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🌐 Collection and storage of myometrium, decidua, fetal membranes, and placenta (RNAlater-treated and snap frozen) tissue samples with cervicovaginal fluid sampling

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We use this protocol and it's working

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Borne

Abstract

This protocol was developed as part of Dr Brendan Browne's 'Origins of Labour' PhD project in collaboration with the Borne Uterine Mapping Project (BUMP) feasibility study; specifically for the goal of storing human tissues of the uterine environment (obtained at Caesarean section) to later analyse using RNA-seq and SWATH-MS. Samples of eight different tissue types were collected; namely myometrium (upper segment basalis region, upper segment parietalis region, and lower segment), decidua (basalis and parietalis region), amnion, chorion, and placenta. Cervicovaginal swab samples were also collected (and cervical length measurements were acquired using a transvaginal ultrasound (TVUS) scanner).

Guidelines

Where ⊕ symbol is present at a protocol step, record the time that the associated step has been reached or what has been stated in the step to be noted for timing.

Materials

- Quantities stated are the recommended minimum - spare items should be made readily available, where possible.
- Items noted to be stored in freezer prior to use need to be stored in a -20°C freezer.
- All reagents and single-use consumables should be sterile-packed and nuclease-free.
- All Dulbecco's phosphate-buffered saline (DPBS) aliquots need to be prepared using aseptic technique in a Class II biological safety cabinet.

	Item	Quantity	Supplier	Product number	Note
	Plastic cooler bucket, rectangular and 4 L capacity	1	Fisher Scientific	11334085	To transport samples on pre-chilled gel packs from clinical area to laboratory
	Cooling gel packs	Enough to line the base of the 4 L plastic ice bucket	n/a	n/a	Store in freezer when not in use
	Tischler-Morgan 22 cm uterine biopsy punch forceps	2	Surtex Instruments	GY-871-22	Ideally keep in operating theatre; need to integrate into hospital sterilisation routines for surgery equipment
	Sterile gauze	3	n/a	n/a	Part of Caesarean section (operating theatre) pack
	Transvaginal ultrasound (TVUS) scanner	1	site-dependent		Clinical equipment to be used in the C-section operating theatre
	Speculum	1	site-dependent		
	Magnifier with built-in lighting	1	site-dependent		Alternative: dissection microscope
	Watchmaker forceps	2	World Precision Instruments	501979-6	
	Blunt end 'filter' forceps	1	Sigma-Aldrich	XX6200006P	
	Tissue dissection scissors	1	World Precision Instruments	14393	
	Microcentrifuge (fixed angle rotor); fits 1.5/2.0 mL	1	site-dependent		Suitable for 2500 g centrifugation

	Item	Quantity	Supplier	Product number	Note
	tubes, adjustable temperature settings				
	Micropipette, P200	1	site-dependent		
	Microfuge / cryovial tube racks	2	site-dependent		
	Polystyrene box with lid (~5 L capacity)	1	n/a		To store wet ice for use in laboratory
	Polystyrene box with lid (~20 L capacity)	1	n/a		To store dry ice for use in laboratory

List of equipment.

	Item	Quantity	Supplier	Product number	Note
	Dacron sterile culture swab	1	Nerbe Plus	09-511-5017	
	Cryovials with internal thread caps; 1.8 mL capacity	21	Starlab	E3110-6122	Pre-determine colour coding system for simple identification of different tissue types stored from each study participant
	Colour cap inserts (for cryovials)	21	Starlab	E1494-6099	
	Resealable (zip lock) specimen bags; 7.5 x 8.5 cm	8	Fisher Scientific	11326924	
	Petri dishes; 10 cm diameter	10	Greiner	632181	
	Sterile-packed scalpels, #22 blade	3	Appleton Woods	MP015	
	Filter paper	~1 pack	VWR	1002-917	
	Absorbent 'inco' pads	2	Medisave	55525	
	Cell scraper	2	Sarstedt	83.3590	
	Microfuge tubes; 1.5 mL capacity	2	Starlab	S1615-5500	

	Item	Quantity	Supplier	Product number	Note
	Pasteur pipettes; 1 mL capacity	2	Starlab	E1414-0111	
	Sterile filter micropipette tips, to fit P200 capacity micropipette	~1 box	site-dependent		
	Placental clinical disposal bags	2	n/a	n/a	Follow waste disposal procedure instructed by labour ward; also use a specimen bucket to transport placenta with fetal membranes to laboratory

List of single-use consumables. Quantities of tubes do not include those for aliquots listed in the 'chemicals and reagents' table.

