SECTION 4.0 – SPECIMEN COLLECTION, PROCESSING, ONSITE STORAGE & SHIPPING

4.0 SPECIMEN COLLECTION, PROCESSING, ONSITE STORAGE & SHIPPING

4.1. SWAN CORE SPECIMENS (Annual Visits)

A common specimen collection protocol was developed in SWAN to ensure quality and consistency among the blood and urine samples collected at all seven clinic sites, beginning at the baseline visit. The detailed protocol outlines steps involved in the blood draw and urine collection, specimen processing and aliquotting, temporary storage at site clinics, and shipping to the Repository. Standardized collection kits, developed at the CLASS lab, contained the appropriate number and type of Vacutainers®, cryovials, and a sterile urine collection cup along with preprinted barcode labels and Specimen Collection Record (SCR) paperwork for all Core SWAN participants. Project Directors (PDs) and other key clinic personnel at all sites participate in hands-on training and certification on an annual basis.

4.1.1. Annual Visit Serum & Plasma Collection, Processing, and Onsite Storage

Overview

Repository blood samples were obtained at each follow up visit, at all seven clinic sites, between days two and five of the participant's menstrual cycle for regularly cycling women who are not using birth control pills or HRT. For women who were no longer cycling regularly or post-menopausal, blood was drawn at their clinic visit, scheduled to be on the anniversary of their previous visit. Samples were collected from patients who had been fasting with nothing but water as intake for previous 12 hours, and between 8:00-10:00 AM. Any deviations from the protocol were noted on the SCR.

Additional details below; also found in the SWAN Manual of Operation (MOO) section 6.1.2

4.1.1.1. Blood Collection Supply Kit

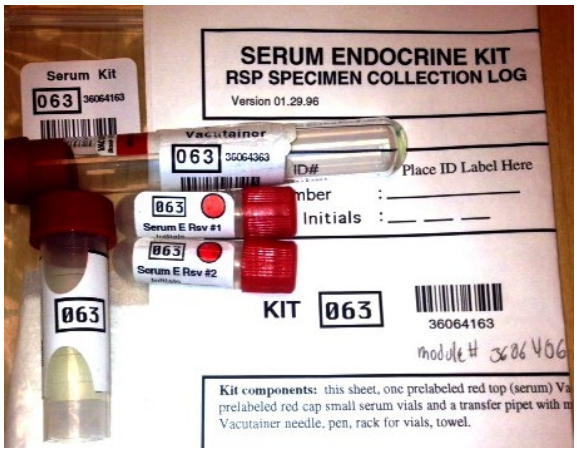
Specimen collection supplies needed for Repository-designated and other SWAN samples were provided to study sites by the CLASS Lab, as one module/packet per subject. The content of each module/packet included the following Repository blood-related supplies:

- Specimen Collection Log /Specimen Collection Record
- Blood Drawing Kit:

- Two (2) pre-labeled 10-mL red-top (serum) Vacutainer tubes (for Repository samples, as well as for measuring endocrine measures, glucose, insulin, bone markers)
- One (1) pre-labeled 7-mL red-top (serum) Vacutainer tube (for Repository samples)
- One (1) pre-labeled 5-mL blue-top (citrate plasma) Vacutainer tube (for Repository samples as well as for measuring clotting factors)
- One (1) Vacutainer Eclipse™ multi-sample needle with safety lock
- One (1) single-use Vacutainer holder

- Blood Aliquoting Kit:
  - Seventeen (17) pre-labeled red-cap small serum cryovials (Repository)
  - Three (3) pre-labeled blue cap small plasma cryovials (Repository)
  - Three (3) disposable absorbent pads

CLASS Central Laboratory staff pre-labeled all Vacutainer and transfer vials using labels containing a unique bar code and highly visible three-digit number common to all materials in the kit (see example below).



4.1.1.2. Blood Collection

SWAN sites followed the protocol below to uniformly schedule visits and collect Repository serum and plasma from all participants:

(a) Scheduling of Blood Draws

1. For a Respondent with regular bleeding (predictable within a week) and no current use of prescription HRT or birth control pills, a fasting blood specimen should be obtained before 10:00 AM., and preferably between 8:00-10:00 AM., during days 2 - 5 of her menstrual cycle window. For Respondents with current use of prescription HRT/BC pills or those who have had a hysterectomy or no bleeding in past 3 months, a fasting blood sample should be drawn between 8:00-10:00 AM within the data collection window.

2. Within the first 60 days of initiation of follow-up data collection, the site will attempt to obtain a fasting blood sample drawn during days 2-5 of the Respondent's menstrual cycle. If the site determines during a blood draw attempt that the Respondent is not fasting or is not in days 2-5 of her menstrual cycle, then the blood sample should not be taken at that time and another attempt made at a later date. Depending on a woman's menstrual regularity, there may be one or more opportunities for a blood draw in the menstrual window during the first 60 days.
3. After 60 days have elapsed, the site staff will attempt to schedule a respondent's blood draw whenever it is convenient and feasible, without regard to day of menstrual cycle. On this last attempt, sites should try to get a fasting blood sample if at all possible, but if this is not possible because the respondent has eaten, the blood sample still should be drawn and the reason for this protocol variation documented.

(b) Blood Collection Protocol

1. Subjects must be fasting for ALL specimen collections. Fasting is defined as nothing to eat or drink except water for at least 12 hours.
2. Have the Subject seated for at least five minutes prior to blood collection. Complete the blood collection preferably between 8:00-10:00 AM. Note on the Specimen Collection Log in what position the Subject is during specimen collection (i.e., sitting, lying down) and use the same position used for drawing blood at baseline and each annual follow-up visit. This information will be printed on the Respondent's Contact Record.
3. Using a Vacutainer multi-sample needle, collect the blood in the following order. After each tube is filled, remove it, replace it with the next tube, and mix each tube 8-10 times gently by inversion

    #1: 10-mL pre-labeled red-top Vacutainer (CLASS, Repository)
    #2: 10-mL pre-labeled red-top Vacutainer (CLASS, Repository)
    #3: 7-mL pre-labeled red-top Vacutainer (Repository)
    #4: 5-mL pre-labeled blue-top (Citrate) Vacutainer (CLASS, Repository)
    #5: 7 mL pre-labeled purple-top (EDTA) Vacutainer (CLASS, Repository)

    Collect a total of 39 mL of blood in 5 Vacutainers. Use a butterfly needle (21 g) and/or 5-mL (pediatric) Vacutainer sizes on women with smaller veins. Collect the samples in this order to prevent the carry-over of Vacutainer additives/ anticoagulants into the red-top (serum) tube.

