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DELIVERABLE 3.1

SOP for tissue collection and processing

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List of Acronyms

CBTM	Cambridge Biorepository for Translational Medicine		**GRL**	Genome Research Limited		**SOP**	Standard Operating Procedure
CCHT	Tervisetehnoloogiate Arenduskeskus AS		**ICH**	International Conference on Harmonization		**sPE**	Severe preeclampsia
CGP	Good Clinical Practices		**INCLIVA**	Hospital Clinic of Valencia Research Foundation		**UEA**	University of East Anglia
CEI	Ethics Committee		**HUTER**	Human Uterus Cell Atlas Project		**UPPSALA**	Uppsala Universitet
DNA	Deoxyribonucleic acid		**RNA**	Ribonucleic acid			

1. PURPOSE

The rationale of this Standard Operating Procedure (SOP) is to serve as a practical guide for the HUTER partners managing samples and with the aim to achieve uniformity of performance during sample collection and processing, with high-standard and reproducible laboratory transcriptome/genetic/epigenetic analysis under controlled conditions. Nevertheless, these SOPs could be subject to minor modifications in order to ensure they stay relevant to the current needs of the Consortium members.

2. INTRODUCTION

HUTER project is a descriptive case series, prospective, competitive, multicenter and international clinical study. All participating centers will be responsible for the recruitment of patients and obtaining the samples described below.

HUTER aims to create the single-cell and spatial reference map of the human uterus by providing unprecedented insight at transcriptomic, genomic and spatial changes of this important female organ not only throughout the menstrual cycle but also across lifespan (pre-menstrual, young adult, adult, post-menopausal). For this purpose, a detailed molecular characterization through RNA/DNA single-cell sequencing and other approaches for the different endometrial and myometrial cell types and stages will be performed.

2.1. Basic requirements

This research project will respect the essential principles established in the Declaration of Helsinki, the International Conference on Harmonization (ICH) Good Clinical Practices (CGP), the Agreement of the European Council concerning Human Rights and Biomedicine, in the Universal Declaration of UNESCO on the Human Genome and Human Rights, as well as fulfilling the requirements established in the local legislation of the biomedical research field, personal data protection, and bioethics.

Signing the informed consent form approved by the Research Ethics Committee (CEI) is required for the inclusion of patients in the study. For this purpose, healthcare practitioners should have enough experience and training according to local research governance procedures to take written informed consent and perform the sampling procedure.

Prior to the invasive sampling procedure, participants (or their representatives when deceased donors) should be allowed to read and understand the participant information sheet, knowing the potential risks, benefits and inconveniences arising from their participation and allowed to ask any related questions. In accordance, they should be asked to complete and sign two copies of the consent form. Participants should be also

informed that they may leave the study at any time, without this implying any consequences for their subsequent medical care.

2.2 Study population and sample type

The population of study will be recruited from 3 different European countries (Estonia, UK and Spain). Type of patient recruited, and sample collected will change according to the participant site and country. The following Table 1 summarises HUTER foresee type, number and place where the samples will be collected:

Table 1. Summary of sample procurement plan

Sample Type (Group)	Study population &Recruitment site	Sample size
WHOLE UTERUS (G1)	***Deceased donors:*** - CBTM (UK) - H. CLINIC VALENCIA (Spain) ***Live individuals:*** -Research volunteers when attending hysterectomies due to pelvic prolapse at Hospital La Fe (Spain).	**n= 40** 10 x girls before menarche (5 - 15 years old) 10 x young donors in reproductive age (18 – 25 years old) 10 x adult donors in reproductive age (26 - 42 years old) 10 x postmenopausal donors (50 to 80 years old)
ENDOMETRIAL BIOPSIES (G2)	***Live individuals:*** From healthy research volunteers attendingObs/Gyn service at Hospital La Fe (Spain), and at South-Estonian Hospital (Estonia).	**n=50** (reproductive age, 18 – 42 years old)
ENDOMETRIAL BIOPSIES sPE (G3)	***Live individuals:*** From patients who developed **sPE** in a previous pregnancy at Hospital La Fe (Spain).	**n=15** (reproductive age, 18 – 42 years old)

3. EXPERIMENTAL PROCEDURES AND WORK-FLOW

Samples will be obtained from three different locations in Europe; H. CLINIC VALENCIA (Spain), CBTM (UK) and SOUTH-ESTONIAN HOSPITAL (Estonia), and up to five different experimental techniques will be performed in order to accomplish the objectives of the HUTER project, aiming to obtain a full single cell characterization of the human uterus along lifespan.

