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ECHO Placenta Collection, Processing and Storage Protocol

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A. Overview (all delivery specimens: maternal blood, cord blood, placenta)

- Fresh cord blood and placental tissue will be collected for the ECHO-wide Cohort Data Collection Protocol (EWCP) and processed (aliquoted) at the site prior to shipping to the biorepository for storage.
- Cohorts are expected to fulfill the placental and cord blood collection requirements for cohort-specific aims and contribute remaining specimens to the EWCP.
- All sites are expected to follow their local or institutional policies when handling placenta and cord blood specimens.
- All or selected collection and processing procedures in this document should be followed based on the cohort’s expertise and availability of resources. The following tables provides an overview of the delivery collections in relation to time of delivery:

<table>
<thead>
<tr>
<th></th>
<th>*Maternal Blood Perinatal: Recommended</th>
<th>**Cord Blood Recommended</th>
<th>Placenta Recommended</th>
</tr>
</thead>
<tbody>
<tr>
<td>Admission to Labor and Delivery</td>
<td>Collect specimen upon admission to Labor and Delivery</td>
<td>Option 1 - Collect cord blood directly from the cord: Study/clinical staff collects 15 mL cord blood in tubes directly from the cord and reclamps cord. Prepare blood spot card.</td>
<td></td>
</tr>
<tr>
<td>Delivery</td>
<td></td>
<td>Option 2 - Collect Cord blood from placental vessels: If 15 mL cord blood was not already collected, study/clinical staff collects blood from placental vessels using syringe within 30 minutes of delivery (preferably after taking the first set of fetal photographs if collecting placenta). Prepare blood spot card, if not already done above.</td>
<td>Photograph (pre-biopsies) Clean away blood clots and blood. Photograph fetal and maternal surfaces of the placenta. Biopsies: • If collected within 3 hours, store tissue for epigenetics in RNAlater and for environmental analysis. • If collected within 12 hours, store tissue for environmental analysis only.</td>
</tr>
<tr>
<td>Post-Delivery</td>
<td>Collect if specimen has not been collected at admission to Labor and Delivery. Attempt to collect within 8 hours of delivery. If not collected within recommended time, note deviation on Specimen Tracking Form (STF).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Re-photograph (post-biopsies) fetal and maternal surfaces after removing blood clots, blood and cord.

Whole or remaining formalin-fixed placenta: Used for morphology/pathology if collected within 48 hours.

| Collection Kit | Blood collection kit | Cord blood collection Kit | Placenta biopsy kit | Placenta RNAlater bulk kit | Placenta formalin kit |

* Follow procedures in the Blood Collection, Processing, and Storage Protocol to collect maternal blood.

** Follow procedures in the Cord Blood Collection, Processing, and Storage Protocol to collect cord blood.

**B. Placenta Collection Overview**

The placenta procedures are divided into 3 tiers based on the time when the placenta is available for processing. Cohorts are encouraged to perform all procedures under each tier.

**Tier 1: Placenta collected within 3 hours of delivery:**

a. Photograph the fetal surface pre-biopsies.
   
   Note: Attempt to collect cord blood, depending upon time from delivery.

b. Photograph the maternal surface pre-biopsies.

c. Biopsies: Collect 100 mg of tissue from the maternal surface; aliquot each 50 mg in 2x 2 mL RNAlater tubes.

d. Biopsies: Collect 5 gm of tissue from the maternal side; aliquot each 1 gm of tissue in 5x 2 mL cryovials.

e. Re-photograph the maternal surface post-biopsies.

f. Re-photograph the fetal surface post-biopsies.

g. Take placental and cord measurements.

h. Place the remaining placenta in formalin for fixation.