4.1.1.3. Onsite Processing of Blood Samples (Serum & Plasma)

Overview

Plasma: Store the blue-top and purple-top tubes in a 4-8°C refrigerator (or in a cooler on wet or blue ice) for a maximum of two hours prior to centrifugation. Immediately centrifuge if possible.

Serum: Allow the red-top tubes to sit at room temperature for a minimum of 30 minutes to a maximum (preferred) of 60 minutes to allow the clot to form. Then, refrigerate the tubes at (4-8 °C) for a minimum of 30 to a maximum (preferred) of 60 minutes before centrifugation.

See details below, or in the SWAN Manual of Operation (MOO) section 6.1.2

Protocol

a. Before Centrifugation

1.) Serum: Allow red-top tubes to sit at room temperature from 30 to (preferred) 60 minutes to allow the clot to form; then refrigerate the tubes at 4-8 °C for 30 to (preferred) 60 minutes before centrifugation.

2.) Plasma: Centrifuge blue and purple-top tubes immediately after collection. If this is not possible, place them in a cooler on blue ice or wet ice, or in a refrigerator (4-8 °C), for no more than 2 hours, then centrifuge them.

b. Centrifuging Blood Samples

Within 2 hours of collection, centrifuge all tubes:

- in a refrigerated centrifuge (4°C)
- at a minimum force of 1300 x g
- for 20 minutes

c. Aliquotting Blood Samples

For the SWAN Study, it is preferable to have fewer aliquots at a given volume than more aliquots at a reduced volume. Do not aliquot less than a 0.5-mL volume into any vial. To avoid unnecessary freeze/thaws and assure optimal use of these specimens, all Repository aliquots are to contain a standard volume of 0.5 mL.

Use the Finnpipette™ pipettor (provided to each site by CLASS) and the plastic, disposable Finnpipette tips to transfer the serum and plasma, changing to a new Finnpipette tip for each new blood tube.

- From red-top Vacutainer #1, pipet 2.0 mL serum to the large (S1), 1.0 mL to the smaller vial (S2) and **0.5 mL serum into each of the four (4) Repository cryovials (S3-6)**. Close vial caps tightly.
- From red-top Vacutainer #2:
  Non-Bone Turnover study sites: Pipet 1.0 mL serum to the larger vial (S7), **and 0.5 mL serum into each of the six (6) smaller cryovials (S9-S14)**. Close vial caps tightly. Note: S8 is not collected.

Bone Turnover study sites: From red-top Vacutainer #2, Pipet 1.0 mL serum into each of the two larger vials (S7, S8) and **0.5 mL serum into each of the six (6) smaller cryovials (S9-S14)**. Close vial caps tightly.

- From red-top Vacutainer #3, **Pipet 0.5 mL serum to each of the other seven (7) smaller cryovials (S15-S21)**. Close vial caps tightly.
- From the blue-top Vacutainer, transfer 1.0 mL plasma to the larger blue-cap tube (P1) and **0.5 mL into each of the three (3) blue-cap Repository cryovials (P2 - P4).** Take care to aspirate NO cells. Close vial caps tightly.
- From the purple-top Vacutainer, transfer three (3) 1.0-mL plasma aliquots to the three (3) larger purple-cap tubes (E1, E2, and E3). Close vial caps tightly.

If cells or platelets are accidentally aspirated in the plasma aliquoting process, centrifuge the tubes a second time and transfer the contents cleanly to prevent the carryover of cells or platelets to the plasma sample.

Carefully match ID labels on the collection tubes with ID labels on corresponding aliquot tubes from the same kit. Write the Subject's three initials and the date of collection on the label of each tube. Attach the subject ID label to the Specimen Collection Record. Record the subject initials onto the Shipping Record. Complete the Specimen Collection Record, checking that ID labels are correctly affixed and noting any problems such as insufficient volume, spills, etc.

4.1.1.4. Temporary Onsite Storage of Blood Samples

Follow the steps below to ensure proper handling and storage of Repository serum and plasma samples before shipment to the Repository facility.

- Check that all aliquot tube caps are securely closed; Check that all labels are securely placed on the tubes.
- Return all aliquots to the appropriate shipping bags, seal bags securely and place the bags with tubes upright in a -20 °C (or lower) non-self-defrosting freezer. Carefully monitor and record the freezer temperature daily in a log.
- Store frozen specimens locally for no more than 30 days, and at least overnight before shipping to the Repository. Distributing specimens among the designated shipping bags prior to freezing will eliminate the need to open frozen bags later, and will avoid partial thawing of specimens when preparing them for shipping.

4.1.2. Annual Visit Urine Collection, Processing, and Onsite Storage

*NOTE: Urine sample collection took place only at the **five SWAN sites** studying bone health – Michigan, MGH, UC Davis, UCLA, and Pittsburgh. The Chicago and NJ sites do not have Repository urine samples.*

Overview

An early morning (before 9:00 AM) sample of urine for measurement of bone markers and Repository was collected from participants at the time of, or immediately prior to, their visit. *See details below, or in the SWAN Manual of Operation (MOO) section 6.1.2*

4.1.2.1. Urine Specimen Collection Supply Kit

In addition to the blood collection supplies described in section 4.1.1.1. above, each supply module/packet from the CLASS Lab included the following urine collection supplies:

- One (1) 50-mL sterile urine collection cup
- One (1) pre-labeled yellow-cap large urine vial
- Eight (8) pre-labeled yellow-cap small urine vials

4.1.2.2. Urine Specimen Collection

At the time of, or immediately prior to, her study visit, ask the Subject to collect an early morning sample of urine. This sample should be collected before 9:00 AM, but should not be the first voided urine, and brought to the clinic study visit. Record the estimated time of urination.

4.1.2.3. Urine Specimen Onsite Processing

a. Handling: While the urine need not be refrigerated between the time of collection and delivery to the site that same morning, it should be protected from direct sunlight by wrapping in aluminum foil or placing in an envelope. Immediately aliquot the urine and place into freezer storage. If not aliquoting the specimen immediately, refrigerate (4-8 °C) the urine upon receiving it from the subject, or place it in a cooler on blue ice for not more than 4 hours.

b. Aliquoting: Using the Finnpipette pipettor and plastic tips, transfer 0.5 mL of urine into each of the eight pre-labeled (Repository) yellow-capped vials. Close vial caps securely. Write the Subject's three initials and the date of draw on the label of each tube.