All the five procedures and the places they will be performed are listed below:

- Single-cell RNA library preparation and/or sequencing

  INCLIVA (Spain), GRL (UK) and CCHT (Estonia)

- Protein atlas

  UPPSALA (Sweden)

- Spatial transcriptomics

  GRL (UK)

- 30× whole genome sequencing

  GRL (UK)

- Single-cell epigenomic analysis

  UEA (UK)

3.1 Whole uterus

3.1.1 Whole uterus (G1) sampling:

As it is shown in the figure 1, **healthy endometrial (E) and myometrial (M) tissues** will be obtained from **whole uterus samples** from deceased donors **(G1)** collected in Spain and UK. As additional source, whole uterus will be also collected from alive healthy live research participants with indication for hysterectomy due to pelvic prolapse (Spain).

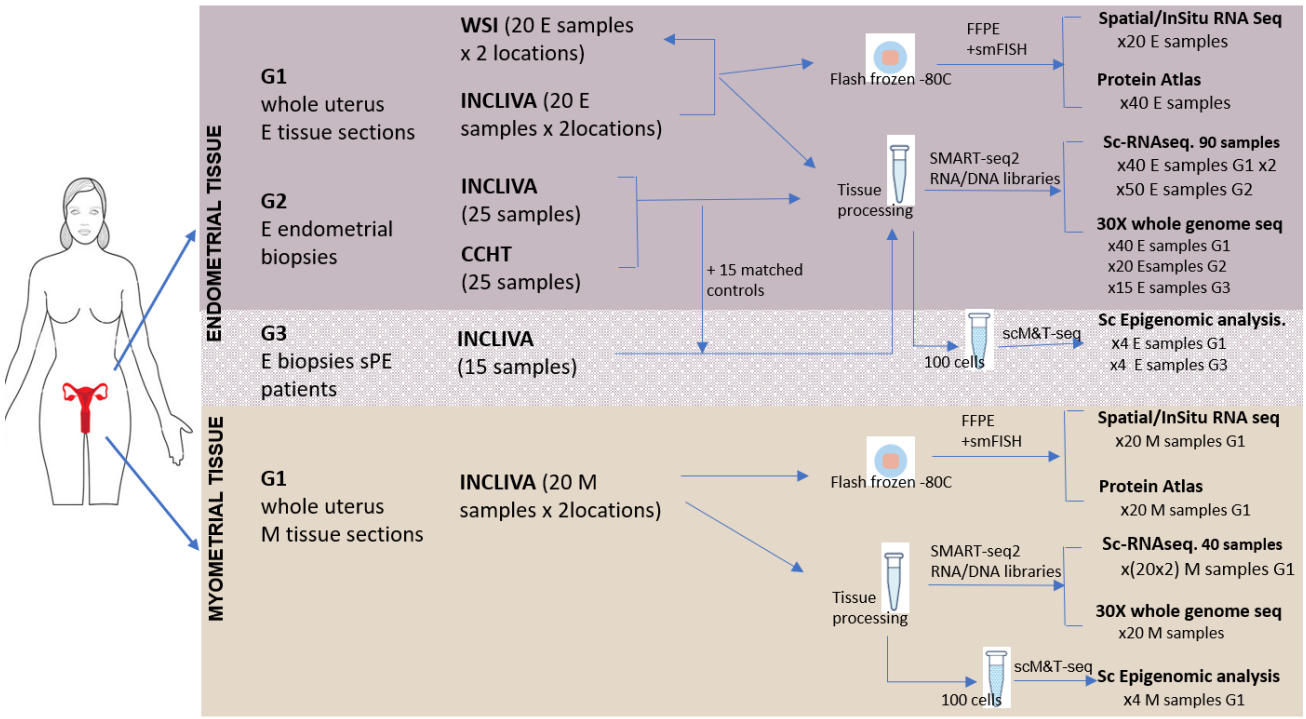

Fig1. Work-flow for processing samples

Collaborating gynecologists or surgeon will surgically remove the whole uterus in the operating room and place the organ in a container with ice and proceed with its quickly transfer to the laboratory in a preservation solution (HypoThermosol® FRS) at 4°C, for their prompt processing, ensuring viability of the organs and cells. In all cases, before whole uterus processing, physical specimens will be registered and imaged for their physical coordinates, and two adjacent sections (3×3 cm) of the same specimen will be isolated and analysed by cellular and spatial approaches. Endometrial (E) and myometrial (M) tissue sections will be sampled in both cases for the performance of the five previous mentioned experimental procedures.

It is important to note, that all the experiments described in this deliverable will be preferably processed fresh. Nevertheless, in order to ensure all the procedures are performed, and the objectives of the HUTER project are fully accomplished, a second portion of **all collected tissue samples will be cryopreservated** for long-term storage. For this purpose, samples will be put into a NalgenewCryo 1°C "Mr. Frosty" Freezing Container (Thermo Scientific, USA) and placed into -80°C freezer overnight and will be stored in liquid nitrogen till transportation on dry ice or laboratory manipulations. Alternative is to put the sample with "Mr. Frosty" to -20°C for max 3h and then place -80°C freezer overnight