**Kits:** Placenta RNAlater Bulk Kit, Placenta Biopsy Kit, and Placenta Formalin Kit

**Tier 2: Placenta collected within 12 hours of delivery:**

a. Photograph the fetal surface pre-biopsies.

b. Photograph the maternal surface pre-biopsies.
c. Biopsies: Collect 5 gm of tissue from the maternal side; aliquot each 1 gm of tissue in 5x 2 mL cryovials.
d. Re-photograph the maternal surface post-biopsies.
e. Re-photograph the fetal surface post-biopsies.
f. Take placental and cord measurements.
g. Place the remaining placenta in formalin for fixation.
   **Kits:** Placenta Biopsy Kit and Placenta Formalin Kit

**Tier 3: Placenta collected within 48 hours of delivery:**

a. Place the whole placenta in formalin for fixation
   **Kit:** Placenta Formalin Kit

**C. Placenta Collection Guidelines**

- Sites that are unable to store formalin-fixed placentas for up to 3 months due to storage limitations are encouraged to ship more frequently to the biorepository. The ECHO Laboratory Manual includes information on shipping and how to order return shipping kits.
- The processes in this document are for singleton deliveries only. If a cohort is expecting multiples, contact the ECHO Coordinating Center (CC) to coordinate consultation with the placenta CPS team.
- If birth occurs at or before 30 weeks gestational age, sites are expected to provide a pathology report only upon request. After 30 weeks, sites should attempt to perform all procedures described in this protocol and a pathology report may be requested if available. Note: Cohorts are responsible for storing pathology reports at the local site until requested by ECHO. Use local standard operating procedures for de-identification of participant information on the pathology report.
- Cohorts are encouraged to photograph fetal and maternal surfaces at the following time points:
  - Fetal surface (pre-cord blood collection and biopsies; cord placement in 2 areas to ensure capture of full fetal surface); 4 pictures
  - Maternal surface pre-biopsies; 2 pictures
  - Maternal surface post-biopsies; 2 pictures
  - Fetal surface post-biopsies; 2 pictures
- While sites are expected to take up to 10 pictures of the placenta, additional pictures can be taken if deemed important, but a description of the pictures must be added on the STF. Note: All pictures should have the same specimen ID label, and the matching label is placed on the STF.
• A smart phone camera, iPad camera, or regular camera with excellent pixilation is recommended. Do not use flash. Follow institutional policy regarding use of personal phones.

D. Placenta Collection Kit and Supplies

1. Placenta RNA-later Bulk Kit (Fisher BioServices)*
   • 2x 2.0mL RNA-later tubes, pre-filled (1.5mL RNA-later volume)
   • 2 twin specimen ID labels
   • 2 sterile 90 mL containers for washing

*NOTE: The RNA-later tubes are used in Tier 1 only hence ordered separately from the Fisher order website. There are 10 tubes with 10 twin-specimen ID labels and 10 wash containers for washing tissue with phosphate buffered saline (PB) in each kit. Cohorts are expected to order according to their enrollment projections as well as the number of anticipated deliveries under Tier 1. Each bulk kit holds supplies for 5 placentas.
2. Placenta Biopsy Kit (Fisher BioServices)
   - 3x 2.0 mL cryovials
   - 2x 2.0 mL FluidX metals-free cryovials
   - *6 twin specimen ID labels
   - * 5 twin specimen ID labels for cryovials and 1 for photographs

3. Placenta Formalin Kit (Fisher BioServices)
   - 3 10% buffered formalin-filled containers; *240 mL each
   - 1 biohazard bag 12 x 15 in.
   - 1 95kpa bag 13 x 18 in. with absorbent sheets
   - **2 twin specimen ID labels

* The formalin container is labeled 480 mL but volume inside is 240 mL.

** 1 twin specimen ID label is for the biohazard bag that holds the placenta, and 1 twin specimen ID label is for the photographs. NOTE: Placenta Biopsy Kit also includes a twin specimen ID label for photographs. If Tier 1 or 2 procedures are performed, then the twin specimen ID label from the Placenta Biopsy Kit can be used and the extra label in the Placenta Formalin kit can be discarded. Only 1 twin-specimen ID label should be used for all pictures.
4. Supplies (Site)

Tier 1, 2, and 3:
- Powder-free gloves, eyewear, and laboratory coat
- Biohazard container
- Work surface or biosafety cabinet
- Fume hood (optional – formalin-fixation step)
- Placenta – Specimen Tracking Form (STF)

