
2. Centrifuge at 2500g, 15 minutes, no brake, at 4°C
3. Transfer the plasma into a conical tube and homogenize it
4. Aliquot the plasma up to 12x220µl in 0.5ml Matrix cryotubes and store aliquots at -80°C

2.11 Whole blood (general laboratory tests)

2.11.1 Complete blood count and HbA1c measurements

Whole blood shipment and storage conditions

1. Blood collection in K2-EDTA blood tubes
2. Temporary storage and shipment at RT
3. <48h between collection and measurement
4. No storage of whole blood for these analyses (only fresh)

2.11.2 aPTT, D-dimer and Fibrinogen measurements

Whole blood shipment and storage conditions

1. Blood collection in 3.2% sodium citrate tubes
2. Temporary storage and shipment at RT
3. <1h between collection and centrifugation start

Whole blood processing, plasma aliquoting and storage conditions

1. Centrifuge at 2000g, 10minutes, soft brake, RT
2. Transfer the supernatant into a conical tube and homogenize it
3. Perform a second centrifugation of supernatant at 16000g, 10 minutes, soft brake, RT
4. Transfer the final plasma in a 2ml cryotube and homogenize it

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

5. Aliquot a maximum of 1ml plasma aliquots and store them at -80°C for maximum 6 months

2.12 Buffy coat from EDTA

2.12.1 Blood processing (EDTA)

Refer to Section **Errore. L'origine riferimento non è stata trovata.**.9 for storage conditions and processing of plasma.

2.12.2 Buffy coat processing and storage conditions

1. Pipette 940µl of buffy coat (or another appropriate volume, as indicated by the DNA extraction kit to be used) into a 15ml conical tube (or another tube, according to the total volume)
2. Add 3ml of lysis buffer (or another appropriate volume of lysis buffer, as indicated by the DNA extraction kit to be used) into the tube
3. Vortex the tube briefly and temporarily store it at 2-8°C up to DNA extraction (to be performed within 7 days). Alternatively, store at -80°C until DNA extraction

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

3 Other Body fluids

3.1 Ascites and pleural fluid

3.1.1 Liquid biopsy for cfDNA purification: shipment and storage conditions

1. Liquid biopsy collection in sterile polypropylene tube
2. Temporary storage and shipment at RT
3. < 2h between collection and centrifugation start

3.1.2 Liquid biopsy processing, supernatant and pellet storage conditions

1. Centrifuge at 2000g, 10 minutes, soft brake, RT
2. Transfer the supernatant into a conical tube and homogenize it
3. Perform a second centrifugation of supernatant at 16000g, 10 minutes, soft brake, RT
4. Add 1.25ml of precooled RNA-protect cell reagent to the cell pellet and mix by pipetting up and down
5. Transfer the whole volume in a 2ml cryotube
6. Once the second centrifugation is finished, transfer the final supernatant into a new conical tube and homogenize it
7. Aliquot a maximum of 400µl supernatant aliquots
8. Store all supernatants and pellet aliquots at -80°C

3.2 Urine

3.2.1 Urine shipment and storage conditions

1. Temporary storage and shipment at 2-8°C
2. < 4h between collection and centrifugation start
3. < 6h between collection and storage at -80°C

3.2.2 Urine processing, supernatant and pellet aliquot storage conditions

1. Perform a urine analysis test using Combur Test Urine test strips
2. If the urine volume is less than 5ml, no urine analysis will be performed
3. Centrifuge at 12000g, 20 minutes, soft brake, 2-8°C
4. Transfer supernatant in another tube and homogenize it
5. Aliquot the urine up to 12x3.8ml in 4 ml cryotubes
6. Add 1.25ml of precooled RNAprotect Cell Reagent to the cell pellet and mix by pipetting up and down
7. Transfer the whole volume in a 2ml cryotube