before liquid nitrogen storage. The usage of proper media and moderate freezing conserves intact cells, providing living cells for further specific antibody labelling and sorting steps (modified according to Krjutškov et al., Human Reproduction, 2016). These cryopreservated portions will serve as a backup in the case they need to be used to repeat some analysis or procedures that could not be correctly performed in fresh samples.

3.1.2 Endometrial tissue dissociation for single cell RNAseq:

The following protocol describes the tissue dissociation procedures from human endometrium samples. Endometrial tissues will be immediatelly procesed following the tissue dissociation procedures adapted from Vento-Tormo et al. 2018 with some modification from Prof. Ashley Moffett (Department of Pathology, University of Cambridge). Part of the biopsy is cryopreserved as for backup. The online version of the protocol is posted here https://www.protocols.io/view/endometrium-dissociation-with-collagenase-76thren.

a) Optional: wash tissue sample twice with PBS solution.
b) Place wet tissue under a petri dish. Take 2 scalpels and roughly mince up the tissue.
c) Transfer contents to 50 ml tube containing the collagenase mix (3 ml/tissue but it will depend on the size of the tissue).
d) Tighten lid and incubate at 37°C for 45 min. Shaking during the incubation is recommended.
e) Resuspend with 20 ml RPMI 10%.
f) Filter sample through 100 µm strainer – **do not discard retained tissue!** The retained tissue will be used for "**Endometrium-Trypsin**" protocol below.
g) Centrifuge filtered material at 450×*g* for 5 min.

h) Wash the pellet twice with 10 ml PBS.

i) Resuspend sample with 2 ml to 4 ml of 1× RBC lysis buffer mix and incubate for 10 min. RLB preparation: Dilute 10X RLB stock with water.

j) After RBC lysis, add 10 ml of RPMI 10% and centrifuge 450×*g* for 5 min.

k) Wash twice with 10 ml PBS.

l) Resuspend with 1 ml RPMI 10%, count cells and proceed with the dead-cell exclusion procedure, that can be performed in two possible ways:

   a. Cells will be stained for flow cytometry and dead cells will be excluded by sorting.

   b. Dead cells will be excluded from the sample using the MACS Dead Cell Removal Kit (*Milteny Biotech*), following manufacture's procedures.