4.1.2.4. Urine Specimen Temporary Onsite Storage

Freeze the vials at -20 °C (or lower) until the monthly shipment to the Repository, when they are packaged with dry ice (8 x 0.5 mL vials). Write in the notes section on the Specimen Collection Record form any comments such as problems with the urine collection, insufficient volume, spills, etc.

4.1.3. Shipping Annual Visit Specimens to the Repository

Ship all Core Annual Visit specimens to the Repository on a monthly on dry ice and according to all IATA shipping regulations. Attach a copy of the Repository Shipping Record to

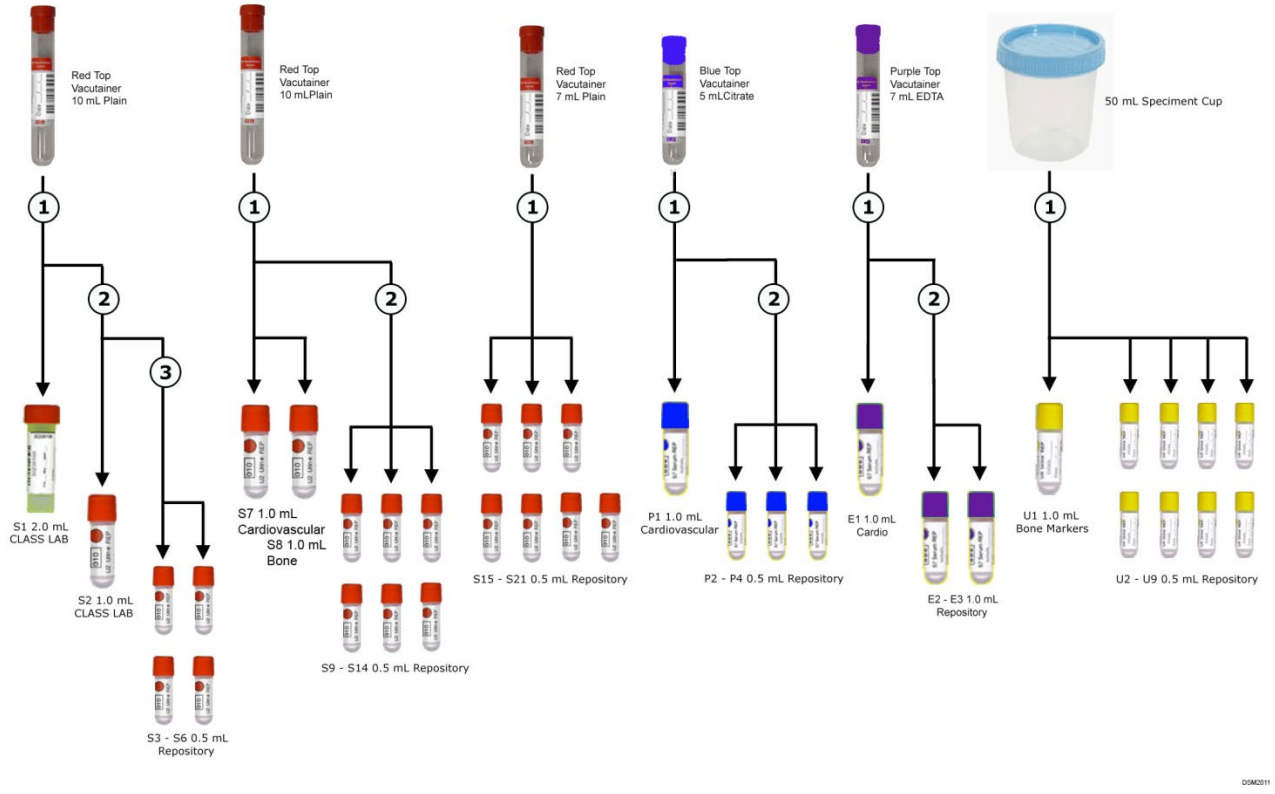
copies of the specimen collection records, and enclose with the shipment. Keep a copy of the completed Repository Shipping Record at the site.

4.1.4. Summary of Annual Visit Blood and Urine Samples Collected for Repository

The schema below, Figure 6.3 from the SWAN MOO, shows the blood and urine collection protocol for Core SWAN Study participants. This protocol was followed for Visit 01 to Visit 13. A change in collection, decreasing serum and increasing plasma, took effect for the final SWAN visit, V15 in SWAN V (see next section, 4.1.5).

In cases of short draws, alternate priorities were outlined consistently for all sites. Specific protocols covered a number of short draw scenarios, most often eliminating the purple-top tube and shifting vials to go to the assays of highest priority. *(see Figures 6.9a – 6.9d, short draw schemas, Section 4 Appendices)*

SWAN MOO Figure 6.3, Full Sampling Schema



The table below shows the number and volume of vials collected for the Repository, for each specimen type between Baseline (V00) and Visit 13, along with tube top colors, vial names and applicable SCR version numbers.

Repository Specimen Collection Protocols, SWAN I-SWAN IV (Visit 00-13)

			SWAN I **Baseline Visit 00** SCR 03/15/1996		SWAN I **Visit 01(A)** SCR 02/01/1997		SWAN I-III **Visits 01(B)–10** SCR 09/01/97 SCR 09/01/00		SWAN IV **Visits 12-13** SCR 05/01/2009	
Specimen Type	**Tube cap color**	**Vial Names** (Visit1b-13)	**# Vials**	**Volume (mL)**	**# Vials**	**Volume (mL)**	**# Vials**	**Volume (mL)**	**# Vials**	**Volume (mL)**
Serum	Red-top	S3-S6 S9-S21	5	1.0	4	1.0	17	0.5	17	0.5
Plasma, Citrate	Blue-top	P2, P3, P4	1	1.0	4	1.0	3	0.5	3	0.5
Plasma, EDTA	Lavender -top	E2, E3	1	1.0	-	-	-	-	2	1.0
Urine	Yellow-top	U2-U9	-	-	4	1.0	8	0.5	8	0.5

Several items to NOTE about Repository specimen collections:
Visits 11 and 14: Follow up Visits 11 and 14 were interim visits with no Repository sample collection.

Visit 01: The SWAN specimen collection protocol changed in the middle of the Visit 01 cycle, and two versions of the Specimen Collection Record (version 02/01/1997 and version 09/01/1997) were used. The number of aliquot tubes collected and the volume collected within each tube differs between the two versions. The collections included the following:
02/01/1997 – 2 red-top tubes, 2 blue-top tubes, and 1 purple-top tube were collected
09/01/1997 – 3 red-top tubes, 1 blue-top tube, and 1 purple-top tube were collected.