	Item	Quantity	Supplier	Product number	Note
	1x Dulbecco's phosphate-buffered saline (DPBS; CaCl ₂ and MgCl ₂ included)	8 × 15 mL aliquots	Sigma-Aldrich	D8662	15 mL aliquots in Universal tubes (30mL capacity; Greiner, 201151)
	10x DPBS (CaCl ₂ and MgCl ₂ included)	1 × 25 mL aliquot (to prepare 250 mL of 1x DPBS)	Sigma-Aldrich	D1283	25 mL aliquots in 50 mL centrifuge tubes (Greiner, 227261)
	Autoclaved deionised water	225 mL	n/a	n/a	Use to dilute 10x DPBS to prepare 1x DPBS to use for washing fetal membranes and placenta
	1x DPBS (no CaCl ₂ , no MgCl ₂)	1 × 1 mL aliquot	Thermo Fisher Scientific (Gibco)	14190169	Supplement 1x DPBS with protease inhibitor by following manufacturer's instruction to obtain 1x working concentration of the latter, then prepare the supplemented DPBS as 1 mL aliquots in sterile cryovials (2 mL capacity; Starlab E1420-2343) to store in freezer
	cOmplete protease inhibitor cocktail	see Note	Sigma-Aldrich	11836145001	
	RNAlater	8 × 1.5 mL aliquots	Sigma-Aldrich	R0901	1.5 mL aliquots in RNase-free Eppendorf tubes (2 mL capacity; VWR 211-2165)



	Item	Quantity	Supplier	Product number	Note
	Wet ice	n/a			Store in polystyrene box (~5 L capacity) to use in laboratory
	Dry ice	n/a			Store in polystyrene box (~20 L capacity) to use in laboratory
	Ethanol (absolute)	variable	VWR	20821.330	Use to prepare 70% v/v ethanol for cleaning equipment and work surfaces, especially for items to be used within a Class II biological safety cabinet
	Virkon disinfectant (tablets)	variable	VWR	115-0021	To use as part of disposal of human tissue-containing waste in laboratory - solution in spray bottle and tablet in 500 mL plastic beaker for tissue dissection bench; follow local safety procedures

List of chemicals and reagents.**Other items (in addition to personal protective equipment)**

- permanent marker pens (for labelling e.g. DPBS aliquot tubes)
- ballpoint pens (for documenting timings of tissue collection, dissection, freezing, and storage)
- printouts of sample collection proforma (for documenting timings, number of tissue pieces and cryovials stored)
- 1x digital watch
- Disinfectant wipes (e.g. Clinell)
- paper towels
- plastic clinical waste sharps bins



Safety warnings

! In addition to assessing risks (**biohazards**) for the handling of human-derived tissues and biofluids, please seek local health and safety guidance for use of dry ice (**hazardous chemical**).

Ethics statement

Ethical approval must be obtained from the Local Research Ethics Committee (LREC), Institutional Review Board, or equivalent body to recruit pregnant study participants for the purpose of acquisition, use, and storage of human tissue and biofluid samples specified in the protocol. Researchers must confirm that written consent has been obtained (by Good Clinical Practice (GCP) certified personnel; or equivalent for outside of the UK) via a LREC approved study consent form before proceeding with the use of this protocol. Seek further guidance from the institution(s) responsible for hosting the study with regards to regulations to abide by for the use of human-derived samples.

This protocol does not involve the use of (non-human) animal tissues.

Before start

Preparation in the laboratory before commencement of C-section (on day of sample collection) - confirm the following:

- There is space available in -80°C cryoboxes to store the upcoming set of tissue samples.
- Tissue dissection bench has been disinfected (by wiping with e.g. Distel) and the items listed in **Table I** are present.
- The microcentrifuge is pre-chilled to 4°C and able to centrifuge at 2500 g for 10 minutes.
- The magnifier/dissection microscope is available to use for dissection of tissues once they have been transported from the operating theatre to the laboratory.
- The 4 L plastic cooler bucket has been packed with frozen gel packs (no earlier than an hour before study participant enters the operating theatre) to use for transporting 3 × 15 mL DPBS aliquots (separate from those listed in Table I) to labour ward for collection of myometrial biopsies in the operating theatre.

