Stillbirth
CENTRE OF RESEARCH EXCELLENCE

Protocol

Human placental tissue collection

This collection protocol is designed as a basic minimal guide to placental tissue collection for biobank storage that meets the agreed standards of the Stillbirth Centre for Research Excellence, Australia. For a comprehensive discussion of placental sample collection for research upon which the current protocol is based, the reader is directed to Burton *et al*. 2014 (*1*).

Box 1 | Ethical clearance

Appropriate ethical clearance from national and local bodies must have been granted for the collection and use of tissues. In addition, site assessments for collection from each locality must have been completed.

Box 2 | Health and safety

As with the collection of all biological samples, appropriate personal protective equipment must be worn/used during the processing of placental tissues. Human tissue should be processed within certified laboratory/clinical space following aseptic procedures. Unutilised tissue should be disposed of following local guidelines for clinical waste, or may be returned to the family or pathology services (see Box 3).

Box 3 | Return of tissues

If requested, tissue not utilised in research may be returned to the family or pathology services at initial processing stages. Approximately one or two teaspoons worth of tissue will be removed when a placenta is processed as described in this protocol, leaving the majority of tissue intact. Procedures for the return of tissue should be optimised at each location through liaising with appropriate stakeholders.

Note, it is not recommended that tissue be processed for research if the family intends encapsulation or other preparation leading to consumption of the placenta. Intended consumption of placental tissue should be considered as exclusion criteria for inclusion in research, as appropriate food-grade processing by researchers cannot be guaranteed.

Box 4 | Recording of sample and patient details

For each sample collected, a de-identified sample ID number should be assigned. We suggest the following scheme: *year date, two letter location ID, numeric assignment*; e.g. *18GC0001*. Samples from multiple (non-singleton) pregnancies should be denoted with a decimal place for each placenta; e.g. *18GC0001.1* and *18GC0001.2*. This does not preclude researchers assigning local sample identifiers, but all sample identifiers should be linked to the standard coding detailed above.

All relevant clinical information should be recoded associated with the sample ID number. A template for the recording of clinical information can be downloaded or see Appendix A. The suggested minimal list of details to be recoded is: *pathology (if any), date collected, time of birth, time of collection, time of processing, mode of birth (vaginal, CS, emergency CS), gestation, parity, maternal age, maternal body mass index (at booking), height (at booking), weight (at booking), mother's ethnicity (self-reported), baby weight (g), baby sex, placental weight (g; note if trimmed), induction method (if any), analgesia (if any), other medication (if any)*.