EDTA Plasma: EDTA plasma was not collected for Repository in Visits 01-10; however, many of the vials sent to the MRL laboratory during this period was recovered in 2007 and added into available Repository inventory. In Visits 12 and 13, while EDTA plasma was being collected for the Repository, it was excluded in cases of a short-blood draw. In these situations, the E1, E2 and E3 vials were processed as serum (not plasma) and sent to CLASS. These exceptions were documented in the specimen logs.

4.1.5. Visit 15 (SWAN V) Updates to Core Annual Visit Specimen Collection

Some changes were made to the specimen collection and processing protocols for Visit 15, the final SWAN clinic visit. These changes are summarized here.

4.1.5.1. V15 Repository Specimens Collected

The number of vials collected for each material type was adjusted to better match Repository utilization and demand. The number of serum vials collected was

reduced, EDTA plasma increased, and urine was collected in fewer but larger vials, as seen in table below.

			SWAN V Visit 15 SCR 04/26/2015	
Specimen Type	**Tube color**	**Vial Names**	**# Vials**	**Volume (mL)**
Serum	Red-top	S3-S6, S10-S13	8	0.5
Plasma, Citrate	Blue-top	P2-P4	3	0.5
Plasma, EDTA	Lavender-top	E4-E10	7	0.5
Urine	Yellow-top	U1-U4	4	2.0

4.1.5.2. Updated Repository Shipping Address and Personnel

All V 15 Repository sample shipments and related questions should be sent to:
SWAN REPOSITORY - PRECISION BIOSERVICES (Frederick, MD)
Shipping Address:
Precision for Medicine
8425 Progress Drive, Suite M
Frederick, Maryland 21701

Contact Person:
Misti Dowell
Project Manager
Phone: 240-306-4104
Fax: 301-668-3416
Misti.Dowell@precisionformedicine.com

4.1.5.3. New Bone Turnover/Urine Collection Sites

Four SWAN sites will participate in the bone turnover protocols in SWAN V and collect Repository urine samples: Michigan, MGH, UCLA and Pittsburgh. (Previously, the UC Davis site also participated as a bone site, but will not in V15.)

4.1.5.4. Specimen Boxes Used to Store and Ship Repository Samples

After collection, Repository samples in Visit 15 are stored locally and shipped in 2" (9x9) cardboard specimen boxes rather than the reclosable plastic bags. This change was made to keep the vials upright during the initial freezing process, provide better protection during shipping, and make the task of inventorying quicker and easier.

4.1.5.5. Electronic Manifests

The Visit 15 Repository samples collected will be entered into an online database by each site. The Coordinating Center will manage and track the samples as they are shipped to CLASS and the Repository. Access to electronic shipping manifests via a secure online site (EDC CloudShare) is available to the Repository personnel responsible for inventorying the incoming shipments. Previously the Repository entered the data from the paper SCR forms which were (and still are) included in each shipment. The electronic forms will eliminate the need for this data entry step at the Repository.

Table 6.1
Integrated Summary of Specimen Shipping for Visit 15

	Purpose	Vol	Medium	Label	Cap	Store	Ship To	Freq	Packing
CLASS Specimens:									
1	Hormones, insulin	1 x 2 mL	Serum S1	CLASS	Red	<-20C	CLASS	Monthly	Dry Ice
2	Endo Mass Spec	1 x 1 mL	Serum S2	CLASS	Red	<-20C	CLASS	Monthly	Dry Ice
3	Glucose; Lipids	1 x 1 mL	Serum S7	CLASS	Red	<-20C	CLASS	Monthly	Dry Ice
4	Inflamm Markers	1 x 1 mL	Serum S8	CLASS	Red	<-20C	CLASS	Monthly	Dry Ice
5	Adipokines	1 x 1 mL	Serum S9	CLASS	Red	<-20C	CLASS	Monthly	Dry Ice
6	Clotting factors	1 x 1 mL	Plasma P1	CLASS	Blue	<-20C	CLASS	Monthly	Dry Ice
7	CV Markers	1 x 1 mL	Plasma E1	CLASS	Purple	<-20C	CLASS	Monthly	Dry Ice
8	Inflamm Markers	1 x 1 mL	Plasma E2-3	CLASS	Purple	<-20C	CLASS	Monthly	Dry Ice
Repository Specimens (Precision Bioservices):									
9	Repository (Serum)	8 x 0.5 mL	Serum S3-6; S10-13	Repository	Red	<-20C	Repository	Monthly	Dry Ice
10	Repository (Citrate)	3 x 0.5 mL	Plasma P2-4	Repository	Blue	<-20C	Repository	Monthly	Dry Ice
11	Repository (EDTA)	7 x 0.5 mL	Plasma E4-10	Repository	Purple	<-20C	Repository	Monthly	Dry Ice
12	Repository (Urine)	4 x 2.0 mL	Urine U1-4	Repository	Yellow	<-20C	Repository	Monthly	Dry Ice

For Visit 15 sampling schema diagrams, see Appendix.

4.2. DAILY HORMONE STUDY (DHS) SAMPLES

4.2.1. DHS Urine Kit, Collection, Processing, and Onsite Storage

4.2.1.1. DHS Urine Collection Kit

Specimen collection supplies for the DHS urine collection were provided to all SWAN study sites by the CLASS laboratory. Supplies were set up in modules, in which one packet included all supplies necessary for the collection of DHS urine for one participant.

Four boxes were provided. Two of the boxes (marked with a red dot to indicate non-frozen storage) contained 50 labeled yellow-cap vials (25 pairs), with each pair arranged in sequence, marked "A" and "B" as indicated by numbers on the inside of the box and on their labels. The other two boxes (marked with a blue dot to indicate that they should be placed in the freezer to receive the samples for frozen storage) contained dividers with spaces for 50 vials, a log sheet, two permanent markers, 50 paper cups and 2 plastic urine collection cups. The log sheet was to be retained by the site and used at the time of kit retrieval. The subject ID number was to be placed on the log sheet immediately upon issuing a kit.

CLASS Urine Collection Kit Contents List:

- 100 pre-labeled Urine Vials each containing 200uL of 50% glycerol
- Two (2) permanent markers
- Two (2) plastic Urine Specimen collection cups
- Fifty (50) paper Urine Specimen collection cups
- Four (4) boxes with dividers
- Urine Collection Log Sheet (to be kept at site)
- A Daily Diary for the participant to fill out each day of collection

4.2.1.2. DHS Urine Specimen Collection

Daily samples of urine for measurements of endocrine markers were obtained each morning throughout a woman's menstrual cycle. Samples were collected from the first day of the cycle until the first day of the next cycle, or for up to 50 days.