Tier 1 and 2:
- 2 pennies
- 10 gauze pads
- 4 blue absorbent underpads (chux); use more as needed
- Dissecting mat or board
- Clean sterile scalpel
- Clean sterile scissors
- Clean sterile dissecting forceps
- Clean sterile hemostat
- 1 disposable ruler (cm or inches)
- 1 smart phone (laptop or iPad is acceptable)
- Tube rack
- Weighing scale (min 3-kg capacity, scaled to nearest 1-g)
- Water ice
- Biocision 1-L mini ice pan
- 2-8°C refrigerated storage
- −80°C freezer
- Cryobox 2.0 mL cryovials (5 x 5 x 2 in.; 81 cells; 9 x 9 grid)

Tier 1 only:
  Note: Need 20 mL/placenta; 10 mL/washing container (2 containers are used per placenta; provided in the RNALater bulk kit)
E. Collection

1. Pre-collection instructions for study staff
   - Print the placenta STF. Fill out the headers and record Biopsy and Formalin Kit Stock numbers.
   - Wear gloves, eyewear, and a laboratory coat.
   - Upon collection, the placenta should be placed at refrigerated temperature until processing, e.g., during transport or awaiting arrival of the collection team.
   - Complete STEP 0, 1, and 2 on the STF.
   - NOTE: Place the placenta on a new chux at any time during the procedure if the chux in use is covered in a pool of blood.
   - If biopsies are performed, prepare an ice bath by placing water ice into a Biocision 1-L mini ice pan. Partially submerge the tube rack into the ice bed. Alternately, tubes can be placed directly on ice in a pan.

2. Photograph fetal surface pre-biopsies
   i. Refer to Exhibit 1 below for an example.
   ii. Lay 2 chux pads (blue side down) inside the biosafety cabinet or on the work surface.
   iii. Gently place the placenta onto the chux pads (fetal side up).
   iv. Clean the fetal surface with gauze pads to eliminate bloods and clots. The cord is still attached at this point.
   v. Place 1 twin specimen ID label within an inch of the placenta so it is visible in each picture taken. Do not have the specimen ID label touch the placenta. Place the matching label in STEP 3 on the STF.
   vi. Place the penny within an inch of the placenta (at approximately 7 o’clock as shown in the Exhibit 1) so it is visible in each picture taken. Do not have the penny touch the placenta.
   vii. Place a ruler next to the placenta as shown in Exhibit 1. Do not have the ruler touch the placenta.
   viii. Remove gloves and take 2 pictures with a smart phone, iPad, or camera (no flash).
ix. Check photos be sure the photos are in focus and contain all information.

x. Put on a second pair of gloves.

xi. Move the cord to new position to obtain two complete pictures of the placenta and check photos. Cord is moved to capture the vascular pattern on the placental fetal surface. **Do not** detach the cord.

xii. Remove gloves and take the photos without flash.

xiii. Record date and time when the first picture was taken in **STEP 3** on the STF and also select the fetal surface pictures taken.

### Exhibit 1: Fetal Surface (Pre-cord Blood Collection and Biopsies)

Note penny, specimen ID label, and ruler placement

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**NOTE:** If collecting cord blood, follow the cord blood CPS protocol for procedural details. Cord blood should be collected after taking *fetal surface pre-biopsy* pictures and before maternal surface *pre-biopsy* pictures.

3. Photograph maternal surface pre-biopsies
   
i. Refer to Exhibit 2 below for an example.

   ii. Wear new gloves.
iii. Flip the placenta around the vertical axis created by the penny (7 o’clock should remain 7 o’clock). Orient the placenta so the maternal side is facing up.

iv. Locate the hole in the membrane and pull it back towards the edges of the placenta.

v. Remove all loosely adherent blood clots. Blot away blood with gauze pads.

vi. Ensure that the specimen ID label and penny are visible in the picture. Be sure the penny matches the location of placement (7 o’clock) where the fetal surface was photographed.

vii. Place the ruler next to the placenta with cm side toward the placenta.

xiv. Remove gloves and take 2 pictures with a smart phone, iPad, or camera (no flash).

xv. Check the photos to be sure the photos are in focus and contain all information.

xvi. Select the maternal surface pictures taken in STEP 3 on the STF.