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

8. Store all urine supernatants and cell-stabilized urine pellet aliquots at -80°C

3.3 Stool

3.3.1 Stool collection

Follow the 'DNA/RNA Shield fecal Collection Tube Quick Protocol' data sheet

3.3.2 Stool shipment and storage conditions (for genetic or metagenomics analysis)

1. Stool collection in 'Zymo Fecal' collection tubes
2. Temporary storage and shipment at RT
3. < 30 days between collection and storage at -80°C

3.3.3 Stool processing and aliquoting

1. Homogenize the contents by shaking the collection tube for at least 30 seconds
2. Tap the bottom of the tube in order to collect any sample adhering on the side
3. Aliquot 3.8ml of stool suspension in a 4 ml cryotube and using a wide bore tip, and store it at -80°C
4. Store the collection tube as well at -80°C

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

4 Tissues (Pathology)

4.1 Tissue collection

This workflow is intended to manage every surgical specimen resected for a diagnosis of colon cancer (histologically proved or not).

To NEVER compromise the diagnosis, research samples must be surplus samples. Under 1 cm, NO SAMPLE is collected for research.

The time of collection and the time when samples were placed in liquid nitrogen and/or formalin MUST be always annotated.

Proceed as follows:

1. Examine the specimen and perform the macroscopy (specimen description, including measurements) according to the specimen type
2. Identify the tumor and, according to its size, estimate the number of aliquots to be collected, processed and frozen/fixed within 20 minutes of collection (ideally)
3. Process tumoral or normal tissue specimens using different utensils and processing supplies to prevent cross-contamination
4. Process tumor tissues before normal tissue in the following order, with the following maximum number of tissue pieces processed:
   - Tumor (T) : 4 cryovials
   - Adjacent Normal Tissue (A) : 4 cryovials
   - Distant Normal Tissue (D) : 4 cryovials
   - Node tissue (if applicable): 1 cryovial

Figure 1: Diagnostic and research sample preparation

Size of the tumor	Diagnostic samples	Research samples	
	Proportion of the biospecimen	Frozen	FFPE
1 - 2 cm	1/2	T1, A1, D1	T1, A1, D1
> 2 cm	1/2	T1, T2, T3, T4 A1, A2, A3, A4 D1, D2, D3, D4	T1, T2, T3, T4 A1, A2, A3, A4 D1, D2, D3, D4

5. Cut a small piece (maximum 2cm x 1cm x 1cm and minimum 1cm x 0,5cm x 0,5cm) in two. One will undergo flash frozen processing and, the second one, formalin fixation
6. Repeat this step as many times as the number of samples collected. The number of aliquots collected per tissue type (tumor, adjacent normal and distant normal) should be the same

4.2 Preparation of flash frozen samples

Process samples for flash freezing before FFPE samples:

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

1. Using forceps, place each piece of tissue in a separate cryovial
2. Cover tissue from the cryovial with Optimal Cutting Temperature compound (OCT)
3. Freeze the cryovials in a Dewar filled with liquid nitrogen or in isopentane
4. Transfer the sample from liquid nitrogen or isopentane bath to cryoshipper, liquid nitrogen tank or -80°C freezer

4.3 Preparation of formalin-fixed paraffin-embedded samples (FFPE)

1. Place the sample, using forceps, inside a flask with neutral buffered formalin
2. Transport, at RT, the formalin flasks to the processing site
3. Fix with 10% buffered formalin (BF10). This procedure depends on the tissue type
   - For biopsies

     The standard fixation time is 6-24h, though 6-8h is enough. A sample ID is given in the laboratory information system (LIS) at the Pathology Department.
     Biopsies are usually delivered inside a container pre-loaded with BF10. Those specimens with less than 5mm width are processed within the same day (12-18h total fixation time).
     If the specimen, due to its size, requires opening or dissection, it will be processed the following day (18-24h total fixation time)
   - For non-fixed (fresh) specimens (except intraoperative biopsy)

     A macroscopy description and 3-5mm-width slices will be carried out and tissue will be kept in fixation. If samples are received:
     a. before 11:00am they will be processed in the same day (6-12h total fixation time
     b. after 11:00am they will be kept in BF10 till next day (18-24h total fixation time)
   - For surgical specimens