Item on tissue dissection bench
1x polystyrene box (with lid) of dry ice (~20 L)
1x polystyrene box (with lid) of wet ice (~5 L)
Thaw on wet ice: 1 × 1 mL frozen DPBS supplemented with protease inhibitor cocktail
Chill on wet ice: 5 × 15 mL DPBS aliquots - 1 labelled for each tissue type (from fetal membranes and placenta) to be collected (temporary storage prior to further dissection)
Chill on wet ice: 250 mL of 1x DPBS - prepared by adding 25 mL aliquot of 10x DPBS to 225 mL autoclaved deionised water in a Duran bottle
5 × 1.8 mL cryovials with colour cap inserts - all labelled for cervicovaginal fluid supernatant samples
8 × 1.5 mL RNAlater aliquots - 1 labelled for each tissue type to be collected (temporary storage prior to transfer into cryovials)
8 x resealable specimen bags (all zip locks closed) - 1 labelled for each tissue type to be collected (temporary storage prior to transfer into cryovials)
10 × 10 cm diameter Petri dishes
1 x Watchmaker forceps
1 x tissue dissection scissors
1 x blunt end (filter) forceps
1 x P200 micropipette + ~1x box of 0.2 mL sterile filter tips
2 (+1 spare) x sterile-packed #22 blade scalpels
70% v/v ethanol (in spray bottle)

	Item on tissue dissection bench
	1 (+1 spare) x cell scraper
	~1 x pack of filter paper (at least 16 sheets will be required)
	2 x absorbent 'inco' pads - lie onto dissection bench as a double layer with absorbent side facing up
	2 (+ spares) x 1.5 mL capacity microfuge tubes
	2 × 1 mL capacity Pasteur pipettes
	2 x permanent marker pens
	1 x Virkon tablet in 500 mL plastic beaker
	Virkon solution (in spray bottle)

Table I. Items to make available (before C-section commences) at tissue dissection bench in laboratory for tissues to be snap frozen on dry ice prior to -80°C storage.

Preparation at labour ward before commencement of C-section (on day of sample collection):

- Transport 3 × 15 mL DPBS aliquots inside the 4 L plastic bucket with semi-frozen gel packs (each wiped clean with disinfectant (Clinell) wipe, along with 1 x Dacron swab (labelled with study participant code) and 1 x Watchmaker forceps, to area within vicinity of the C-section operating theatre.
- Confirm written consent to study participation has been obtained from participant, and the surgeon is aware that they have consented to upper and lower segment myometrial biopsies.
- Ensure transvaginal ultrasound (TVUS) scanner, cardiotocograph (CTG), and speculum (along with the 1x Dacron swab) are available for use in the C-section operating theatre.
- Check the attending scrub nurse is aware that they need to (i) provide the surgeon with the Tischler-Morgan forceps after fetal delivery, (ii) 3 x sterile gauze is ready for placing the upper segment punch biopsies on it to pass to researcher, and (iii) the researcher will provide the saline tube (i.e. 15 mL DPBS aliquot) for them to place the lower segment biopsy into using forceps.
- Inform the attending midwife that the placenta with fetal membranes will need to be given to the researcher immediately after their routine clinical checks; they are to be left in the kidney dish wrapped with the inco pad at room temperature.

Acquisition of cervicovaginal fluid sample and transvaginal scanning prior to start of C-section.

- 1 ⊕ Record **contraction frequency** from CTG during preparation for C-section in the operating theatre.
- 2 ⊕ Undertake a speculum examination.
- 2.1 Record **fetal membrane rupture status** and **Bishop score** metrics.
- 2.2 Use the Dacron swab (and fetal fibronectin swab if available for analysis) to obtain a **cervicovaginal fluid sample**. Insert the swab(s) into their tubes and rest on the cooling gel packs.
- 3 ⊕ Undertake a TVUS and record 3x **cervical length measurements**.

Acquisition of upper and lower uterine segment biopsies at C-section.