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Exhibit 2: Maternal Surface (Pre-biopsies)
Note penny, specimen ID label, and ruler placement

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4. Collect each 50 mg tissue in 2x 2.0 mL RNAlater tubes from maternal surface side
   i. See Exhibits 3, 4, & 5 for removal of the maternal decidua, providing access to the placental villous tissue used for RNA/DNA collection in RNAlater.
ii. There are twin-specimen ID labels for each RNAlater tube. Apply the first label in **STEP 4** on the STF and the second label on the RNAlater tube.
   
   a. Repeat the labeling step for the second RNAlater tube.

iii. Open the wash containers and set next to the gauze pads. Fill each wash container with 10 mL of the PBS solution.

iv. Remove the basal plate from the surface of a lobule associated with normal appearance.

v. Using sterile forceps and scissors/scalpel, remove a 100 mg of villous tissue from the underlying placental tissue (amount is roughly the size of Lincoln’s head or about a ½-cm cube) See Exhibit 3 below:

Exhibit 3: Removal of tissue

vi. Using sterile scissors or scalpel, cut a 100 mg sample into 2x 50 mg pieces.

vii. Blot the tissue gently on a gauze pad. See Exhibit 4 below:

Exhibit 4: Tissue blotted on a gauze pad

viii. Dip each piece of tissue in the first wash container and swish around to remove excess blood.

ix. Blot the tissue gently on a gauze pad again.

x. Dip the tissue in the second wash container and swish around.

xi. Blot the tissue gently on a gauze pad for the last time.

xii. Place each 50 mg of tissue in a RNAlater tube x 2 (see exhibit 5 below), and place on a tube rack.
xiii. When finished, recap the wash containers and discard in a biohazard container.

xiv. Place all used supplies in a biohazard container.

xv. RNAlater tubes containing placental tissue **should NOT be frozen at −80°C temperature immediately.** Place them on an ice bath following collection and then at refrigerated temperature (4°C) for 24 hours before placing in a labeled cryobox to store in a −80°C freezer.

   a. If ice or refrigerator is not immediately available, RNAlater tubes can be kept at room temperature (25°C) for up to a week prior to placing in a −80°C freezer.

xvi. Record date and time when tubes were stored at refrigerated or room temperature in **STEP 4** on the STF. Record the date and time tubes were removed from the selected temperature.

**5. Collect 5 gms of villous tissue divided as 1gm tissue in each of 5x 2.0 mL cryovials**

   i. Place 1 gm of tissue or 1 cc (1 cm x 1 cm x 1 cm) in each of 5 cryovials as follows:

      a. 1 gm of tissue in each of 2x 2.0 mL metals-free cryovials.
      
      b. 1 gm of tissue in each of 3x 2.0 mL cryovials.

   ii. **Metals-free cryovials:** There are twin-specimen ID labels for both cryovials. Apply the first label in **STEP 4A** on the STF and the second label to the cryovial.

      a. Repeat the labeling step for the second metals-free cryovial.

   iii. **Cryovials:** There are twin-specimen ID labels for three cryovials. Apply the first label in **STEP 4B** on the STF and the second label to the cryovial.

      a. Repeat the labeling step for the remaining two cryovials.

   iv. Using sterile forceps and scalpel/scissors, cut the tissue in 1 gm pieces from the least damaged placental lobules. The specimen should be blotted, but no need to be washed.

      a. Use volume markings on the vial representing weight, but the volume does not have to be exact.
b. Place approximately 1 gm tissue in a cryovial so it reaches the 1 mL mark, recap, and place the cryovial on a tube rack.

v. Place the cryovials on an ice bath following collection.

6. Re-photograph maternal surfaces post-biopsies: villous sampling pictures
   i. Orient the placenta so the maternal side is facing up. The position should be consistent with that outlined in Exhibit 1.
   ii. Place all 1 gm tissue cryovials on the placenta from where the tissue was removed, as shown in Exhibit 6. Note: Exhibit 6 shows only 4 cryovials instead of 5 since the 100 mg of tissue for the metals-free cryovials was removed from 1 area and cut into 2 halves to place in each of the 2 metals-free cryovials.
   iii. Ensure that the specimen ID label and penny are visible in the picture.
   iv. Place the ruler next to the placenta with cm side toward the placenta.
   v. Remove gloves and take 2 pictures of the maternal side with cryovials of the placenta (as seen in Exhibit 6 below) with a smart phone, iPad, or camera (no flash), and check photos for focus and clarity. Ensure that the specimen ID labels are visible in the pictures.
   vi. Remove the cryovials from the placenta for storage.
   vii. Select the maternal surface pictures taken in STEP 3 on the STF.

