Manual of Procedures (MOP)
Section 9. Laboratory Procedures

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9.1 OVERVIEW

Particpants enrolled in D2d have blood and urine samples collected as part of their participation in the study. Specimens collected at screening and certain safety labs during the study will be analyzed locally at the collaborating sites' local laboratories. The remaining specimens will be collected on site, prepared for shipping on site and shipped to the D2d Central Laboratory for analyses and long-term storage. The Laboratory for Clinical Biochemistry Research (LCBR) at the University of Vermont serves as the D2d Central Laboratory and is responsible for performing assays and reporting results. The LCBR is responsible, in collaboration with the D2d Coordinating Center, for developing and implementing protocols for specimen collection and handling.

Throughout this manual, the D2d Central Laboratory will be referred to as LCBR.

The primary outcome of the D2d study is time to development of diabetes, which is determined in real-time by laboratory measures (fasting plasma glucose [FPG], plasma glucose 2 hours after a 75-gram oral glucose tolerance test [2hPG] and Hemoglobin A1c [HbA1c]). Therefore, one of the most important steps, and also potentially the most variable, in implementing the protocol is the collection and processing of blood samples.

- ✓ For the laboratory results to be precise and valid, which maximizes the power of the study to detect an effect, biological samples need to be collected, processed, shipped and analyzed in a consistent fashion.
- The study involves the collection of blood from participants during a fasting morning venipuncture (after an 8 hour overnight fast), during the screening, baseline, 3, 6, 12, 18, 24, 30, 36, 42 and 48-month research visits.
- A morning spot urine sample will be collected while the participant is fasting at baseline, 3, 12, 24, 36, and 48 months visits.
- An oral glucose tolerance test is conducted at yearly scheduled visits.
- Women of reproductive potential will have additional urine or blood testing at screening to rule out pregnancy and during the study if there is reason to suspect pregnancy.
- Some blood and urine tests will be repeated outside of the scheduled visits, as needed, for safety and to confirm the diagnosis of diabetes.

This laboratory manual provides study staff with the information necessary to collect, process (prepare for short-term local storage and shipping) and ship specimens to LCBR for measurements and long-term storage. For any questions, study staff can call or e-mail one of the laboratory staff listed in Section 9.2 and on the web portal at www.D2dstudy.org.

9.2 D2d CENTRAL LABORATORY (LCBR) CONTACT INFORMATION

9.2.1 LCBR Address

University of Vermont-Pathology
Laboratory for Clinical Biochemistry Research
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9.3 SITE START UP

Prior to the first screening visit, each collaborating clinical site will have the following in place:

- Equipment and supplies for processing and short-term storing of specimens locally:
    - Refrigerated centrifuge (4°C), with a swinging bucket, capable of spinning at a minimum of 2000g
    - Freezer -70°C or colder
    - Refrigerator (4°C)
    - Dry ice
    - Blood collection supplies (see section 9.4)
    - Vacutainer tube mixer (optional)

- Standard laboratory supplies (see section 9.4)

- An established procedure for sending D2d laboratory research specimens to the clinical site's local laboratory (e.g. transport, use of appropriate forms, arrangements to ensure that the cost of D2d research tests are not billed to the participants' health insurance).

- Staff working with specimens will have completed all training and meet all requirements set forth by the local institution for universal precautions and biological specimen shipment.

9.4 LABORATORY SUPPLIES

Prior to the first screening visit, each site should review its inventory to ensure that adequate laboratory supplies for the D2d study are available, as described below.

The LCBR will provide the following supplies to the sites for D2d visits that include testing by the Central Laboratory. The sites are responsible for supplies needed for tests that are analyzed at the site's local laboratory (e.g. all labs at screening, certain safety labs during the study).