- 4 ⊕ Record time of fetal and placental deliveries.
- 5 After delivery of the placenta, confirm the **uterine site of placenta implantation** and ability to proceed with excising upper and lower segment myometrial biopsies (after applying Green-Armitage forceps to the bleeding uterine edge) with the operating surgeon.
- 5.1 ⊕ Excise 4-6 x **upper segment basalis region** myometrial biopsies by applying the Tischler-Morgan forceps within a $\sim 4 \text{ cm}^2$ uterine area underlying the site of placenta implantation. Place them on the first piece of gauze.
- 5.2 ⊕ Excise 4-6 x **upper segment parietalis region** myometrial biopsies by applying the Tischler-Morgan forceps within a $\sim 4 \text{ cm}^2$ uterine area that is away from the site of placenta implantation and was instead in direct contact with the chorion. Place them on the second piece of gauze.
- 5.3 ⊕ Excise a $\sim 2 \times 1 \text{ cm}$ **lower segment** myometrial biopsy from the upper edge of the C-section incision using surgical scissors; avoid serosa and decidua where possible.

- 6  Use the Watchmaker forceps (wipe clean with a disinfectant (Clinell) wipe and dry with paper towels) to transfer the biopsies from the gauze into their pre-labelled 15 mL DPBS aliquots (one for each type of myometrial tissue). Secure the lids of each DPBS aliquot tube and return to the cooling gel packs. 
- 7 Transport the myometrial biopsies and cervicovaginal fluid samples in the 4 L plastic cooler bucket to the laboratory, along with the placenta with fetal membranes in a double-layered (clinical waste disposal) bag after confirming with the midwife that they have completed all their clinical routine checks.

Isolation of decidua, amnion, chorion and placenta tissue samples.

- 8  In the laboratory, transfer all the containers (DPBS aliquot tubes) of myometrial biopsies from the cooling gel packs to wet ice. Also chill the resealable specimen bags on wet ice (with all zip locks closed). 
- 8.1 Transfer the placenta with fetal membranes from the bag onto the absorbent side of the top 'inco' pad on the tissue dissection bench; basal plate (greyish top layer) of the placenta and chorion (textured decidua parietalis side) of the fetal membranes need to be facing up.
- 9 **Decidua basalis.**
- 9.1 Identify a lobe of placenta with basal plate visible at the site of cord insertion.
- 9.2 Use dissection scissors (wiped clean with 70% v/v ethanol and air dried) to cut a ~3×3 cm region of decidua basalis off the lobe of the placenta identified at the previous step. Keep attachment of trophoblast (villi) to the sample of decidua basalis tissue to a minimum.
- 9.3  Use a pair of blunt end (filter) forceps (wiped clean with 70% v/v ethanol and air dried) to transfer the sample of decidua basalis tissue into its pre-labelled and pre-chilled (on wet ice) 15 mL DPBS aliquot. Securely cap before inverting the aliquot tube to dislodge blood attached to the tissue before returning to chill on wet ice. 
- 9.4 Clean the dissection scissors and blunt end (filter) forceps with a disinfectant (Clinell) wipe and wipe dry with paper towels before using with the next tissue type.
- 10 **Placenta (trophoblast).**

- 10.1 Use the dissection scissors to sample a ~3×3 cm region of trophoblast tissue exposed at the site where the decidua basalis was sampled at step 9.2.
- 10.2 ⊕ Repeat steps 9.3 and 9.4 with the sample of placenta (trophoblast) tissue.
- 11 **Fetal membranes: decidua parietalis, amnion, and chorion.**
 - 11.1 Separate the **amnion** and **choriodecidua**, and use the dissection scissors to cut a ~8×8 cm piece of each to lie flat on the 'inco' pad with the side at which they were attached to each other facing up.
 - 11.2 Place an upturned Petri dish (lid) onto the 'inco' pad and transfer the choriodecidua (separated from the amnion at the previous step), with its decidua parietalis side still facing up, onto the upturned Petri dish (lid).
 - 11.3 Add ~20 mL fresh pre-chilled DPBS (from the 250 mL stock bottle on wet ice) to the decidua parietalis side of the choriodecidua on the Petri dish (lid) to keep it hydrated.
 - 11.4 ⊕ Repeat step 9.3 with the sample of amnion tissue.
 - 11.5 Use the cell scraper to detach ~1 g **decidua parietalis** (cream in colour; often loosely packed tissue) from the chorion without tearing the latter. The chorion will appear increasingly clear (resembling the amnion) as the decidua parietalis is scraped off it.
 - 11.6 ⊕ Repeat step 9.3 with the sample of decidua parietalis tissue.
 - 11.7 Use the dissection scissors to cut a ~5×5 cm piece of **chorion** cleared of decidua parietalis (at step 11.5).
 - 11.8 ⊕ Repeat step 9.3 with the sample of chorion tissue.
 - 11.9 Repeat step 9.4. Additionally, dispose the used Petri dish (lid) and cell scraper into a clinical waste bin, and transfer the placenta with fetal membranes remaining on the 'inco' pad back into the double layered (clinical waste disposal) bag to be disposed along with the 'inco' pad.
 - 11.10 Wipe down the cleared area of the tissue dissection bench with a disinfectant (Clinell) wipe, dry, and line with clean paper towels.