If the participant went to sleep before midnight and woke up with her period having started, she collected her first day's urine upon awakening that morning; if her period began during the day, she began collecting urine the next morning upon awakening. (Daily urine samples were all to be obtained from the first urination in the morning upon awakening.) Collection of urine every morning continued until the first day of her next menstrual period, or through the fiftieth consecutive day, whichever occurred first. The participants were informed to call the site on the last day of urine collection to schedule phlebotomy and specimen retrieval.

After collection, the urine vials were promptly (within an hour) placed in the box in the freezer to match the numbers on the bottom of the box. If the participant

collected her urine specimen somewhere other than her home one morning, the specimen was to be refrigerated within 3 hours and frozen within 24 hours. The woman was to write on the Daily Diary, "Not Cold".

At the time of kit pick-up, kits were placed in a styrofoam or plastic insulated box (like an igloo), containing 2 pieces of previously frozen blue ice. The box was taped (if foam) or snapped (if plastic) shut. The transportation time was not to exceed 1 hour. If participants refused a home visit to retrieve the kits, they could transport the specimens to the site in the styrofoam box or a cooler packed with at least 2 trays of ice cubes or 2 pieces of previously frozen blue ice. Again, transportation time was not to exceed 1 hour.

4.2.1.3. DHS Urine Specimen Processing & the Urine Collection Log

Site clinicians kept the samples on ice in the container in which they were received while they filled out the Urine Collection Log. Recorded on the form was: participant ID number, visit number, participant initials, and site. Each tube was visually inspected for fill level and integrity of the tube. The date was written in the Urine Collection Log box to the left if the specimen was present, and the corresponding date was checked in the box for the "B" series of tubes as long as its date matched the "A" tube. If the date on the "B" tube did not match, the correct date on the "B" tube was recorded in the box on the Urine Collection Log. Comments were added to the right of the tube number and letter. Missing tubes, uncapped tubes with urine in them, partially filled tubes or other tube integrity problems with the specimen were recorded in the comments box. *NOTE:* If a tube was missing or empty, staff recorded the date as '–9/-9/-9-9' and recorded 'EMPTY' in the related comments field.

DHS URINE PROCESSING NOTE: In 2007, the majority of the DHS urine samples from Visits H1-H6 were aliquoted down from 5-mL tubes to 0.5-mL tubes, creating 10 aliquots of each daily sample. This activity was performed at SeraCare (now Precision Bioservices) following a detailed protocol outlined in SeraCare's SOP25347.

4.2.1.4. DHS Urine Temporary Storage at Sites

As soon as the Urine Collection Log was completed, the urine vials were maintained in a freezer at -20°C or below until they were shipped to the Repository. Repository DHS shipments occurred monthly.

4.2.2. DHS Serum Kit, Collection, Processing, and Onsite Storage

4.2.2.1. DHS Serum Collection Kit

DHS serum specimen collection supplies were also provided to study sites by CLASS as part of a module. Each module included all supplies for the collection of serum specimens for endocrine markers and Repository samples.

The content of each module was as follows:

One (1) pre-labeled red-top (serum) Vacutainer tube
One (1) pre-labeled red cap large serum cryovial (CLASS)
Six (6) pre-labeled red cap small serum cryovials (Repository)
One (1) transfer pipet with markers at 0.5 mL and 1.0 mL
Two (2) absorbent pads
Two (2) shipping bags (one each for CLASS and Repository)

4.2.2.2. DHS Serum Specimen Collection

a. Specimen Labeling/Logging Procedures

The following steps were followed when preparing for DHS serum collection:

1. Remove the pre-labeled CLASS tubes.
2. Enter the date of the collection and three Subject initials on all the tube labels, corresponding to the first, middle and last name. Use a hyphen if no middle name is given. Print the initial letters clearly in plain block letters.
3. The draw date must correspond with the date of the visit. Therefore, **DO NOT** enter draw dates ahead of time.
4. **DO NOT** use a vial (i.e., 20B) intended for another collection (i.e., 25B) and manually change the vial number. This manual change will cause an error when entering the barcode data into the Repository database.

b. Blood Collection Protocol:

Using a Vacutainer multi-sample needle, collect one 10 mL red-top Vacutainer tube of blood, and immediately invert it 8-10 times for gentle mixing after collection.

A butterfly needle (21 g) and/or 5-mL ("pediatric") Vacutainer may be used on women with smaller veins.

4.2.2.3. DHS Serum Specimen Processing at Sites

Sites followed a standard protocol for processing DHS serum onsite, as follows:

a. Before Centrifugation

Allow red-top tubes to sit at room temperature for 30 to 60 minutes maximum (preferred) to allow optimal clot formation, then refrigerate the tubes (4-8 °C) for 30 to 60 minutes (preferred) before centrifugation. If collection is occurring at a field site, the 30 to 60 minutes of transport time (in the car) will count towards the 30-60 minutes at room temperature for clotting. If more than 1

hour transportation time is needed, put the tube on ice after 60 minutes (if possible).

b. Centrifugation

Within 2 hours of collection, centrifuge the red-top tube:

- in a refrigerated centrifuge (4 -8 °C)
- at a minimum force of 1300 X g
- for 20 minutes.

c. Aliquotting

Using the plastic, disposable pipet provided in the kits, transfer the serum as follows:

- From the red-top Vacutainer, pipet 2.0 mL serum to the large vial (D1) and pipet remaining serum into the smaller vials (D2-D7) in 0.5-mL aliquots. If insufficient serum exists to make all aliquots 0.5-mL, distribute the serum evenly across the tubes (D2-D7).
- Should cells or platelets be aspirated in the aliquoting process, centrifuge the tube a second time to prevent the carryover of cells or platelets to the next sample vial.

d. Complete the Specimen Collection Record

- Check that ID labels are correctly affixed to the Specimen Collection Record.
- Note any problems such as insufficient volume, spills, etc. on the Specimen Collection Record.

4.2.2.4. DHS Serum Temporary Storage at Sites

After checking that all aliquot tube caps are secure, and that all labels are secure on the tubes, separate the samples into two shipping bags: 1) the original bag that held the Serum Specimen collection supplies (for CLASS) and 2) a second bag provided in the module (for Repository). Seal the bags securely and place them upright in a -20 °C (or lower) non-self-defrosting freezer. Monitor and record freezer temperature daily and record in freezer temperature log. Store frozen specimens locally at least overnight and for no more than 30 days. Separate specimens into the designated shipping bags prior to freezing to eliminate the need to open frozen bags later and to avoid partial thawing of specimens when preparing them for shipment to the laboratory or Repository.