Materials

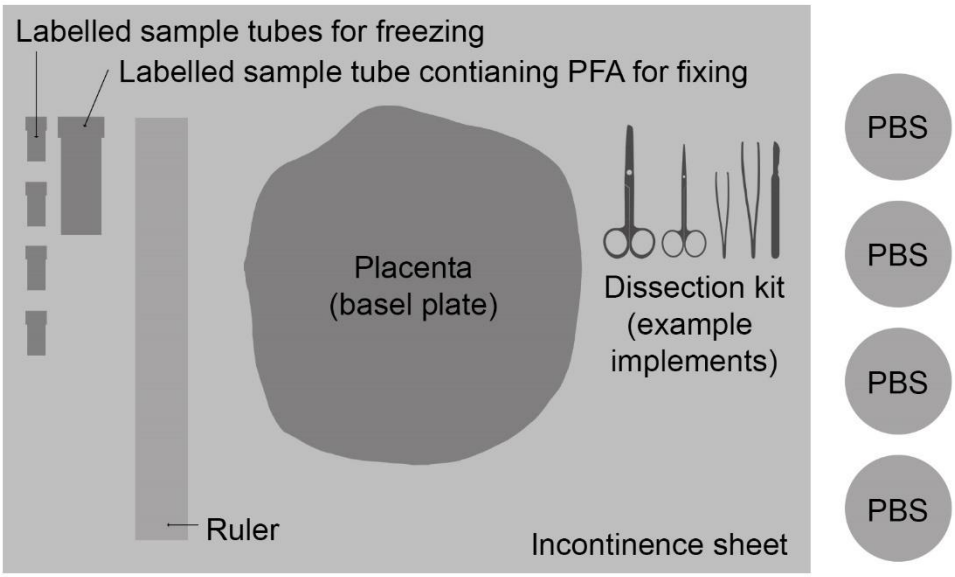
Reagents

- Phosphate buffered saline (PBS) or similar physiological buffer
- Liquid nitrogen
- Isopentane and dry ice; optional
- Absolute methanol, chloroform, glacial acetic acid; optional
- 4% paraformaldehyde (PFA) or similar fixative
- 70% ethanol

Equipment

- Biosafety cabinet
- Personal protective equipment
- Balance/scale capable of measuring between 100 g to 1 kg to one decimal place
- Incontinence sheets or similar
- Gauze swabs; optional
- Hair clip; optional (for PFA fixed samples of membrane)
- Ruler or similar equipment to use as scale bar, camera
- Dishes such as deep petri dish for the rinsing of placental samples
- Dissection equipment, e.g. scissors, forceps, biopsy punch, scalps
- Tubes and sample pots, and labels for tubes or pen to write labels

Box 4 | Processing station set up



Procedure

This procedure describes the collection of snap frozen (for RNA [Note 1], protein, or DNA isolation) and PFA fixed (for histology or immunohistochemistry) human villous placental tissue, and the additional collection of umbilical cord and membranes. The video version of this protocol can be found online via the Mothers and Babies Research Centre.

All procedures should be carried out as quickly as possible to minimise degradation (*1*). Typically, sample processing should be completed within 15 minutes of birth. If there is a delay in processing the placenta, this should be noted against the clinical information recorded.

1. Prior to sampling, set up Health and Safety, Ethical Clearance, and the Recording of Sample and Patient Details as described in **Boxes 1–3**.
2. Set up a sample processing station in the biosafety cabinet as described in **Box 4**.
3. Photograph the placenta from the chorionic (fetal) and basal (maternal) aspects against a scale bar (Figure 1).
4. Weigh the placenta to the nearest decimal place. If trimming [Note 2] is carried out, this should be noted with the clinical information recorded.
5. Place the placenta in the processing station with the basal plate uppermost (Figure 1B).
6. For frozen samples of villous tissue, identify a random sampling site [Note 3] with no frank pathology then dissect a ~1–5 mm^3 sample of villous tissue, avoiding the basal plate. Villous tissue can be accessed by (a) trimming away the basal plate with a pair of scissors or a scalpel then cutting out a piece of the exposed villous tissue, or (b) cutting or punching out a ~1 cm deep section of tissue, moving this away from the main placenta, and then cutting away ~2 mm from the basal aspect. Discard the basal plate tissue.
7. Move the dissected villous tissue to a dish containing PBS.
8. Repeat the dissection (Step 6) of another three to seven samples taken from additional random sampling sites. A total of at least four separate sites should be sampled. Move the dissected villous tissue to a dish containing PBS (Step 7).
9. Wash the villous tissue samples thoroughly but gently to minimise maternal blood carry over. Several (3–5) dishes may be required to adequately remove maternal blood. Keep tissue immersed in PBS as much as feasible.
10. For each villous tissue sample, section the main sample into subsamples (~5–10). Combine the subsamples into a single sample pool.
11. Snap freeze washed tissue in at least three separate identically labelled replicate tubes (sample aliquots). Tissue may be frozen by (a) placing randomly selected subsamples of tissue into a 1–2 mL tube suitable for snap freezing, and immediately immersing in liquid nitrogen, or (b) dabbing off excess liquid on a gauze swab, dropping randomly selected subsamples of tissue into isopentane then transferring to a 0.5–2 mL tube and placing on dry ice. [Note 4] The tissue is now stably temporarily stored, and samples for histology can be processed.