Dissection, RNAlater treatment, and snap freezing on dry ice of myometrial tissues.

- 12 Fill an upturned lid of the wet ice (~5 L) polystyrene box with wet ice itself to act as a cooling tray, which needs to be placed under the magnifier/dissection microscope.
- 13 Separate the lid and base of a new Petri dish. Chill both in the wet ice tray prepared at the previous step; put the Petri dish lid on wet ice while upside down and fill with ~10 mL fresh DPBS.
- 14 Prepare two sheets of filter paper (as a double layer) on the paper towel lining the tissue dissection bench for each type of tissue to be stored (i.e. 8 × 2 sheets in total).

15 **Upper segment basalis region.**

- 15.1 Transfer all of the upper segment basalis region biopsies with its 15 mL DPBS aliquot from their tube to the Petri dish base on wet ice (from step 13). Keep the empty tube for storing waste tissue.
- 15.2 Use a scalpel (and magnifier/dissection microscope) to dissect off any blood clots and traces of decidua at each biopsy in the ice-cold Petri dish base.
- 15.3 Use a pair of Watchmaker forceps (wiped clean with 70% v/v ethanol and air dried) to transfer half of the washed (and dissected) biopsies from its ice-cold DPBS in Petri dish base into the upturned Petri dish lid of ~10 mL fresh DPBS on wet ice (from step 13).
- 15.4 Use the Watchmaker forceps to transfer HALF the number of all biopsies in the ice-cold DPBS in upturned Petri dish lid onto one **double layer of filter paper** (from step 14) to briefly remove excess DPBS (without drying out the biopsies), then into their pre-labelled 1.5 mL **RNAlater** aliquot tube.

Note

Check manufacturer's protocol for the maximum amount of tissue to be treated with 1.5 mL RNAlater. One ~3 mm³ piece of tissue typically weighs ~30-50 mg; myometrial tissue is denser than fetal membranes and placenta.

- 15.5 **⊕** Securely cap the RNAlater aliquot tube and confirm the biopsies are fully submerged in the reagent; they will have a tendency to float just beneath the liquid surface until the RNAlater has had several hours to penetrate the tissue sufficiently.

- 15.6 Promptly use the Watchmaker forceps (wiped clean with 70% v/v ethanol to remove residual RNAlater) to transfer the biopsies still in the ice-cold DPBS in upturned Petri dish lid onto a dry area of the **double layer of filter paper** (used at step 15.4) to briefly remove excess DPBS (without drying out the biopsies).
- 15.7 Use the Watchmaker forceps to promptly transfer the biopsies from the filter paper into their pre-labelled and pre-chilled (on wet ice from step 8.0) resealable specimen bag. Position the biopsies so that they are well-separated from each other (to avoid forming clumps during snap freeze).

Note

Tissues snap freeze on dry ice in specimen bags faster than (thick walled) tubes.

- 15.8 ⊕ Close the zip lock and promptly bury in dry ice for ~3-5 minutes to snap freeze the biopsies. DO NOT ALLOW THE BIOPSIES TO THAW from here onwards.
- 15.9 Dispose the used DPBS (in the ice-cold Petri dish base and lid) into the beaker of Virkon (tablet) and dispose both parts of the used Petri dish into a clinical waste bin. Store any large amounts of tissue remains back into its DPBS aliquot tube (from step 15.1) and disinfect using Virkon solution. Wipe clean the forceps with 70% v/v ethanol and air dry.
- 15.10 Leave the biopsies in RNAlater and snap frozen specimen bags in wet ice and dry ice, respectively, and promptly proceed to the next type of tissue sample to be stored.