     The standard fixation time is 24-48h.
     All surgical specimens (except during weekends or holidays) will be received as 'fresh'. Once registered in the LIS, macroscopic description and dissection are carried out and tissue is maintained under fixation until the next day.
     Representative slices are then obtained, introduced in cassettes and left in BF10 till automatic processing. However, if:
     a. fixation is complete for surgical specimens obtained the same day, then the total fixation time will be 24-30h
     b. due to its large size, the specimen requires more fixation, then total fixation time will be extended to up to 48h
4. Start the automated processing (the total time required to complete it is around 14h):
   a. fixation with BF10
   b. washing with distilled water to remove BT10 remnants
   c. dehydration (alcohol: 75% up to 100%)
   d. clearing with xylene substitute
   e. embedding in paraffin at 62°C
5. Include tissue inclusion in paraffin blocks:

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

   a. use melted paraffin at 56-58°C to fill the mold containing tissue
   b. snap-cool at -20°C

6. Perform histological sections
   a. cut sections in rotary motorized microtomes 3-4µm width using low profile blades
   b. place sections to float on a water bath at 40-45°C to avoid formation of ruffles
   c. use glass slides with positive electrostatic charge to take the sections from the bath
   d. incubate slides containing the sections at 60°C to remove the humidity
7. Stain slides with hematoxylin and eosin (H&E)
8. Assess, for each H&E slide, the sample quality (tissue sample characterization: percent of tumor, necrosis, stroma, normal tissue, inflammation, fibrosis, meta- and/or dysplasia, tumor infiltration, etc.)
9. Assess the immunoscore, via IHC for CD3 and CD8 using an automatic immunostainer and following antibody manufacturer instructions
10. Store FFPE blocks and slides in an archive at RT. Unstained slides can be stored at -20°C

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

5 Tumor organoids

5.1 Tissue preparation for organoids culture

1. Cut the tumor into small pieces while adding 1-2 ml of 20% FBS in 1XPBS solution
2. Cut off the tip of a 1ml pipette, to obtain a wider bore, and aspirate both tissue pieces and solution for transfer into a 15ml tube
3. Add:
    a. 1ml DNase (300 IU/ml)
    b. 1ml Hyaluronidase (1 mg/ml) and 1ml Collagenase (5 mg/ml)Bring the final volume to 10 ml by adding more solution: 20% FBS in 1x PBS.
4. Put the vial on a rotator inside the incubator (37°C and 5% CO_2) for 30-45 minutes, until a single cell suspension will be obtained
5. Spin down at 300g for 5 minutes, aspirate and discard the supernatant
6. Add RPMI1640 (10% FBS, PSN, L-Glut) and resuspend the cell pellet
7. Spin the pellet once more at 300g for 5 minutes, aspirate and discard the supernatant
8. Wash the pellet 2-3X in 1XPBS, by centrifuging at 300g for 5 minutes. Aspirate and discard the supernatant
9. Add RPMI 1640 (10% FBS, PSN, L-glut) to resuspend the pellet and count the cells
10. Prepare the organoid culturing medium by add the following agents to RPMI 1640 (10% FBS, PSN, L-glut):
    a. 2XB27 final concentration (stock: 50X)
    b. 40ng/ml EGF final concentration (stock: 200μg/ml)
    c. 40ng/ml FGFb final concentration (stock: 50 μg/ml)
11. Aspirate, with a 1ml syringe, the cell suspension volume needed to obtain the desired numbers of cells
12. Add, drop by drop, the cell suspension to 35mm dishes. Every dish should contain 10^3-10^4cells/2ml medium, depending on the cell line (each cell line consists of their own amount of cancer stem cells)
13. Place the 35mm dishes inside 100mm dishe. Normally, three 35mm dishes will fit into a one 10mm dish. Do not stack the dishes
14. Culture the tumor organoids in an incubator at 37°C, 5% CO_2, from two to four weeks