- Six different D2d Laboratory Kits, which are visit-specific:
  - ***Baseline – Annual visit Kit*** **(BAS, M12, M24, M36, M48):**
    - Sarstedt 10 mL pre-labeled, color-coded transfer tubes (9)
    - Urine collection container and wipe (1)
    - Conical tubes (2)
    - Transfer pipettes (11)
    - Additional barcoded Kit ID labels for draw tubes, e-CRFs, sample boxes, and extras.
  - ***Month 3 visit*** **Kit (M03):**
    - Sarstedt 10 mL pre-labeled, color-coded transfer tube (1)
    - Urine collection container and wipe (1)
    - Transfer pipette (1)
    - Additional barcoded Kit ID labels for e-CRFs, sample boxes, and extras.
  - ***Month 6 visit*** **Kit (M06):**
    - Sarstedt 10 mL pre-labeled, color-coded transfer tubes (3).
    - Conical tube (1)
    - Transfer pipette (1)
    - Additional barcode Kit ID labels for draw tubes, e-CRFs, sample boxes, and extras.
  - ***Semi-annual visit*** **Kit (M18, M30, M42):**
    - Sarstedt 10mL pre-labeled, color-coded transfer tube (1)
    - Conical tube(1)
    - Transfer pipette (1)
    - Additional barcode Kit ID labels for draw tubes, e-CRFs, sample boxes, and extras.
  - ***Unscheduled-Confirmatory visit*** **Kit (UNCO) (as needed)**
    - Sarstedt 10 mL pre-labeled, color-coded transfer tubes (3)
    - Conical tube (1)
    - Transfer pipettes (2)
    - Additional barcoded Kit ID labels for draw tubes, e-CRFs, sample boxes, and extras.
  - **End of Study Visit Kit (EOS)**
    - Sarstedt 10 mL pre-labeled, color-coded transfer tubes (3)
    - Conical tube (1)
    - Transfer pipette (1)
    - Urine collection container and wipe (1)
    - Additional barcoded Kit ID labels for draw tubes, e-CRFs, sample boxes, and extras.

- 8.5 mL PaxGene DNA tubes. PaxGene tube is used at baseline visit only.
- A kit that contains extra Sarstedt transfer tubes and caps, urine collection containers and wipes, and transfer pipettes.

- Specimen shipping supplies. The following must be used:
  - Specimen storage bags
  - Absorbent strips (250 mL capacity)
  - 5” x 3” x 3“ fiberboard sample boxes (with 7x7 grids)
  - Specimen Styrofoam shipping containers.
  - Cold packs (for refrigerated shipments)
  - Labels for shippers (e.g. dry ice labels and UN3373 Biological Substance Category B labels)
  - FedEx Airbills

The site will provide the following supplies:

- Standard laboratory procedure and safety supplies
    - Lab coats
    - Gloves
    - Timers
    - Face shield or eye goggles
    - Blood tube racks
    - Biohazard / sharps container
- Phlebotomy supplies
    - Venous access supplies (e.g. saline locks or butterfly needles (21G) with luer adapter)
    - Vacutainer barrels
    - Tourniquet
    - Alcohol prep pads
    - Gauze pads
    - Tape (preferably paper)
- Ice bucket and ice
- Dry ice (for frozen shipments)
- Thermo Scientific 75 gram Trutol® Glucose Tolerance Test Beverage (10 ounce bottle) in one of the following flavors: orange (Cat. 401223P); lemon-lime (Cat. 401025P) or fruit punch (Cat. 401526P). *No substitutions can be made.*
- Urine collection container and wipe (1) for the screening visit
- Blood collection vacutainer tubes
    - 4 mL Sodium Fluoride (BD 367922)
    - 10 mL Serum Separator tubes (BD 367985)
    - 3 mL K_2 EDTA (BD 3678856)
    - 10 mL K_2 EDTA (BD 366643)

9.5 OVERVIEW OF SAMPLE COLLECTION

9.5.1 Safety Issues and Precautions for Handling Specimens

In accordance with the Occupational and Safety Health Administration (OSHA) regulations on blood borne pathogens, the LCBR recommends the following laboratory safety protocol for blood collection:

- Use of non-permeable lab coats, latex gloves, and face shields when handling any blood in any situation where splashes, spray, spatter, or droplets of blood may be generated, and eye, nose, or mouth contamination can be reasonably anticipated.
- Use of aerosol containers in all centrifuges.
- Following 'Universal Precautions' when handling any blood or urine products.
- Transferring contaminated needles and sharps immediately into a puncture-resistant, leak proof container.
- No recapping or breaking needles.
- The Hepatitis B vaccine should be offered to all unvaccinated technicians handling blood, and documentation of vaccination, or technician's refusal to be vaccinated, should be kept on file.