16 Upper segment parietalis region.

- 16.1 Repeat step 13.
- 16.2 Transfer all of the upper segment parietalis region biopsies with its 15 mL DPBS aliquot from their tube into the new Petri dish base on wet ice (from the previous step). Keep the empty tube for storing waste tissue.
- 16.3 Repeat steps 15.2 to 15.10 with the upper segment parietalis region biopsies.

17 Lower segment.

- 17.1 Repeat step 13. Additionally, fill the new ice-cold Petri dish base with ~20 mL fresh pre-chilled DPBS (from the (<)250 mL stock bottle on wet ice).
- 17.2 Use the Watchmaker forceps to transfer the lower segment biopsy from its 15 mL DPBS aliquot tube into the ~20 mL fresh DPBS in ice-cold Petri dish base (from the previous step). Gently agitate to wash as much blood off the biopsy as possible. Keep the DPBS aliquot tube on wet ice.
- 17.3 Use a new scalpel with the Watchmaker forceps to dissect five tissue strips (~12×3×3 mm) from the core of the biopsy, where there is maximal amount of smooth muscle and minimal amount of decidua and blood. Striated appearance of longitudinal muscle should be visible at each tissue strip.
- 17.4 Use the Watchmaker forceps to transfer the five tissue strips from its ice-cold DPBS in Petri dish base into the fresh ~10 mL DPBS in upturned Petri dish lid on wet ice (from step 17.1). Transfer the remains of the lower segment biopsy back into its ice-cold DPBS aliquot tube (from step 17.2).
- 17.5 Use the scalpel and Watchmaker forceps (from step 17.3) to dissect each tissue strip into three ~4×3×3 mm pieces.
- 18 Repeat steps 15.4 to 15.10 with the 15 x ~4×3×3 mm pieces of lower segment myometrial tissue.

Dissection, RNAlater treatment, and snap freezing on dry ice of decidual and placental tissues.

- 19 **Decidua parietalis.**
- 19.1 Repeat step 13.
- 19.2 Transfer all of the decidua parietalis tissue sample with its 15 mL DPBS aliquot (from step 11.6) into the Petri dish base on wet ice (from step 19.1). Keep the empty tube for storing waste tissue.
- 19.3 Use the dissection scissors (wiped clean with 70% v/v ethanol and air dried) with blunt end (filter) forceps to cut out 20 x ~3-5 mm² pieces and transfer them into the fresh ~10 mL DPBS in upturned ice-cold Petri dish lid (from step 19.1).
- 19.4 Repeat steps 15.4 to 15.10 with the 20 x ~3-5 mm² pieces of decidua parietalis tissue.

20 **Decidua basalis.**

- 20.1 Repeat step 13.
- 20.2 Transfer all of the decidua basalis tissue sample with its 15 mL DPBS aliquot (from step 9.3) into the Petri dish base on wet ice (from step 20.1). Keep the empty tube for storing waste tissue.
- 20.3 Use the dissection scissors (wiped clean with 70% v/v ethanol and air dried) with blunt end (filter) forceps to trim off any remaining trophoblast tissue (more red than grey in colour and villous texture) while this side of the sample faces up.
- 20.4 Use the blunt end (filter) forceps to transfer the dissected sample of decidua basalis tissue from its ice-cold DPBS in Petri dish base into the fresh ~10 mL DPBS in upturned Petri dish lid on wet ice (from step 20.1).
- 20.5 Use the dissection scissors with blunt end (filter) forceps to cut out 20 x ~3-5 mm² pieces.
- 20.6 Repeat steps 15.4 to 15.10 with the 20 x ~3-5 mm² pieces of decidua basalis tissue.

21 **Placenta (trophoblast).**

- 21.1 Repeat step 13. Additionally, fill the new ice-cold Petri dish base with ~20 mL fresh pre-chilled DPBS (from the (<)250 mL stock bottle on wet ice).
- 21.2 Swirl the capped DPBS aliquot with placenta tissue sample (from step 10.2) before uncapping to retrieve the tissue using the Watchmaker forceps and transferring it into the ice-cold fresh ~20 mL DPBS in Petri dish base (from step 21.1). Keep the DPBS aliquot tube on wet ice.
- 21.3 Use the dissection scissors with Watchmaker forceps to cut out 20 x ~3-5 mm² pieces.
- 21.4 Use the Watchmaker forceps to transfer the dissected sample of placenta (trophoblast) tissue from its ice-cold DPBS in Petri dish base into the fresh ~10 mL DPBS in upturned Petri dish lid on wet ice (from step 21.1).