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

6 Next generation sequencing (NGS)

Exceptional precision and reliability of GenXPro's NGS procedures outlined here rely much on extensive use of the TrueQuant (= Unique Molecular Identifier, UMI) which was invented by GenXPro's co-founder Dr. Björn Rotter and patented by the company (Patent DE102008025656B) already in 2008.
The UMI is a unique tag of random sequence that is added to each molecule, either DNA or RNA, present in the original mix of nucleic acids from any source. The nucleic acids can then be PCR-amplified at any step during preparation for NGS. Molecules that are duplicated during the process and thus are PCR artefacts contain the same combination of sequence and UMI and are bioinformatically removed from the data set. Therefore, this technology allows reliable quantification of any nucleic acid from even very low amounts of material as e.g. from FFPE-material or blood plasma. The UMI is contained in all GenXPro's NGS procedures and kits applied in the frame of the REVERT project for Exome-Seq, MACE-Seq, small RNA-Seq and Methyl-Seq. Detailed manuals for application of the latest version of commercially available kits accompany each of the kits and are available from the company on request. In the following we therefore outline only a rough overview of the technologies used and refer the reader to these manuals and relevant literature citations for extensive detail. Also, we roughly describe the bioinformatics workflows we use to process the sequencing data and the output of these procedures. Again, we refer the reader to the respective literature cited for more detail.

6.1 Sample preparation and shipment

6.1.1 Fresh-frozen tissue

Fresh-frozen tissue samples should be flash-frozen in liquid nitrogen and stored at -80°C. Shipment should be performed in dry-ice and with overnight carrier.

6.1.2 Blood (plasma and buffy coat)

1. Collet whole blood in 7.5 mL EDTA tubes
2. Gently mix tube content by inverting the tubes
3. Process EDTA tubes within 1h
4. Centrifuge samples at 300g, for 10 min, at RT and collect carefully the upper plasma layer
5. Collect carefully the buffy coat and resuspend it in ammonium salt containing red-blood cells lysis buffer to selectively lyse red blood cells and centrifuge (300g,10 min, RT) to remove the hemoglobin
6. Remove supernatant and repeat the resuspension and centrifugation
7. Lyse the remaining nucleus-containing cells by removing the supernatant and add 500µl of guanodinium containing lysis buffer (RLT plus) per each millilitre of EDTA-blood
8. Mix by pipetting and vortex thoroughly
9. Store at -80°C and ship in dry-ice with overnight carrier
10. Centrifuged once more EDTA-plasma at 1800g, for 10 min, at RT
11. Collect carefully the supernatant and store it at -80°C
12. Ship in dry-ice and with overnight carrier

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

Do NOT use Heparin containing vacutainers for any blood samples dedicated for NGS.

6.1.3 Cell culture material and organoids

Cell culture material should be processed differently according to the type of cells.

1. Pellet cell suspension by gentle centrifugation at 1200g, for 5 min, at 4°C
2. Remove the supernatant and add 500µl of lysis buffer (RLT plus) per each 10^6cells
3. Mix by pipetting and vortex thoroughly
4. Store samples at -80°C
5. Lyse adherent cells directly on the culture well or flask by adding lysis buffer and mixing thoroughly by pipetting
6. Store samples at -80°C
7. Ship in dry-ice and with overnight carrier

Samples derived from organoids must be treated according to its size and correspondent number of cells.

For small organoids with less than $5*10^4$ cells:

1. Lyse by means of lysis buffer and thorough mixing
2. Store samples at -80°C

For samples with more than $5*10^4$ cells, see Section 6.1.1.

Do NOT use RNAlater.

6.1.4 FFPE material

For FFPE material, the need is stacked 10 µm FFPE-slides per sample with at least 2 mm² of tumor or peritumoral/stroma tissue per sample to reach about at least 10 mm² of total surface.

In case matched tumor and normal samples are to be analysed, a stack of samples in which the first slide is stained and marked with tumor (t) and normal (n) for a macro-dissection, should be provided.