The following protocol is for enrichment of epithelial glands on **endometrium**, following the step from "Endometrium dissociation with collagenase". Use material from step "**f)"** above.

https://www.protocols.io/view/endometrium-dissociation-with-trypsin-72dhqa6?step=3

Collect pieces retained on the filter by inverting the filter into a new 50 mL tube and adding 45 ml of PBS with a 1 ml pipette. Continue with following steps:

a) Centrifuge at 450×*g* for 5 min together with collagenased sample. Discard supernatant.

b) Resuspend the pieces with 10 ml Trypsin/EDTA 0.25% and incubate for 20 min at 37°C while shaking.

c) Add 20 ml RPMI with 10% FBS.

d) Filter material slowly and carefully (very dens liquid) through a 100 µM strainer. Discard retained tissue.

e) Centrifuge at 500×*g* for 5 min at 4°C. Discard supernatant.

f) Resuspend sample with 5 ml RLB mix and incubate for 10 min.

g) After RBC lysis, add 10 ml PBS and centrifuge at 500×*g* for 5 min.

h) Resuspend cells in 1 ml PBS in 2 ml clean tube.

i) Centrifuge at 500×*g* for 5 min at 4°C and discard supernatant.

j) Resuspend with 250 µl PBS (final volume depending on cell number).

k) Filter cells trough a cell strainer snap cap tube.

l) Count live cells using tryphan blue and haematocytometer.

3.1.3. Myometrial tissue dissociation for single cell RNAseq:

Myometrial tissues will be rinsed in wash buffer solution containing Hank's Balanced Salt Solution and 1% antibiotic-antimycotic solution for the removal of remaining blood and mucus. Afterward, they will be immediately procesed following the tissue dissociation procedures.

The following protocol describes the tissue dissociation procedures from human myometrial samples. This protocol is adapted from Mas et al., 2012 with minor modifications. The online version of the protocol is posted here: dx.doi.org/10.17504/protocols.io.bb5miq46

a) Uterine tissues should be transported in preservation solution (HypoThermosol® FRS) at 4°C from the surgery room to the lab.
b) Rinse the sample with PBS, removing blood or mucus.
c) Place wet tissue under a petri dish. Isolate with a sterile scalpel the myometrial layer by removing the endometrium, serosa and laser-burnt zones.
d) Mechanical disaggregation by chopping and thoroughly mincing up the tissue into small pieces (<1 mm3).
e) Transfer contents to 50 ml tubes containing 30 ml of enzyme buffer. Tighten lid, seal with parafilm and incubate at 37°C overnight horizontally.
f) Afterwards, cell suspensions should be filtered through 100 to 50-µm polyethylene filters to remove cellular clumps and undigested tissue.
g) Centrifuge the filtered medium 400×*g* 5 min. Remove supernatant and add at least 1 ml of resuspension buffer (depending of the pellet content).
h) Optional: if after centrifugation there is a ring of red blood cells, add ACKL lysis buffer, consisting in hypotonic shock, and incubate at 37°C for 5 min. Then, follow step 7 again.
i) Add 400 µl Tryple Select Enzyme to resuspended media containing cells and mix thoroughly by pipetting. Incubate for 15 min at 37°C and centrifuge at 400×*g*.
j) Add 100 µl DNAse I to digest the extracellular genomic DNA and mix thoroughly by pipetting. Incubate for 5 min at room temperature.
k) Add at least 1 ml of resuspension buffer. Filter through a 40 µm cell strainer and centrifuge at 400×*g* for 5 min.
l) Remove supernatant and resuspend the content in at least 1 ml of resuspension buffer (depending of the pellet content).
m) Proceed with the dead-cell exclusion procedure, using the MACS Dead Cell Removal Kit (*Milteny Biotech*), following manufacture's procedures.

n) Count the total number of cells using tryphan blue and haematocytometer (at least twice on two different cell counters).

o) Proceed to 10x experiment.

3.1.4. Uterine tissue fixation with formaldehyde for Protein Atlas purposes:

Selected tissue samples (endometrium: n=40, myometrium: n=20) will be fixed in 4% formaldehyde in PBS and paraffin embedded. Samples will be stored at room temperature and shipped to UPPSALA lab to perform the **protein atlas through spatial immunohistochemical image maps**.

Formaldehyde fixation is the first step in preparing the sample (from whole uterus/endometrial biopsy). Formaldehyde is a cross-linking fixative which acts by creating covalent chemical bonds between proteins in tissue. This anchors soluble proteins to the cytoskeleton, and lends additional rigidity to the tissue. Normally this method uses 4% formaldehyde in PBS.