4.2.3. Shipping DHS Specimens to the Repository

DHS Samples were shipped to the Repository on a monthly basis, and included:

- DHS URINE – Up to 50 yellow-capped urine vials marked "B" (01B – 50B). Each vial contained 5 mL of urine and a small amount of cryoprotectant (glycerol).
- DHS SERUM – 6 red-capped serum vials (D2 - D7) , vials at 0.5mL in volume

The Repository Shipping Record was included in each shipment, along with copies of the entire DHS Specimen Collection Record and the entire DHS Urine Collection Log for each ID/Visit included in the shipment. A copy was filed as part of the site records.

Complete shipping instructions for the DHS Repository samples are as follows:

Place the yellow-capped "B" urine vials and six red-capped serum vials (D2 - D7) into an empty box (or reclosable plastic bag). Place pre-affixed Dri Mop™ absorbent pads in each box (bag). Assemble all boxes awaiting shipment, with only one box (bag) per subject in a shipment, complete the Repository Shipping Record form, and transfer boxes (bags) to a reusable Styrofoam shipping container (e.g. Thermosafe™ #399). Add approximately 10-15 lb dry ice, close the Styrofoam lid securely, then close and seal the outer container. Remember to include the Repository Shipping Record and copies of the DHS Specimen Collection Record and the DHS Urine Collection Log for each ID/Visit included in the shipment. Attach to the shipping box the red and black biohazard label and a shipping label addressed to:

Address:
McKesson BioServices
685 Lofstrand Lane
Rockville, MD 20850
Email for sites: swan@mckesson.com

Contact Person:
Christine Bravo
(301) 424-7658
(301) 340-3275 Fax
Christine.Bravo@mckesson.com

Affix a Dry Ice Label (9, International Goods) reading: "Dangerous goods, shipper's declaration not required. Dry Ice 9, UN 1845, 1 x 6.6 kg" and add your name and address as sender.

Fax the Repository Shipping Record to McKesson (prior to sending the shipment). McKesson BioServices will confirm receipt of shipment notification messages and inform sites whether it is okay to proceed with shipment.

Ship prepaid (by Repository) for overnight delivery by the shipper (FedEx).

Attach the shipper's copy of the air bill to a copy of the completed Repository Shipping Record and file the forms locally.

McKesson BioServices will confirm receipt of shipment by e-mail.

4.3. DNA SAMPLES

In 2002-2003, spanning Visits 04-06, SWAN participants had the option of donating blood and buccal cells for DNA extraction and B-cell immortalization. Participation was voluntary and the participants signed an Informed Consent Form (see section 3.3).

A one-time collection of blood served as the substrate for the preparation of genetic and cellular materials for the DNA Repository. This collection typically took place at the time of the follow up clinic visit. Two 10-mL ACD Vacutainers of whole blood were drawn for this purpose. Additionally, a mouthwash/buccal cell sample was collected. Participants did NOT have to meet requirements for fasting or time of month cycling for these collections. Specific collection and storage protocols were as follows:

4.3.1. DNA Specimen Collection and Onsite Activities

4.3.1.1. DNA Specimen Collection Supplies Kit

DNA specimen collection supplies were provided to study sites in modules created by McKesson BioServices. Each packet included all supplies for collection of specimens for the DNA Repository. For each subject, McKesson provided the following:

- Two (2) 10-mL Vacutainer tubes with ACD preservative
- Labels for the Vacutainer tubes
- Scope™ Mouthwash (Original Mint flavor) - 1.5 fl. oz.
- Bitran™ reclosable plastic bag - 6" x 6"
- Reclosable plastic bag - 6" x 9" bag with outer pouch
- Nalgene™ sterile 15-mL cryovial
- Shipping instructions
- Temperature Tracker datalogger

Specimen collection records were also provided. The subject ID number was placed on the specimen collection record immediately upon issuing a kit. The site retained one copy, while the other copy was shipped with the biological specimens.

4.3.1.2. DNA Specimen Collection

DNA samples were to be drawn, whenever possible, Monday through Friday for shipping no later than the following day to ensure sample quality and integrity.

<u>Blood Collection protocol:</u>

Using a Vacutainer multi-sample needle, collect two tubes of blood:

Two (2) – 10-mL ACD Vacutainer tubes. Immediately after collection, invert each tube 8-10 times for gentle mixing.

<u>Mouthwash/spit Collection Instructions for Participants:</u>

- Do not eat, drink, or rinse your mouth for one hour before collecting the saliva sample.
- Open the collection container and fill it half full of mouthwash (to the red fill line).
- Swish the mouthwash from the container around in your mouth vigorously for 45 seconds. We'll watch the clock while you do this. Do not gargle or clear your throat.
- Holding the container close to your mouth, spit all of the mouthwash back into the container. Replace the top on the container, and screw it on tightly.

4.3.1.3. DNA Collection Record/Log and Specimen Processing at Sites

Because the DNA study only required the collection of 3 specimens (2 blood tubes and 1 mouthwash/saliva specimen), the Specimen Collection Record and Specimen Collection Log were combined into one form. The technician filled out this form at the clinic site. The form was completed with information including date, participant ID and date of birth, and the corresponding specimen labels were affixed to each specimen collected. Each tube was inspected. Missing tubes, partially filled tubes, or other problems with the specimen were recorded on the Log.
NOTE: If a tube was missing or empty, staff recorded the date as '–9/-9/-9-9' and 'EMPTY' in the related comments field.

While the sites were responsible for effective collection, as listed above, no specimen processing for these samples took place at study sites. All processing occurred at McKesson.

4.3.1.4. DNA Specimen Temporary Storage at Sites

DNA specimens required NO REFRIGERATION OR FREEZING at the sites.

Clinic personnel were to: visually inspect each tube for fill level and vial integrity; check that all ACD tube caps are secure; and check that all labels are secure on the tubes.

DNA Specimens were to be retained at the site for no more than 24 hours prior to shipment to the Repository. (If a Saturday draw could not be avoided, it was retained until the following Monday.)