If the tissue consists only of tumour tissue, the FPPE sections can be shipped as 10µm thick curls with at least 10 mm² of surface.

All samples can be shipped via standard shipping at room temperature.

6.2 Sequencing of nucleic acid

6.2.1 mRNAs: massive analysis of cDNA ends (MACE-Seq)

MACE-Seq performs gene expression profiling by sequencing part of the 3' end of mRNA transcripts. Samples with 10-500 ng of total RNA are used for library preparation.

MACE-seq is performed using the GenXPro MACE-Seq kit. A recent, detailed manual accompanies each kit. Briefly, RNA is fragmented using GenXPro fragmentation buffer and subsequent synthesis of cDNA is performed by reverse transcription using barcoded oligo(dT) primers containing TrueQuant unique molecular identifiers, followed by template switching.

Library amplification is done using polymerase chain reaction (PCR), purified by solid phase reversible immobilization beads and subsequent sequencing is performed using the Illumina platform.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

6.2.2 Small RNAs: sRNA-Seq

Small RNA libraries are prepared using the TrueQuant sRNA-Seq Kit.

Samples with 1-500 ng of total RNA are used for library preparation. A recent, detailed manual accompanies each kit. Briefly, 3' and 5' TrueQuant adapters are ligated to small RNA. Adapter-ligated RNA is reverse transcribed and amplified by polymerase chain reaction.

The amplified libraries are purified by solid phase reversible immobilization beads and subsequent sequencing is performed using the Illumina platform.

6.2.3 Genomic DNA: Exome-Seq

Exome-Seq performs genome-wide analysis of protein-coding regions of genes in a genome.

Samples with 10-500ng of genomic DNA are used for library preparation. A recent, detailed manual is under preparation and will be availbale by the end of 2021.

Currently, genomic DNA is randomly sheared using a Bioruptor (Diagenode), TrueQuant adapters are ligated, amplified by polymerase chain reaction (PCR) and purified by solid phase reversible immobilization beads. The targeted enrichment is performed by solution-based hybrid capture. Briefly, the libraries are hybridized with biotinylated probes, captured by streptavidin beads and enriched by polymerase chain reaction (PCR).

The amplified libraries are purified by solid phase reversible immobilization beads and subsequent sequencing is performed using the Illumina platform.

6.2.4 Genomic DNA: Methyl-Seq

Methyl-Seq performs genome-wide analysis of CpG DNA methylation using the GenXPro Methyl-Seq kit. A recent, detailed manual accompanies each kit.

Samples with 10-500ng of genomic DNA are used for library preparation. In brief, HpaII is used as the methylation-sensitive enzyme, recognizing non-CpG-methylated CCGG sites. After digestion with HpaII, TrueQuant adapters are ligated to the digested DNA molecules. Ligated DNA is randomly sheared using a Bioruptor (Diagenode) and TrueQuant adapters are ligated.

Library amplification is done using polymerase chain reaction (PCR), purified by solid phase reversible immobilization beads and subsequent sequencing is performed using an Illumina platform.

6.3 Bioinformatics

6.3.1 Bioinformatics processing and statistical analysis of MACE-Seq data

Adapter sequences are removed using Cutadapt v2.1 (Martin 2011). PCR-derived duplicates are removed using TrueQuant technology (GenXPro, Frankfurt am Main, Germany). Using Bowtie2 in "local" mode (Langmead et al. 2012), MACE reads are mapped onto the Genome Reference Consortium Human Build 38 (hg38 / GRCh38). Mapped reads are being counted using HTSeq R/Bioconductor package (Anders et al. 2014) and annotated on exon based features derived from ENSEMBL, version 100.38. Normalization and testing for differential expression is calculated using the DESeq2 R/Bioconductor package (Love et al. 2014).