12. For PFA fixed samples of villous tissue, [Note 5] identify a random sampling site with no frank pathology then dissect a ~1–2 cm full thickness sample of villous tissue, including the chorionic and basal aspects. Alternatively, villous tissue can be dissected as described in Step 6.
13. Move the dissected villous tissue to a dish containing PBS.
14. Wash the full thickness or villous tissue sample thoroughly but gently to minimise maternal blood carry over.
15. Transfer washed tissue to a labelled 10–30 mL tube containing PFA (approximately four times the volume of tissue to be fixed). The tissue is now stably temporarily stored, and further processing will be required after 12–24 hours. [Note 6]
16. Optional: For PFA fixed samples of membrane, cut a strip of the membranes (~4 cm wide) and roll this around a hair clip. [Note 7] Use only the middle section of the clip and do not roll the membrane too tightly, it needs to easily slide off the clip once fixed. Put the clip in a labelled tube of PFA. The tissue is now stably temporarily stored, and further processing will be required after 12–24 hours. Note, the membrane should be removed from the clip after PFA fixation; pinch the clip and gently slide the membrane down the clip with forceps (the membrane should remain rolled up).
17. Dispose of unutilised tissue and disposable equipment following local guidelines for clinical waste. Clean work area and all non-disposable equipment following local guidelines.
18. For storage, transfer tubes containing snap frozen tissue to a -80^{o}C freezer. The sample for fixation should be left in PFA for 12–24 hours then transferred to 70% ethanol and processed for histology via standard methods. [Note 6]
19. Complete the placenta biobank with relevant clinical information, including maternal and fetal information.

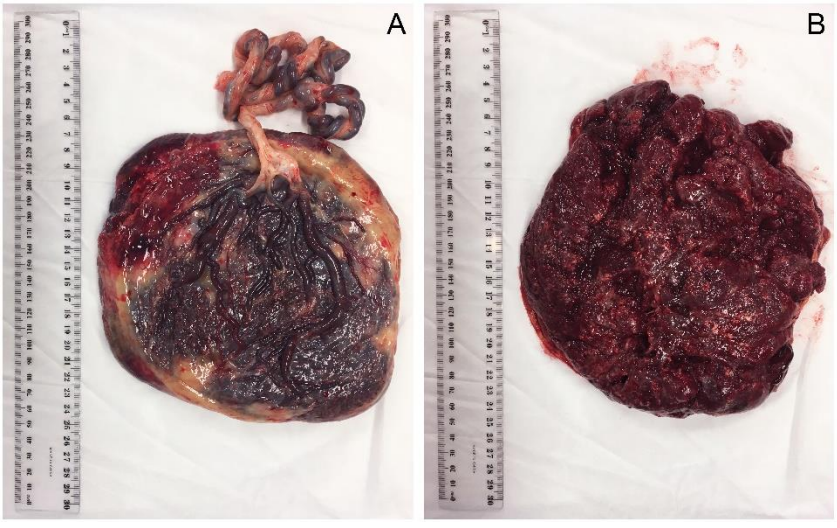


Figure 1. Photographs of a placenta from the (A) chorionic or fetal aspect and (B) basal or maternal aspect. A ruler is included for scale.

Note 1: **Processing placental villous tissue for later RNA extraction**: RNA can be isolated from snap frozen tissues; however, the quality of this RNA is typically low. It is recommended that if RNA extraction is likely to be performed, additional samples of washed tissue should be preserved in RNAlater or similar preservative following the manufacturer's protocol.

Alternatively, methacarn fixation can be used for analysis of gene expressions in paraffin-embedded tissue specimens using the following protocol or refer to published methodology (*2, 3*).

Methacarn fixative (prepare in fume hood):

- 60% absolute methanol (1800 mL)
- 30% chloroform (900 mL)
- 10% glacial acetic acid (300 mL)

Store fixative away from light and use within four weeks.