9.5.2 General Information

1. Research specimens are collected at all visits except the randomization visit.
2. **At the screening visit, no samples are sent to LCBR**. All blood and urine samples are analyzed at the site's local laboratory, so results are rapidly available to determine participant preliminary eligibility.
3. At baseline and during the study, laboratory tests required for the primary outcome (FPG, HbA1c and 2hPG), urine calcium and urine creatinine are measured by LCBR upon receipt and reported in real-time (via the EDC). Other laboratory tests (insulin, 25OHD etc.) will be performed at a different time and results will not be reported to the sites in real-time.
4. During the study, safety laboratory blood tests are analyzed at the site's local laboratory. Safety tests include: serum calcium and serum creatinine for estimated creatinine clearance (GFR, calculated centrally). Complete blood count, liver function tests and pregnancy tests are done as needed at the medical judgment of the site physician(s).
5. All sample collection is conducted in the morning after an 8-hour overnight fast.

9.5.3 Specimen Labeling

LCBR will provide clinical sites with uniquely identified visit specific Kits, containing, color-coded pre-labeled transfer tubes, and additional Kit ID barcode labels. All labels in a kit have the same 6-digit Kit ID on them (e.g. 700350), and the labels on the Sarstedt transfer tubes have an additional 3-digit tube identifier appended to the 6-digit Kit ID (e.g. 700350 907) that is used by the lab to identify visit (e.g., annual) type of specimen (e.g., SST) and time point (e.g., 120 min). The 9-digit barcode (Kit ID + tube ID; example: 700350 901) uniquely identifies the specimen in each transfer tube (see sample labels below).

⇒ **Each Kit ID is unique and is to be used <u>only</u> for a single participant at a single visit.**

⇒ *Please note that the Kit ID number is different from the participant D2d enrollment identification number (E-ID).*

Figure 1. Example of a 6-digit Kit ID label attached to the blood collection tube

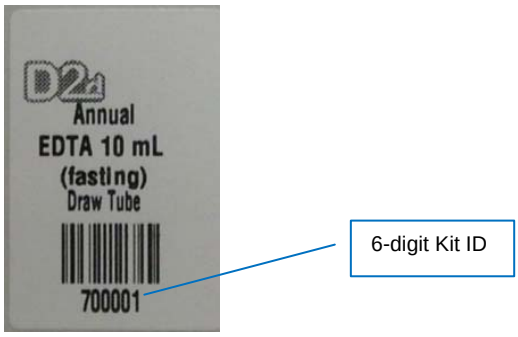


Figure 2. Example of D2d Central Laboratory Sarstedt transfer tube label

To ensure accurate labeling, it is highly recommended that study staff pre-label sets of collection tubes *before* the participant's visit (see picture on the right).

- ✓ For tubes that will be shipped to LCBR, please do not tape over the barcode labels unless labels are not adhering securely to the tubes. Tape may interfere with the scanning of the transfer tubes at the LCBR.
- ✓ At the beginning of each visit, the visit-specific 6-digit Kit ID label is placed on the source document, which also includes the participant enrollment ID.
- ✓ The Kit ID number on the barcode label is entered into the EDC on the Central Laboratory e-CRF for that visit.
- ⇒ *This Kit ID links the specimen to the participant and visit, and allows the LCBR to identify the specimen upon receipt and link the specimen to the appropriate participant and visit.* The participant ID does not go on any tubes; therefore, it is critically important that Kit ID labels are applied accurately and carefully, especially if more than one participant is seen on the same day.
- ⇒ Two Kit ID labels are saved and applied to the EDC-generated printout(s) of the completed Central Laboratory e-CRF. These printouts, which also show the participant enrollment ID, will be included in the refrigerated and frozen sample shipments to LCBR.

After all of a participant's specimens for a particular visit have been shipped to the LCBR, any extra labels from that visit should be discarded. This action will prevent labels from mistakenly being used for a different participant or visit.

9.5.4 Preparation for specimen collection

Tube racks should be prepared with all vacutainer blood collection tubes and pre-labeled color-coded Sarstedt transfer tubes. Collection tubes should be labeled with Kit ID and arranged in the appropriate working order (i.e. set up in a way that corresponds to the blood collection).

9.5.5 Source Documentation of Specimen Collection

D2d source documents are available in MOP Section 13. These forms should be used during the study visit to facilitate appropriate collection and efficient processing of the required specimens. These source documents also facilitate monitoring of phlebotomy and other quality assurance parameters.