- 21.5 Repeat steps 15.4 to 15.10 with the 20 x ~3-5 mm² pieces of placenta (trophoblast) tissue.

Dissection, RNAlater treatment, and snap freezing on dry ice of amnion and chorion tissues.

22 Chorion.

- 22.1 Repeat step 13. Additionally, fill the new ice-cold Petri dish base with ~20 mL fresh pre-chilled DPBS (from the (<)250 mL stock bottle on wet ice).
- 22.2 Swirl the capped DPBS aliquot with chorion tissue sample (from step 11.8) before uncapping to retrieve the tissue using the blunt end (filter) forceps to cut off 20 x ~3-5 mm² pieces and transferring them into the ice-cold ~20 mL fresh DPBS in Petri dish base (from step 22.1).
- 22.3 Repeat steps 15.4 to 15.10 with the 20 x ~3-5 mm² pieces of chorion tissue.

23 Amnion.

- 23.1 Repeat step 13. Additionally, fill the new ice-cold Petri dish base with ~20 mL fresh pre-chilled DPBS (from the (<)250 mL stock bottle on wet ice).
- 23.2 Swirl the capped DPBS aliquot with amnion tissue sample (from step 11.4) before uncapping to retrieve the tissue using the blunt end (filter) forceps to cut off 20 x ~3-5 mm² pieces and transferring them into the ice-cold ~20 mL fresh DPBS in Petri dish base (from step 23.1).
- 23.3 Repeat steps 15.4 to 15.10 with the 20 x ~3-5 mm² pieces of amnion tissue.

Processing of cervicovaginal fluid samples for -80°C storage.

- 24 Remove all of the used filter paper and paper towels that lined the bench for tissue dissection, and wipe down the surface with disinfectant (Distel).



- 25 Confirm that the 1 mL aliquot of DPBS supplemented with protease inhibitor cocktail has completely thawed on wet ice before uncapping its cryovial to insert the **Dacron swab** (from step 2.2); carefully snap the Dacron swab head off its stick handle and securely cap the cryovial.

Note

Confirm that the cryovial that stores the protease-supplemented DPBS will fit the microcentrifuge. If it does not, transfer the thawed protease-supplemented DPBS into a 1.5 mL microfuge tube (chilled on wet ice) using a 1 mL Pasteur pipette BEFORE inserting the swab into the DPBS.

- 25.1 Use a pair of Watchmaker forceps (wiped clean with 70% v/v ethanol and air dried) to transfer the swab from the protease-supplemented DPBS tube into a new empty tube of the same size (to allow it to balance the DPBS tube in the microcentrifuge) with the swab head pointing towards the lid of its new tube once capped.
- 26 Centrifuge both tubes (from the previous step) at **2500 g for 10 minutes at 4°C**. 
- 26.1 After centrifugation, use a 1 mL Pasteur pipette to transfer any fluid that has gathered in the swab-containing tube into its paired DPBS-containing tube (without disturbing any pellets formed at both tubes) to provide the final pool of cervicovaginal fluid supernatant.
- 27 Use a P200 micropipette (wiped clean with a disinfectant (Clinell) wipe) with sterile filter tips to aliquot ~110 µL of the cervicovaginal fluid supernatant into each of 5 x pre-labelled and pre-chilled (on wet ice) cryovials.
- 27.1 Transfer all 5 x ~110 µL cervicovaginal fluid supernatant aliquots in their cryovials onto dry ice to snap freeze and transport into their designated cryoboxes for -80°C storage. 

Short-term and long-term storage of all RNAlater-treated and snap frozen tissue samples.

- 28 **Short-term storage of all tissues.**
- 28.1  Confirm tissue pieces in **RNAlater** are fully immersed in the reagent and their labelled microfuge tubes are securely capped before storing them in a rack to refrigerate (for 12-72 hours; ideally 24 hours).  