4.3.1.5. Shipping of DNA Specimens to the Repository

To package the shipment, these instructions were followed:

Place Vacutainer tubes and mouthwash/saliva collection containers into the appropriate sections of the DNA shipping container (up to 4 sets/participants). Place both sections into the reclosable plastic bag. Remove all air from the bag and seal it. Place both specimen sections back into the styrofoam shipping kit.
Take the temperature tracker and hold down the start button until a star appears in the window. Once the star appears, the tracker is activated and should be placed between

the ACD tubes and the buccal cell collection, to monitor temperature in the shipper during shipment, until it is opened for processing.

Complete the Repository Shipping Record. Remember to include the Repository Shipping Record and copies of the DNA Specimen Collection Record and the Informed Consent Form for each Participant included in the shipment.

No blue ice or dry ice is added. Close the Styrofoam lid securely and then close and seal the outer container. Attach to the shipping box the shipping label addressed to:

Address:
McKesson BioServices
685 Lofstrand Lane
Rockville, MD 20850
Email for sites: swan@mckesson.com

Contact Person:
Christine Bravo
(301) 424-7658
(301) 340-3275 Fax
Christine.Bravo@mckesson.com

Ship via FedEx WITHOUT BLUE ICE OR DRY ICE, prepaid (by Repository) for overnight delivery by the shipper. Attach the shipper's copy of the airbill to a copy of the completed Repository Shipping Record and file the forms locally at the site. Confirmation of receipt of samples will be sent by McKesson by e-mail.

4.3.2. DNA Specimen Activity at McKesson

SWAN clinic sites shipped specimens for DNA preparation and B-cell transformation to McKesson BioServices within 24 hours of specimen collection. At McKesson, any necessary discrepancy reconciliation between samples and consent forms received was completed. Also, the buccal cell samples and one of the 10ml whole blood samples were processed into DNA Pellets and stored for future use. The temperature tracker information was downloaded and stored in the study processing data at McKesson.

4.3.2.1. Consent – Sample Discrepancy Reconciliation

Upon arrival at McKesson BioServices, the receiving technician matched the participant's Informed Consent Form with the blood tubes and buccal cell collection cups. In the event that the specimens did not match the information on the Informed Consent Forms, the specimens were still processed and stored for a period of seven days and an Inconsistency Form was sent to the site for clarification. The possible specimen dispositions are listed below:

Disposition	Specimen	Processing Lab
DNA from Buccal Cells	1 collection cup	McKesson BioServices
DNA from Blood	1 Vacutainer	McKesson BioServices
B-cell Immortalization	1 Vacutainer	BBI Biotech

If the Informed Consent Form did not specify a process, but the specimen was received in the shipment, the specimen was processed using the following guidelines.

A.	Problem:	DNA from Buccal Cell not specified
	Temporary solution:	Specimen processed into a pellet and frozen.
		No DNA extraction until problem resolved.
B.	Problem:	DNA from Blood and B-cell Immortalization not specified
	Temporary solution:	Vacutainer #1 processed up to the buffy coat stage and frozen.
		No DNA extraction until problem resolved
		Vacutainer #2 transferred to BBI Biotech for processing.
		B-cells not transferred to repository until problem resolved.
C.	Problem:	B-cell Immortalization and DNA from Blood specified.
		Only one vacutainer received.
	Temporary solution:	Specimen processed following blood DNA extraction procedure.

The SWAN DNA Repository Inconsistency Form was faxed to the SWAN site for resolution. Possible resolutions included: Missing specimen(s) shipped, or missing Informed Consent Form that matched received samples sent.

If the problem could not be resolved, the specimen was destroyed, and the comment documented. McKesson BioServices removed the specimens from the freezer and destroyed them by autoclaving. If the specimens were being processed for B-cell Transformation, BBI Biotech was notified that the specimens were to be destroyed. The SWAN McKesson DNA Repository Inventory Database was updated, showing that the specimens were destroyed.

4.3.2.2. Buccal Cell Sample Processed and Stored as DNA Pellet

Buccal cell samples were spun for 15 minutes at 2700 rpm. The supernatant was removed, leaving only the buccal cell pellet, which was then resuspended in 1.0 mL of Tris EDTA pH 8.0, transferred to a 2.0-mL cryovial, and stored at –80 ^{o}C.

4.3.2.3. One Whole Blood Sample Processed and Stored as DNA Pellet

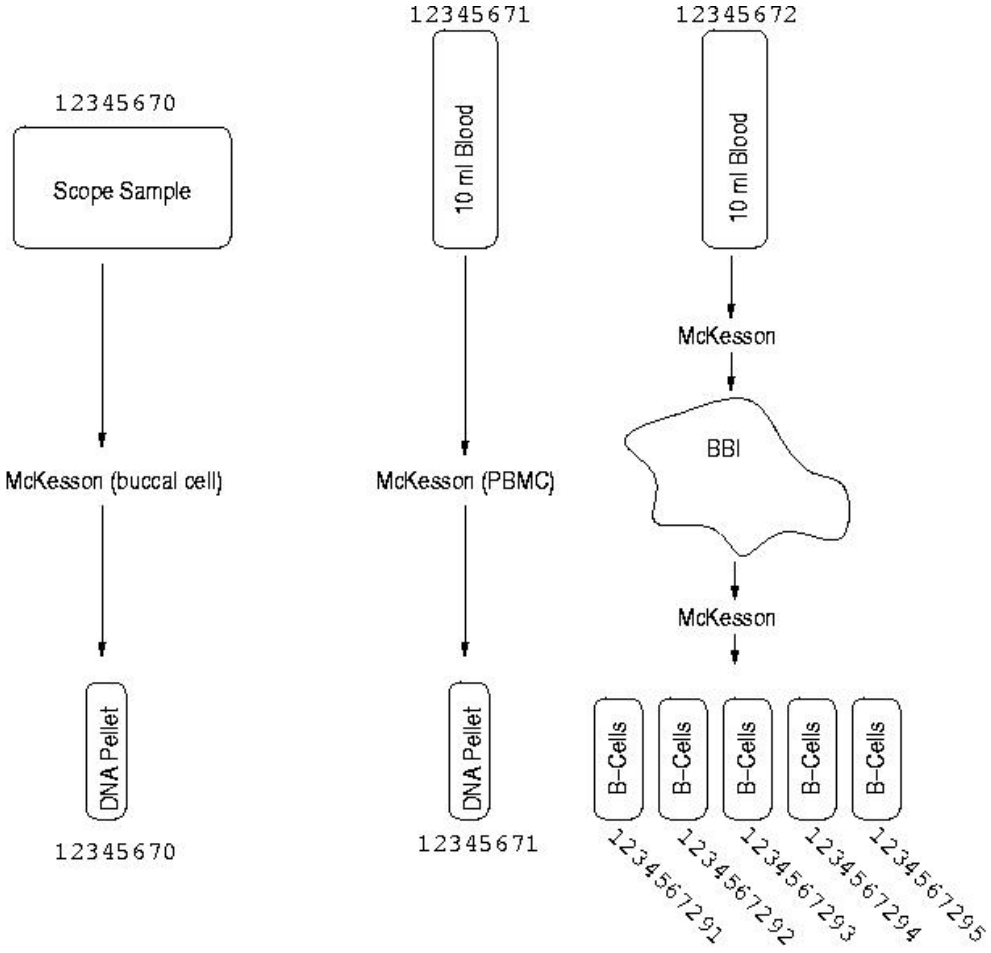
One whole blood was processed to a pellet state. The contents of the tube were pipetted into a Lymphoprep™ tube and the empty tube washed with Hank's balanced salt solution at a volume equal to that of the whole blood contents. The Hank's solution was then pipetted into the Lymphoprep tube, and the contents centrifuged for 10 minutes at 2500 rpm. The white blood cells were carefully aspirated,