The visit-specific source document, which includes specimen collection questions, should be printed from the MOP prior to each visit and completed during the visit. For each visit that requires specimens for the LCBR, the specimen barcoded Kit ID label will be placed on the source document.

In the EDC system, there are Local Laboratory and Central Laboratory e-CRFs that need to be completed for each visit when specimens are collected for the Local or LCBR Laboratory respectively. After the visit, the information recorded on the source document will be entered into the appropriate e-CRF. The (Local or Central) Laboratory e-CRF provides information critical to the interpretation of the assay results and maintenance of the sample repository.

9.6 ORAL GLUCOSE TOLERANCE TEST (OGTT)

The 75-gram oral glucose tolerance test (OGTT) is a key procedure for the D2d Study. Therefore, it is essential that the procedure be consistently followed at each site at every visit. Correct timing of blood draws is essential to the accurate interpretation of the OGTT. Utmost care should be taken in adhering to the blood draw times. *Actual times, not expected times must always be recorded.* Deviations to the procedure need to be avoided and if they occur, they must be documented, so the results can be interpreted.

Participants will have an OGTT done at the following visits:

- *Baseline (BAS) and annually (M12, M24, M36, M48).* Plasma for glucose and serum for insulin at 0, 30 minutes and 120 minutes after the 75-gram glucose beverage will be collected and sent to LCBR.
- *Confirmatory*, as needed – in between scheduled visits – to confirm the diagnosis of diabetes per protocol section 9.1. At confirmatory visits, only plasma for glucose at the 0 and 120 minute time points after the 75-gram glucose beverage will be collected and sent to LCBR.

9.6.1 Instructions for the OGTT

1. It is important to emphasize to the participant that she should not change her physical activity or diet during the 3 day period prior to the OGTT. The participant should also not reduce her intake of carbohydrates (e.g. potatoes, pasta). If a participant is on an extreme low-carbohydrate diet, she should resume carbohydrate intake during the 3 day period prior to the OGTT.

2. Reschedule the OGTT if the following have occurred:

   - Illness or major surgery within the past 7 days.
   - Concomitant condition present or medication taken in the past 2 weeks (such as use of steroids) that would interfere with the glucose results.
   - Worked night before test (it is okay participant regularly works the nightshift).
   - Fasted >18 hours or <8 hours (see bullet 4)
   - Alcohol consumed within 24 hours prior to the test.
   - Exercised vigorously within the past 8 hours.
   - Smoked within 1 hour of test.

3. Chill the Trutol® for at least a few hours prior to participant arrival to enhance palatability.

   => Note the flavor used for the screening visit and use the same flavor for all OGTT, unless the participant requests otherwise.

4. Participants must fast for a minimum of 8 hours prior to the first blood draw of the test at time 0 minutes. To confirm, participants need to be asked the following:

   "Did you eat or drink anything, other than plain water, after (*insert time*) last night? This includes coffee, tea and chewing gum"

   If the participant responds "yes," ask her "when was the last time you ate or drank anything other than plain water?"

   If the response is less than 8 hours, the start of the OGTT can be rescheduled for a different day or postponed for later that day if the participant is willing to stay in the center until 8 hours have elapsed since the last time she ate. For example, if the participant ate something less than 7 hours prior, the visit will be postponed for 1 hour. If the OGTT cannot be started before 11 am, it will be rescheduled for another day.

5. Instruct the participant that you will provide her with a cup of water (8 ounces) that she may drink during the OGTT and that she cannot have anything else to eat, drink, smoke or chew during the 2-hour test. Inform the participant that you will provide her with a snack after the last sample is drawn. Staff should be sensitive to the discomfort related to fasting and should take care to not have others eating or drinking in the participant's presence.

6. *The use of indwelling venous access (saline lock or butterfly needle) is strongly encouraged*. This will reduce the number of needle sticks the participant needs to undergo and promote comfort. ***Please note*** *after every blood draw, the catheter/needle should be flushed with normal saline*

solution, per your institutions policy, and prior to each specimen collection, blood should be drawn into a plain (red top) tube or syringe and discarded per your institutions policy. If the participant expresses a strong preference to have phlebotomy performed at each draw or if the site does not have personnel to insert indwelling venous access, multiple phlebotomy procedures may be performed.