Protocol:

1. Excise and wash placental villous tissue as described above (Steps 6–10).
2. Immerse tissue in 2 mL of methacarn fixative at room temperature. Tissue can be fixed in methacarn in as little as four hours. Specimens can be stored in methacarn fixative for several weeks.
3. For further processing, remove the fixed tissue and rinse with 70% ethanol then proceed with standard paraffin embedding.

Note 2: **Placental trimming**: Placental trimming involves the removal of membranes and the umbilical cord, leaving primarily villous tissue (*1*).

Note 3: **Identifying random sampling sites**: Sampling sites can be selected from four placental 'quadrants' arranged in a north-south-east-west pattern. Alternatively, use the cord insertion as a guide: Mid-way between where the cord was inserted and the edge of the placenta take samples from each of the four quadrants of the placenta.

Note 4: **Additional methodology for preparation of frozen sample aliquots**: After snap freezing, sample aliquots may be made following this methodology: Transfer snap frozen samples to a ceramic pestle and mortar, and crush the tissue to a powder (adding liquid nitrogen as needed) before redistributing the powdered sample to new tubes.

Note 5: **Alternative protocol for fixation of villous tissue**: Take a sample of villous tissue, ensuring it has the fetal and maternal sides, and wash (Step 9). Place the washed tissue into a labelled pot containing ~30 mL of 10% buffered formalin. Put this tube into wet ice prior to storage. After 24 hours, replace the buffered formalin with fresh buffered formalin. Process the samples to histology for paraffin wax embedding.

Note 6: **Fixed sample processing timing**: The time tissues are stored in fixative (e.g. PFA) and ethanol before being processed into paraffin blocks should be recorded.

Note 7: **For PFA fixed samples of membrane**: Cut a strip of the membranes (~4 cm wide) and roll this around a hair clip (Figure 2).

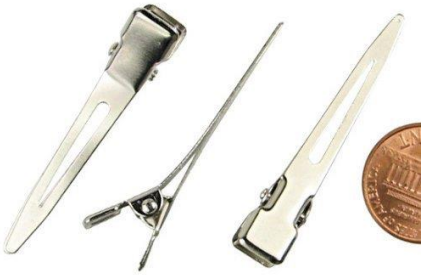


Figure 2. Example of hair clip for use in preparation of fixed placental membranes.

References

1. G. J. Burton, N. J. Sebire, L. Myatt, D. Tannetta, Y. L. Wang, Y. Sadovsky, A. C. Staff, C. W. Redman, Optimising sample collection for placental research. *Placenta* **35**, 9-22 (2014).
2. M. Shibutani, C. Uneyama, K. Miyazaki, K. Toyoda, M. Hirose, Methacarn fixation: a novel tool for analysis of gene expressions in paraffin-embedded tissue specimens. *Laboratory investigation; a journal of technical methods and pathology* **80**, 199-208 (2000).
3. H. Akane, F. Saito, H. Yamanaka, A. Shiraki, N. Imatanaka, Y. Akahori, R. Morita, K. Mitsumori, M. Shibutani, Methacarn as a whole brain fixative for gene and protein expression analyses of specific brain regions in rats. *The Journal of toxicological sciences* **38**, 431-443 (2013).

Appendix A: Template for the recording of clinical information

Note: number of rows can be expanded and table displayed over three pages.

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			Time of			Maternal (weight at booking/first trimester)					Baby		Placenta	
CoRE ID#	Pathology (if any)	Date	Birth	Collection	Processing	Age	Height (cm)	Weight (kg)	BMI	Ethnicity	Weight (g)	Sex	Weight (g)	Trimmed

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	Birth (mode=vaginal, CS, emergency CS)						
CoRE ID#	Mode	Induction	Analgesia (if any)	Other medication (if any)	Gestation (wks.days)	Parity	Notes

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	Placenta (additional information)				Cord			Samples (give numbers)		
CoRE ID#	Length (cm)	Width (cm)	Depth (cm; four locations)	Abnormalities (if any)	Position	Coils in 5 cm	Photo (y/n)	Frozen (-80°C)	Fixed (PFA)	Other