Exhibit 6: Pictures of Maternal Surface Post-biopsies. Note the ruler, penny, and specimen ID
7. Placental and cord measurements; re-photograph fetal surface post-biopsies

i. Flip the placenta 1 more time so the fetal side is visible.

ii. Ensure that the cord is still clamped.

iii. Remove the membranes and detach the cord from the fetal/chorionic surface of the placenta. Blot with gauze.

iv. Record the following information in STEP 5 on the STF:
   a. Cord length (cm): Lay the cord flat, in a straight line. Do not stretch. Measure the cord length with a disposable ruler. If another detached cord piece is available, measure that as well and record the sum of both lengths on the STF. See Exhibit 7 below.
   b. Coil count: Start at the cord end that was attached to the placenta, and count the number of coils in the first 10 cm.
   c. Number of vessels: There are usually 3.

viii. Remove gloves and take 2 pictures with a smart phone, iPad, or camera (no flash). Ensure that the specimen ID label, penny, placenta cord, and ruler are visible in the picture. Select the fetal surface pictures taken in STEP 3 on the STF.

v. Record the following information in STEP 5 on the STF:
   a. Weigh (in grams) of the trimmed and blotted placenta with the basal plate
   b. Date and time measurements completed.

Exhibit 7: Pictures of Fetal Surface Post-biopsies with Cord Removed
8. **Formalin-fixation**
   i. The twin specimen ID labels should be used in this step. Place 1 label on the biohazard bag and the matching label in **STEP 6** on the STF.
   ii. Preferably under a chemical fume hood, place the placenta with the membrane and cord **flat** in a biohazard bag.
   iii. Pour 3 bottles of 10% buffered formalin (total 720 mL) in a biohazard bag.
   iv. Lightly squeeze the bag to release excess air prior to sealing.
   v. Change gloves.
   vi. Place the labeled biohazard bag (with placenta) into a 95kpa bag that already includes absorbent sheets.
      a. The kpa bag is considered a storage container, hence should be labeled using the triplet storage container ID labels. As such, it should be labeled on the inside of the bag with 1 label and on the outside of the bag using the remaining 2 labels from the triple set.
   vii. Lightly squeeze the kpa bag to release excess air prior to sealing.
   viii. Store the kpa bag with the placenta for a minimum of 72 hours at room temperature or in a refrigerator. Longer fixation is fine. The placenta should lie flat, with the fetal side up.
   ix. Record date and time placenta is placed in formalin in **STEP 6** on the STF.

9. **Post-Collection Instructions**
   - Instructions of uploading pictures in Bio-Track are found in the STF Completion Instructions.

F. **Storage**
   - **RNAlater** tubes should be stored in a labeled cryobox (5 x 5 x 2 in.; 81 cells; 9 x 9 grid) at −80°C after 24 hours, but no more than a month, at refrigerated temperature (4°C). Alternatively, storage at room temperature (25°C) is acceptable for up to a week prior to placing in −80°C.
      o Record date and time when RNAlater tubes were stored at −80°C in **STEP 4** on the STF.
   - 2 mL cryovials should be stored in a labeled cryobox (5 x 5 x 2 in.; 81 cells; 9 x 9 grid) at −80°C immediately.
      o Record data and time when cryovials were stored at −80°C in **STEP 4A and 4B** on the STF.
   - After 72 hours of formalin-fixation, place the doubled-bagged placenta on a shelf or stack in a return shipper provided by Fisher for storage until ready to ship.
   - Enter data from the STF into Bio-Track immediately upon storing the specimen or no later than 48 hours.
G. Shipping to Biorepository

- Formalin-fixed placentas can be shipped to the biorepository as often as needed if there are storage limitations. All other cryovials are shipped every quarter with the remaining specimens.
- Information on how to prepare shipments and order shipping kits is available in the Laboratory Manual.

H. Supporting Documents

1. Placenta – Specimen Tracking Form (STF)
2. Placenta STF Completion Instructions