=> The method used will be documented in the source document and must be applied consistently at each visit.

7. While participant is fasting, draw blood in the following tubes in **the order shown below** (note: if using indwelling venous access, draw blood for discard prior to drawing the study collection tubes).
   - 4 mL Sodium Fluoride tube for glucose (all visits requiring OGTT: BAS, M12, M24, M36, M48 and Confirmatory)
   - 10 mL Serum Separator tube for insulin (BAS, M12, M24, M36, M48) *
   - Additional tubes may be drawn for non-OGTT tests, depending upon the visit.
   - If using indwelling venous access, flush with normal saline
   - Record the actual time of the fasting blood draw on the source document.

8. As soon as possible after the fasting venipuncture is completed:
   - Provide the participant with the 75-gram Trutol® (10 ounce) Glucose Tolerance Test beverage.
   - Instruct the participant that he has 5 minutes to finish drinking the Trutol®. If the glucose drink is not consumed within 10 minutes the OGTT must be re-scheduled.
   - Record the time of the completion of the Trutol® beverage on the source document.
   - The time of completion of Trutol® beverage is considered time 0.

=> If the full contents of the glucose drink are not consumed, or if any is vomited, the OGTT needs to be rescheduled.

9. Immediately after the participant has finished the Trutol® beverage, set a timer (according to local practice) to facilitate appropriate timed collection of specimens.

10. Begin to process fasting (time 0 minutes) tubes, as described in section 9.8
    ⇒ *If this is a Confirmatory OGTT,* skip steps 9 and 10 and go to step 11.*

11. Exactly 30 minutes after the Trutol® beverage is finished, draw blood in the following tubes **in the order shown** (note: if using indwelling venous access, draw blood for discard prior to drawing the study collection tubes).
    - 4 mL Sodium Fluoride tube for glucose (BAS, M12, M24, M36, M48)
    - 10 mL Serum Separator tube for insulin (BAS, M12, M24, M36, M48)
    - If using indwelling venous access, flush with normal saline.
    - Record the actual time of the blood draw on the source document.

12. Begin to process the 30-minute tubes, as described in section 9.8.

13. Exactly 120 minutes after the Trutol® beverage is finished, draw blood in the following tubes in the order shown (note: if using indwelling venous access, draw blood for discard prior to drawing the study collection tubes):