Procedure:

a) Normal pathological examination is performed with measurements and weight.
b) Whole tissue (eg. uterus) is opened up from the cervical opening to the fundus, not cutting all the way through, this helps to increase fixation speed.
c) Next, the uterus is fixed on a cork plate using needles, this ensures that the uterus is fixated in a stable way and not twisting around. Remember to fixate the fallopian tubes and ovaries in the same way if they are present.
d) Place cork plate with uterus (upside down to ensure that the uterus is fully submerged) in a suitable container and fill with approximately 10-20 times the volume of tissue with formaldehyde.
e) Leave at room temperature for at least 48 h.

Gross-sectioning refers to when the pathologist section the diagnostically interesting parts from the main sample to smaller samples. During this stage the pathologist removes the sample from the formaldehyde containers and sections the diagnostically important parts to smaller samples for Vacuum Infiltration Process (VIP). The smaller samples are placed in an appropriate tissue cassette for VIP. If the samples are not completely fixated they can be left in formaldehyde for another 24h before VIP.Remember to check that sample number and cassette number is the same.

For core and small biopsy preparation, samples will be placed in a small container with formaldehyde for 24 h before VIP.The biopsies are placed in an appropriate tissue cassette together with a tissue embedding sponge and folded filter paper to ensure that the core does not fold or break during VIP. If there are more than one

core from the same patient in a container, place them in separate cassettes and name them e.g. 1 & 2 or a & b. Remember to check that sample number and cassette number is the same.

Finally, tissue embedding refers to the mounting of the tissue sample into a block of paraffin for fine sectioning using a paraffin embedding machine.

Procedure:

a) Fill metal form with a small amount of paraffin.
b) Place sample (previously dehydrated) in the form and start cooling the form while pressing down on the sample. This is to ensure that the sample is placed in an even position.
    a. **This is especially important for core biopsies.**
c) When the paraffin starts to solidify, you place the cassette base on top of the form and fill it up with paraffin.
d) Move the whole form quickly to the cooling plate to ensure that the paraffin does not melt.
e) When the paraffin is completely cooled, it can be removed from the form and the tissue block sectioned.

3.1.5. Tissue Optimisation for Spatial Transcriptomics:

For 20 selected samples (both endometrial and myometrial), 5×5 mm fresh biopsies will be sectioned and inmediatelly cryopreservated (isopentane flash-frozen tissue) onto the Spatial Transcriptome slides for flash frozen at -80°C to study the tissue architecture through **Spatial Transcriptomics analysis** at WSI.

3.1.6 Tissue Optimisation for 30× Whole genome Seq:

Fresh biopsies of approximately 1 x 1 cm in size will be flash frozen and stored at -80 ^{o}C for whole genome sequencing.

3.1.7. Tissue Optimisation for Epigenomics Analysis:

Approximately 100 cells from the obtained cell suspensions in the previous procedures will be collected directly in lysis buffer and will be shipped to the UEA's laboratory for **epigenetics analysis at single-cell resolution.**

3.2 Endometrial biopsy

3.2.1. Endometrial biopsy (G2&G3) sampling:

As it is shown in the Figure 1, **healthy endometrial biopsies (G2) will be** collected in Spain and Estonia. On the other hand, **pathological endometrial biopsies (sPE, G3)** will be collected from Spain.

The essential equipment for endometrial biopsy collection includes; plastic gloves, pipelle catheter (Genetics, Belgium), cryotube with 2 ml cryopreservation medium, NalgenewCryo 1°C "Mr. Frosty" Freezing Container (Thermo Scientific, USA), cryotube with HypoThermosol® FRS solution and a 4°C cooler for quick transportation from clinic to laboratory.

The biopsy collection procedure will consist in obtaining endometrial samples by using a Pipelle catheter under sterile conditions; once the catheter is in the endometrial cavity, a scratch (or slight scraping) can be performed from the bottom to the cervix in each endometrial wall. The aspiration should be maintained during this scratching process, until the tissue enters the catheter.