6.3.2 Bioinformatics processing and statistical analysis of sRNA-Seq data

A total of 2 to 5 million sRNA-seq reads per sample are sufficient to obtain a comprehensive smallRNA-seq profile. Adapter sequences are removed using Cutadapt v2.1 (Martin 2011). PCR-derived duplicates are

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

removed using TrueQuant technology (GenXPro, Frankfurt am Main, Germany). Using Bowtie2 (Langmead et al. 2012), sRNA-seq reads are first mapped to mature miRNA (miRBase version 22, Kozomara et al. 2019), followed by mapping of piRNA (piRNABank, Lakshmi et al. 2008), transfer RNA (tRNA) (GtRNAdb 18.1 (Chan et al. 2012), ncRNA (Ensembl 100, Yates et al. 2020]) and finally to the human genome (HMG19/39). Only unaligned reads from each mapping step are used for the following set. Mapping of mature miRNA is performed in "end-to-end" mode and the following alignments are performed in "local" mode. The number of reads per detected transcript is counted using a GenXPro in-house script. Normalization and testing for differential expression is calculated using the DESeq2 R/Bioconductor package (Love et al. 2014).

6.3.3 Bioinformatics processing and statistical analysis of Exome-Seq data

Adapter sequences are removed using Cutadapt v2.1 (Martin 2011). Using Burrows-Wheeler-Aligner (BWA-MEM, version 0.7.12, Li 2013), Exome-Seq reads are mapped onto the Genome Reference Consortium Human Build 37 (hg19 / GRCh37). SNP/InDel-Detection is performed by using the Genome Analysis Toolkit from Broad Institute (GATK, version 3.5, McKenna et al. 2010). GATK/MuTect2 is being used for calling somatic mutations. Annotation of variants was performed using SnpEff (version 4.2, Cingolani et al. 2012). Annotation of actionable mutations is performed using CIViC database (https://civicdb.org).

6.3.4 Bioinformatics processing and statistical analysis of Methyl-Seq data

Adapter sequences are removed using Cutadapt v2.1 (Martin 2011). PCR-derived duplicates are removed using TrueQuant technology (GenXPro, Frankfurt am Main, Germany). Read data is filtered by the starting sequence "CGG", which is the recognition site of HpaII. Frequencies of reads are determined. Normalization and testing for differential expression is calculated using the DESeq2 R/Bioconductor package (Love et al. 2014).

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

7 Essential References

Standard Procedures and Guidelines

1. World Health Organization. WHO Guidelines on Drawing Blood: Best Practices in Phlebotomy. World Health Organization press 2010; Geneva
2. Procedures for the collection of diagnostic blood specimens by venipuncture. Approved standard, H3-A5. Wayne, PA, National Committee for Clinical Laboratory Standards, 2003
3. Clinical Laboratory Standards Institute. Procedures for the collection of diagnostic blood specimens by venipuncture. CLSI H3-A6 document. 6th ed. Wayne, PA: Clinical Laboratory Standards Institute; 2007
4. Clinical Laboratory Standards Institute. Procedures for the handling and processing of blood specimens for common laboratory tests. CLSI H18-A4 document. 4th ed. Wayne, PA: Clinical Laboratory Standards Institute; 2010
5. Clinical Laboratory Standards Institute. Collection, Transport, and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays and Molecular Hemostasis Assays. CLSI H21-A5 document. 5th ed. Wayne, PA: Clinical Laboratory Standards Institute; 2008

CAT Serum and EDTA plasma

6. Canadian Tumor Repository Network (CTRNet)
7. Betsou F. et al., Standard preanalytical coding for biospecimens: defining the sample PREanayltical Code, Cancer Epidemiol Biomarkers Prev, 2010
8. Preanalytical guidelines for clinical proteomics investigations of biological fluids, Ann Biol Clin, vol 67, n°6
9. Molecular Medicine Ireland Guidelines for Standardized Biobanking, Biopreservation & Biobanking, Vol 8, n°1, 2010
10. Tuck MK et al., Standard operating procedures for serum and plasma collection: early detection research network consensus statement. Standard operating procedures integration working group, J Proteome Res., Vol 8, n°1, 113-117, 2009
11. Cryopréservation de tissus, cellules et liquids biologiques, Haute Autorité de Santé/service des bonne pratiques professionnelles, September 2009
12. Trezzi JP et al., LacaScore: a novel plasma sample quality control tool based on ascorbic acid and lactic acid levels, Metabolomics, 12:96, 2016