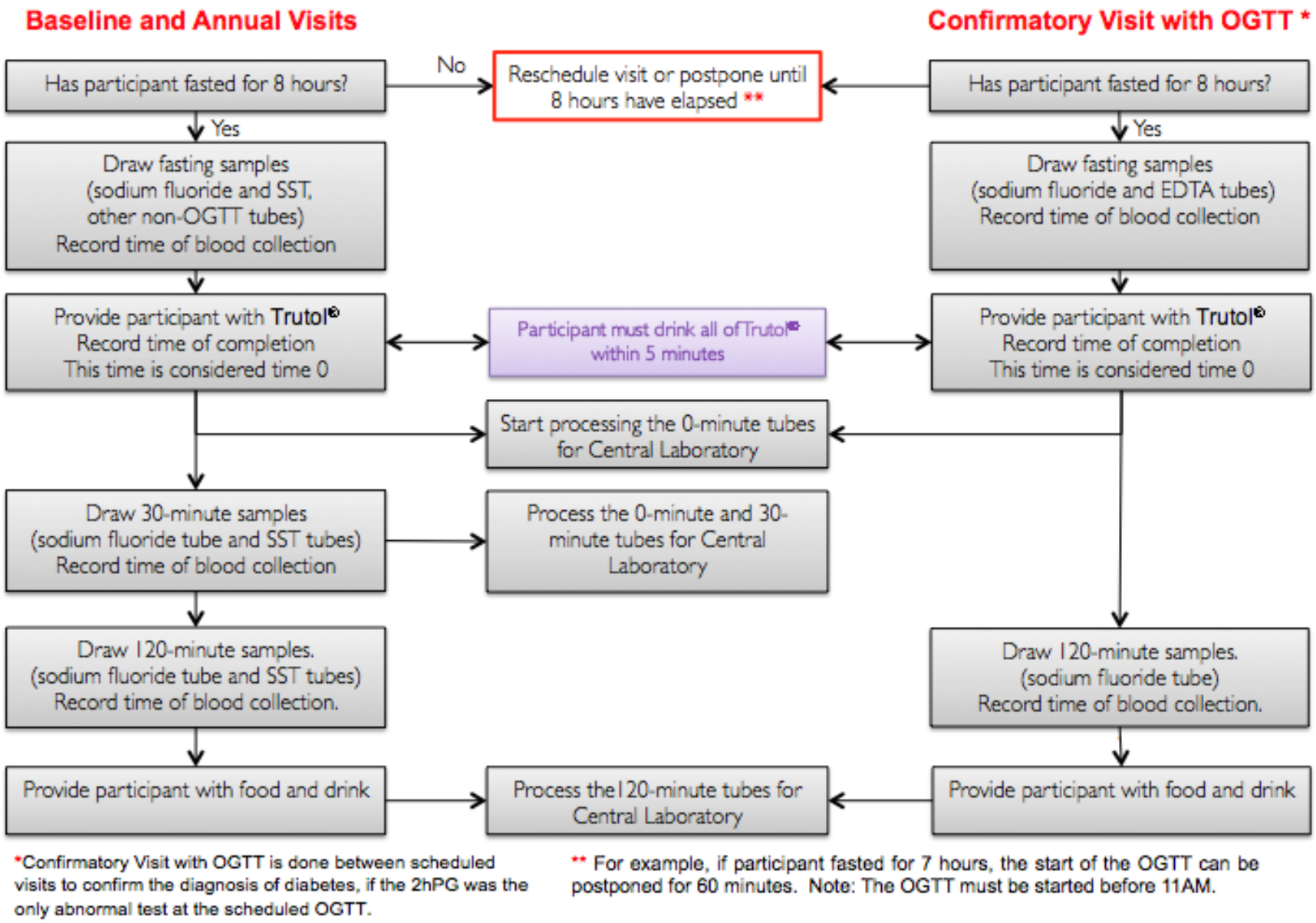
- 4 mL Sodium Fluoride tube for glucose (all visits requiring OGTT: BAS, M12, M24, M36, M48 and Confirmatory)
- 10 mL Serum Separator tube for insulin (BAS, M12, M24, M36, M48)*
- Record the actual time of the blood draw on the source document.

14. Provide the participant with food and drink.

15. Process the 120-minute tubes, as described in section 9.8.

* PLEASE NOTE: the 10 mL Serum Separator tube for insulin is *not required* for the Confirmatory OGTT.

Flow diagram for OGTT procedure



*Confirmatory Visit with OGTT is done between scheduled visits to confirm the diagnosis of diabetes, if the 2hPG was the only abnormal test at the scheduled OGTT.

** For example, if participant fasted for 7 hours, the start of the OGTT can be postponed for 60 minutes. Note: The OGTT must be started before 11AM.

9.7 STUDY VISIT SPECIMEN COLLECTION AND PROCESSING

9.7.1 Screening Visit Specimen Collection

Laboratory specimens collected at the screening visit will be analyzed at the site's local laboratory. The results of these tests will be used to determine participant's preliminary eligibility for the study. Therefore, it is essential for site personnel to be aware of the local laboratory's policies and procedures.

The following procedures and tests will be performed during the screening visit:

1. Collection of urine specimen, while fasting, for:
   a. Pregnancy test for women of reproductive potential. Testing will be point-of-care urine testing followed by confirmatory blood testing, if urine test is positive. If point-of-care urine testing is not an option, a urine (or serum) test will be done at the site's local laboratory.
2. Collection of blood, while fasting, for:
   a. Complete blood count (white blood cell count without differential, hemoglobin/hematocrit, platelet count).
   b. Liver function tests (AST, ALT).
   c. Serum calcium.
   d. Serum creatinine
   e. HbA1c.
   f. Plasma glucose.

9.7.1.1 Supplies Needed at Screening

At screening, all supplies are provided by the site.