The biopsy is placed on petri dish. Using a sterile scarpel the endometrial tissue is extracted from mucosa, blood traces, and divided as following;

a) Approx. 50% of the tissue is cryopreserved by in 2 ml cryopreservation medium using NalgenewCryo 1°C "Mr. Frosty" Freezing Container (Thermo Scientific, USA). This is **cryopreserved backup**.
b) Approx. 50% of the tissue is placed in HypoThermosol® FRS solution and a 4°C cooler and transported quicky to laboratory. This is **fresh biopsy**.

Fresh healthy- and pathological biopsies will be processed for sc-RNAseq, 30× whole genome seq and epigenomic analysis.

3.2.2. Endometrial biopsy dissociation for single cell RNAseq:

Fresh endometrial tissues will be immediatelly procesed following consecutive digestion with collagenase and trypsin. The online version of the protocols are posted here https://www.protocols.io/view/endometrium-dissociation-with-collagenase-76thren; dx.doi.org/10.17504/protocols.io.76thren

a) Optional: the tissue sample was washed twice with PBS solution.
b) Place wet tissue under a petri dish. Take 2 scalpels and roughly mince up the tissue.
c) Transfer contents to 50 ml falcon containing the collagenase mix (3 ml/tissue but it will depend on the size of the tissue).
d) Tighten lid and incubate at 37°C for 45 min. Shacking during the incubation is recommended.
e) Resuspend with 20 ml RPMI 10%.

f) Filter sample through small strainer (100 µm) – **do not discard retained tissue!** The retained tissue will be used for "**Endometrium-Trypsin**" protocol below.

g) Filtered material: centrifuge at 450×*g* for 5 min.

h) Wash twice with 10 ml PBS.

i) Resuspend sample with 2 ml to 4 ml of 1× RBC lysis buffer mix and incubate for 10 min. RLB preparation: Dilute 10× RLB stock with water.

j) After RBC lysis, add 10 mlof RPMI 10% and centrifuge 450×*g* for 5 min.

k) Wash twice with 10ml of PBS.

l) Resuspend with 1 ml RPMI 10%, count cells and proceed with the dead-cell exclusion procedure, that can be performed in two possible ways:

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Collect pieces retained on the filter by inverting the filter into a new 50 mL tube and adding 45 ml of PBS with a 1 ml pipette

m) Centrifuge at 450×*g* for 5 min together with collagenased sample. Discard supernatant.

n) Resuspend the pieces with 10 mlTrypsin/EDTA 0.25% and incubate for 20 min at 37°C while shaking.

o) Add 20 ml RPMI with 10% FBS.

p) Filter material slowly and carefully (very dens liquid) through a 100 µM strainer. Discard retained tissue.

q) Centrifuge at 500×*g* for 5 min at 4°C. Discard supernatant.

r) Resuspend sample with 5 ml RLB. Mix and incubate for 10 min.

s) After RBC lysis, add 10 ml of PBS and centrifuge at 500×*g* for 5 min.

t) Resuspend cells in 1 ml of PBS in 2 ml clean tube.

u) Centrifuge at 500×*g* for 5 min at 4°C and discard supernatant.

v) Resuspend with 250 µl of PBS (final volume depending on cell number).

w) Filter cells trough a cell strainer snap cap tube.

x) Count live cells using tryphan blue and haematocytometer.

3.2.3. Tissue Optimisation for Whole Genome Seq:

Tissue DNA and RNA will be extracted from fresh- frozen samples using the AllPrep DNA/RNA/miRNA kit (Qiagen), following the manufacturer's instructions. Short insert (500-bp) genomic libraries were constructed, flowcells were prepared and 150-bp paired-end sequencing clusters generated on the Illumina HiSeq X platform, according to Illumina no-PCR library protocols, to an average of 30× coverage.

3.2.4. Tissue Optimisation for Epigenomic Analysis:

Approximately 100 cells from the obtained cell suspensions in the previous procedures will be collected directly in lysis buffer and will be shipped to the UEA's laboratory for **epigenetics analysis at single-cell resolution.**