Fragile analytes (i.e. inflammatory biomarkers)

13. Dugué B. et al., Preanalytical factors and the measurement of cytokines in human subjects, Int J Clin Lab Res; 26(2):99-105, 1996
14. Dittadi R. et al., Validation of blood collection procedures for the determination of circulating vascular endothelial growth factor (VEGF) in different blood compartments, Int J Biol Markers, Apr-Jun;16(2):87-96, 2001
15. Rai AJ et al., Effects of preanalytical variables on peptide and protein measurements in human serum and plasma: implications for clinical proteomics, Expert Rev Proteomics; Aug;3(4):409-26, 2006

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

16. Skogstrand K. et al., Effects of blood sample handling procedures on measurable inflammatory markers in plasma, serum and dried blood spot samples, J Immunol Methods, Jul 20;336(1):78-84, 2008

17. Agrawal L. et al., Understanding preanalytical variables and their effects on clinical biomarkers of oncology and immunotherapy, Semin Cancer Biol, Oct;52(Pt 2):26-38, 2018

18. Technical Datasheet – R&D systems™ a Bio-Techne Brand, Magnetic Luminex Performance Assay (Human Cytokine Premixed Kit A)

EDTA buffy coat

19. Sherwood JL et al., Optimised Pre-Analytical Methods Improve KRAS Mutation Detection in Circulating Tumour DNA (ctDNA) from Patients with Non-Small Cell Lung Cancer (NSCLC), PLoS ONE, Feb 26;11(2), 2016

Urine

20. Sherwood JL et al., Optimised Pre-Analytical Methods Improve KRAS Mutation Detection in Circulating Tumour DNA (ctDNA) from Patients with Non-Small Cell Lung Cancer (NSCLC), PLoS ONE, Feb 26;11(2), 2016

21. Human urine and kidney proteome project (HUKPP) and European urine and kidney proteomics: standard protocol for urine collection (www.hukpp.org)

22. Hohemberger E. and Kimling H, Compendium urinalysis , urinalysis with test strips, Roche Diagnostics GmbH, 2008

23. Medeiros M. et al., Optimization of RNA yield, purity and mRNA copy number by treatment of urine cell pellets with RNAlater, J Immunol Methods 279,135-142, 2003

Stools

24. Falony G. et al., Population-level analysis of gut microbiome variation. Science 352, Issue 6285, 2016

25. Nechvatal JM et al., Fecal collection, ambient preservation, and DNA extraction for PCR amplification of bacterial and human markers from human feces, J Microbiol Methods; 72: 124-132, 2008

26. Lewis SJ and Heaton KW, Stool form scale as a useful guide to intestinal transit time, Scand J. Gastroenterol; 32 (9): 920-4, 1997

Tumor organoid

27. Semenas J. et al., Targeted inhibition of ERa signaling and PIP5K1a/Akt pathways in castration-resistant prostate cancer, Mol Oncol; doi: 10.1002/1878-0261.12873; 2020

Tissues (Pathology)

28. Hewitson TD and Darby IA, Histology Protocols, Springer-Humana Press, 2010

Next Generation Sequencing

29. Anders S. et al., HTSeq — A Python framework to work with high-throughput sequencing data. Bioinformatics, in print, online at doi:10.1093/bioinformatics/btu638, 2014

30. Chan PP and TM Lowe, GtRNAdb 2.0: An expanded database of transfer RNA genes identified in complete and draft genomes, Nucleic Acids Res. 44, D184–D189, 2016