28.2 Transport zip locked specimen bags of **snap frozen tissues** still in the dry ice polystyrene box (do not allow to thaw) to the freezer for -80°C storage (for 12–72 hours; ideally 24 hours).

29 **Transfer of RNAlater-treated tissues into cryovials for long-term -80°C storage.**

29.1 Make available fresh boxes of ~5 L wet ice and ~20 L dry ice on a bench wiped clean with disinfectant (e.g. Distel), along with 70% v/v ethanol (for cleaning), ~1 pack of filter paper, a pair of scissors, and a pair of Watchmaker forceps.

29.2 Line the bench (once dry after disinfecting) with clean paper towels. Arrange two sheets of filter paper (as a double layer) for each type of tissue to be stored (i.e. 8×2 sheets in total) on the paper towels.

29.3 Chill 8 x pre-labelled 1.8 mL cryovials (for RNAlater tissues) on wet ice.



29.4  Transfer the rack of RNAlater-treated tissues in microfuge tubes (from step 28.1) onto wet ice.

29.5 For the first tissue type to be stored (upper segment basalis region by following the order of RNAlater immersion), use a pair of Watchmaker forceps (wiped clean with 70% v/v ethanol and air dried) to transfer tissues from its RNAlater aliquot briefly onto a double layer of filter paper (prepared at step 29.2) to remove excess RNAlater.

29.6 Use the Watchmaker forceps to transfer the RNAlater-treated tissues from the filter paper into their pre-labelled and pre-chilled cryovial (from step 29.3). **IMPORTANT:** ensure that each piece of tissue sticks to the inner wall of the cryovial and they are well-separated from each other to avoid the formation of clumps.

29.7 Once the cryovial is filled with all pieces of RNAlater-treated tissues (for the first type of tissue to be stored), securely cap the cryovial and carefully lie it horizontal on dry ice to snap freeze the tissue pieces into position.



29.8 Record the number of tissue pieces present in the cryovial. Wipe clean the Watchmaker forceps with 70% v/v ethanol.

29.9 Repeat steps 29.5 to 29.8 for the next type of RNAlater-treated tissue to be stored.

29.10  Once all 8 cryovials have been filled with RNAlater-treated tissues, keep them on dry ice to transport to the freezer for -80°C storage in their designated cryoboxes.



30 **Transfer of snap frozen tissues into cryovials for long-term -80°C storage.**

30.1 Chill 8 x pre-labelled 1.8 mL cryovials (for snap frozen tissues) on dry ice until frozen. 

30.2  Transfer the zip locked specimen bags of snap frozen tissues (from step 28.2) onto the dry ice and transport to the bench. Flick each bag (whilst still in close proximity to dry ice - do not allow tissues to thaw) to detach its contents of tissue pieces from the inner walls of the bags. 

30.3 For the first tissue type to be stored (upper segment basalis region by following the order of snap freezing on dry ice), use the scissors (wiped clean with 70% v/v ethanol and air dried) to cut off the zip lock edge of the bag, uncap the frozen pre-labelled cryovial (from step 30.1), and carefully pour in its frozen tissue pieces.

Note

Alternative method of transferring snap frozen tissues from a specimen bag to cryovial:
[i] pre-chill a Petri dish (base or upturned lid), along with a pair of Watchmaker forceps (wiped clean with 70% v/v ethanol and air dried) inside a clean 50 mL centrifuge tube, on dry ice.

[ii] empty the frozen tissue pieces from its specimen bag into the Petri dish and use the pre-chilled Watchmaker forceps to promptly transfer them into their frozen pre-labelled cryovial.

[iii] Change to a new Petri dish (base or upturned lid) and wipe clean the Watchmaker forceps with 70% v/v ethanol before proceeding to the next snap frozen tissue type to be stored.

30.4 Securely cap the cryovial now filled with its snap frozen tissue pieces and keep on dry ice (do not allow tissue pieces to thaw).

30.5 Record the number of tissue pieces present in the cryovial. Wipe clean the Watchmaker forceps with 70% v/v ethanol and return to its 50 mL centrifuge tube to chill on dry ice.

30.6 Repeat steps 30.3 to 30.5 for the next type of snap frozen tissue to be stored.

30.7  Once all 8 cryovials have been filled with snap frozen tissues, keep them on dry ice to transport to the freezer for -80°C storage in their designated cryoboxes. 