transferred to a conical centrifuge tube and spun for an additional 10 minutes at 1200 rpm. Then, these steps were repeated. After the final washing, the cell pellet was resuspended in 1.0-mL of Freeze Medium™ (80% DMEM, 10% Fetal Bovine Serum, 10% DMSO) and aliquoted into a 2.0-mL cryovial labeled "PBMC", leaving at least 50 µL of resuspended cells in the 15-mL centrifuge tube. This 50 µL of cells in the 15-mL centrifuge tube was transferred to a microcentrifuge tube to determine cell concentration by adding 50 µL of Trypan Blue dye and counting live and dead cells with a hemocytometer. The sample was frozen and stored in the vapor phase of liquid nitrogen (VP-LN_2, -150 to -180 °C).

4.3.2.4. Second whole blood sample sent to BBI Biotech

The second whole blood sample was shipped to BBI Biotech (now Precision Bioservices) for EBV Transformation (immortalization), see section below.

4.3.2.5. DNA Specimen Processing Overview



4.3.3. BBI Biotech – EBV Transformation

The second whole blood specimen was shipped to BBI Biotech (now Precision Bioservices). There, the whole blood samples were transformed to an immortalized state utilizing the Epstein-Barr virus, using the protocol as follows:

Phosphate-buffered mononuclear cells (PBMCs) were extracted from the second whole blood sample and centrifuged to pellet form. The PBMCs were washed, counted, isolated and frozen, with $5x10^6$ viable cells set aside for Epstein-Barr virus (EBV) transformation. PBMCs were resuspended in 2.5 mL of Cyclosporin-Containing Transformation Medium (CS) and transferred to a slant tube. 1.0 mL of frozen EBV-containing supernatant was then quickly thawed in a 37°-water bath. The PBMC pellet was inoculated with the EBV-containing supernatant and placed in a dual incubation chamber ($37^0C/5\%$ CO_2).

Seven days (+/-2 days) following innoculation, 1.0 mL of culture fluid was removed from each slant tube. 1.0 mL of EBV-containing supernatant was acquired and thawed in a 37 ^0C water bath. For each mL of EBV supernate, 10 µL of 100X Cyclosporin-A stock was added and mixed. The slant tube culture was then inoculated with 1.0 mL of the EBV+Cyclosporin-A mixture. Any pellet growth and bacterial or fungal contamination was recorded. After four weeks (+/-3 days), if the slant tube culture showed pellet growth, medium yellowing, and absence of bacterial or fungal contamination, the culture was transferred to a T25 flask and 6.5 mL of warmed culture medium was distributed to each T25 flask. The pellet was resuspended in a slant tube, transferred to the appropriate T25 flask, and cultures returned to a 37 $^0C/5\%$ CO_2 incubator.

When the T25 culture showed significant cell cluster formation, medium yellowing and absence of bacterial or fungal contamination, it was passed to a T75 flask along with 20 mL of the warmed culture medium. The new T75 flask cultures were placed into a 37 $^0C/5\%$ CO_2 incubator. This process was repeated and incorporated the placement of 60 mL of warmed culture medium into the existing T75 culture flask. Afterward, 45 mL was recollected and transferred to a new, paired flask. Prior to harvesting, a hemocytometer count was performed on all paired T75 flasks suspected of having more than $60x10^6$ viable cells. If the flasks contained the required number of viable cells, they were repooled into a single flask and centrifuged at 1500 rpm (450xG) for 15 minutes, and the supernatant was discarded. For each culture harvest, the remaining cell pellet was re-suspended in 5.0 mL of freezing medium and **dispensed into ten pre-labeled vials** and frozen. *Mycoplasma* testing was done later. Transformed cells were aliquoted into individual cryovials holding approximately 1 million cells each and frozen in VP-LN_2.

4.3.4. University of Pittsburgh Genomics and Proteomics Center – DNA Extraction & Genotyping

One of the10 aliquots of transformed cells was shipped from BBI Biotech to the University of Pittsburgh Genomics and Proteomics Center for extraction, purification, dilution and genotyping. Because of the high cell density, the Puregene™ system was used. In the first

1020 samples, the average yield was more than 210 μ/g with an average purity (260/280 nm ratio) of 1.87.

Single nucleotide polymorphisms (SNPs) associated with six genes related to reproductive hormones were selected for evaluation. The genes included CYP19 (aromatase) located on 15q21.1, 17α-HSD, two cytochrome P450 enzymes related to estrogen catabolism from estrone to hydroxylated estrone urinary excretion products (CYP1A1 and CYP1B1), and two estrogen receptors (ER-alpha and ER-beta receptor). There were 4-5 SNPs selected per gene. SNPs were selected based on a review of the literature and evaluation of information from the National Center for Biotechnology SNP database (www.ncbi.nlm.nih.gov/SNP) and the Celera database (www.celera.com). The specific SNPs and their associated genes are listed in the Section 4 Appendices.

Genotyping was performed through TaqMan™ technology, a high-throughput approach that uses two fluorogenic probes with a "minorgroove binder" that is matched for the SNPs being assayed. When a SNP match was achieved, the fluorescent probe was cleaved, producing a signal. In the absence of a match, displacement caused the quencher to remain in proximity to the fluorophor, and there was no fluorescence signal. Reactions were read on an ABI 7900HT™ and the allele discrimination module software on the ABI SDS™.

NOTE: Currently, Precision Bioservices holds all SWAN genetic samples – pellets, extracted DNA, immortalized cells and buccal cells.