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

31. Cingolani P. et al., A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3.", Fly (Austin); 6:80-92, 2012
32. Dweep H. and N. Gretz, MiRWalk2.0: A comprehensive atlas of microRNA-target interactions, Nat. Methods. 12; 697, 2015
33. Enright AJ et al., MicroRNA targets in Drosophila, Genome Biol. 5, 2003
34. Francisco DMF et al., Advancing brain barriers RNA sequencing: guidelines from experimental design to publication, Fluids Barriers CNS. 17; 51, 2020
35. Hashim A. et al., RNA sequencing identifies specific PIWI-interacting small noncoding RNA expression patterns in breast cancer, Oncotarget 5; 9901–9910, 2014
36. Engler A. et al., Establishment of an easy and straight forward heparinase protocol to analyse circulating and myocardial tissue micro-RNA during coronary artery-bypass-graft surgery, Scientific Reports 8, 1361, 2018
37. Lakshmi SS and Agrawal S., piRNABank: A web resource on classified and clustered Piwi-interacting RNAs, Nucleic Acids Res. 36, D173, 2008
38. Langmead B., S.L. Salzberg, Fast gapped-read alignment with Bowtie 2, Nat. Methods. 9; 357–359, 2012
39. Li H., Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM, arXiv: 1303.3997v2, 2013
40. Lipps CP et al., Non-Invasive Approach for Evaluation of Pulmonary Hypertension Using Extracellular Vesicle-Associated Small Non-Coding RNA, Biomolecules, 2019
41. Love MI et al., Moderated estimation of fold change and dispersion for RNA-seq data with ESeq2, Genome Biol. 15; 550, 2014
42. Martin M., Cutadapt removes adapter sequences from high-throughput sequencing reads, EMBnet.Journal. 17; 10 2011
43. McKenna A. et al., The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res, 20:1297-303, 2010
44. Rapado-González Ó. Et al., Circulating microRNAs as Promising Biomarkers in Colorectal Cancer. Cancers (Basel); 11:898, 2019
45. Rozowsky J. et al., exceRpt: A Comprehensive Analytic Platform for Extracellular RNA Profiling, Cell Systems, 24; 8(4):352-357, 2019
46. Kozomara A. et al., MiRBase: From microRNA sequences to function, Nucleic Acids Res. 47, D155–D162, 2019
47. Raudvere U. et al., Profiler: A web server for functional enrichment analysis and conversions of gene lists (2019 update), Nucleic Acids Res. 47, W191–W198, 2019
48. Szklarczyk D. et al., STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets, Nucleic Acids Res. 47, D607–D613, 2019
49. Yates AD et al., Ensembl 2020, Nucleic Acids Res. 48, D682–D688, 2020.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

8 Revision History

Version number	Date	Action
2.4	12-Apr-2021	Add-on NGS SOPs
2.3	16-Mar-2021	Document revision and approval by UMU and UCAM
2.2	11-Mar-2021	Add-on clinical pathology SOPs (UCAM)
2.1	08-Mar-2021	Add-on organoids SOPs (UMU)
2.0	25-Feb-2021	Document formatted into the official REVERT template for documents (LIH). AULSS3 full draft editing/revision and approval
1.6	01-Feb-2021	Final draft to SR for final revision
1.5	26-Jan-2021	Add-on of fragile biomarkers SOPs (AULSS3). AULSS3 full draft editing/revision
1.4	25-Nov-2020	Release to AULSS3 and SR partners of 'Post-survey unified SOPs (bullet points)', unified tables for liquid and solid biospecimens
1.3	19-Nov-2020	Bibliography add-on (LIH) to 'Clinical Platforms Fitness-for-Purpose'
1.2	17-Nov-2020	Internal (LIH) final revision of 'Post-survey unified SOPs (bullet points)'
1.1	06-Nov-2020	Final table 'Clinical Platforms Fitness-for-Purpose' (LIH)
1.0	28-Oct-2020	Draft 'Post-survey unified SOPs (bullet points)' (LIH)

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".

End of D4.1

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 848098".