- Urine collection container (1) and wipe, for women of reproductive potential
- Urine point-of-care pregnancy test (1), for women of reproductive potential
- Supplies as listed in section 9.4
- Vacutainer tubes *(sites may use smaller volume tubes, if they wish*)
  - 4 mL Sodium Fluoride tube (1), for plasma glucose.
    PLEASE NOTE: Sodium Fluoride tubes must be used for glucose tests.
  - 10 mL Serum Separator tube * (1) for liver function tests, calcium, creatinine
  - 3 mL EDTA tube (1) for complete blood count
  - 3 mL EDTA tube (1) for HbA1c
  - Ice bucket and ice

  **If required by the local laboratory, a green top tube maybe used in place of the serum separator tube for the liver function tests, calcium and creatinine analyzed at the local laboratory. The same tube type used at screening must be used for all subsequent local laboratory calcium and creatinine analyses.*

9.7.1.2 Procedures for Urine Specimen Collection at Screening

If a urine pregnancy test is required, the following should be done:

1. The urine sample should be collected as an early morning sample, preferably the first or second void of the day, before the fasting blood samples are collected.

2. Instruct the participant to:
    a. Wash hands before and after voiding.
    b. Cleanse area (vulva) with antiseptic wipes prior to voiding.
    c. Remove the cap from the urine collection container and void into the container.
    d. Carefully seal the cap of the urine container so that it is tight and leak proof.
    e. Notify the staff to collect the sample.
3. Perform the point of care urine pregnancy test per institution procedures, and record on the results on the source document.

9.7.1.3 Procedures for Blood Specimen Collection at Screening

1. Confirm that the participant has fasted for at least 8 hours.
2. While the participant is fasting, draw blood in the following tubes **in the order shown**.
    - Tube 1 (4 mL Sodium Fluoride): invert 8-10 times, then place in ice bath.
    - Tube 2 (10 mL SST): let sit upright at room temperature for 30 minutes.
    - Tubes 3 and 4 (3 mL EDTA): gently invert tube for ~30 seconds then refrigerate only if there is a delay in sending to the local laboratory.
    - Record the actual time of the blood draw on the source document.
3. Follow local procedures to send tubes to the local laboratory for analyses.

9.7.2 Baseline Visit Specimen Collection

Laboratory specimens collected at the baseline visit will be processed and shipped to LCBR. Therefore, careful attention to the processing and shipping instructions is essential.

The following procedures and tests will be performed during the baseline visit:

1. Collection of urine specimen, while fasting, for:
   - Urine calcium and urine creatinine measurement
   - Storage of urine (with and without acid preservative)
2. Collection of blood, while fasting, for:
   - Plasma glucose
   - Serum insulin
   - HbA1c
   - Plasma 25-hydroxyvitamin D
   - Storage of plasma and serum *
   - Whole blood for DNA **
3. 75-gram oral glucose tolerance test, followed by collection of blood for:
   - Plasma glucose and serum insulin, 30 minutes after the glucose load
   - Plasma glucose and serum insulin, 120 minutes after the glucose load

* Collected from participants who provided specimen repository informed consent for the storage of blood and urine.
** Collected from participants who provided specimen repository informed consent for the storage of DNA from blood.

9.7.2.1 Supplies Needed at Baseline

All supplies are provided by the site, except for the Laboratory Kit and the PaxGene DNA tube, which are provided by LCBR.

- Baseline-Annual visit Kit, provided by LCBR
- Chilled Thermo Scientific 75 gram Trutol® Glucose Tolerance Test beverage
- Supplies as listed in section 9.4
- Vacutainer tubes (sites will pre-label tubes with barcoded Kit ID labels provided by LCBR)
  - 4 mL Sodium Fluoride tubes (3) for plasma glucose at 0', 30' and 120'
  - 10 mL Serum Separator tubes (4) for insulin at 0,' 30' and 120' and for storage*
  - 3 mL EDTA tube (1) for HbA1c
  - 10 mL EDTA tube (2) for 25OHD and for storage *
  - 8.5 mL PaxGene DNA tube (1) ** (provided by LCBR)
  - Ice bucket and ice

* If participant declined specimen repository informed consent for the storage of blood and urine, only three SST tubes, and only one 10mL EDTA tube is needed.
** If participant declined specimen repository informed consent for the storage of DNA from blood, the PaxGene DNA tube is not collected.

Prior to the start of the visit, a tube rack should be prepared with all of the blood collection vacutainer tubes, and pre-labeled color-coded Sarstedt transfer tubes, arranged in the order that the specimens will be collected.

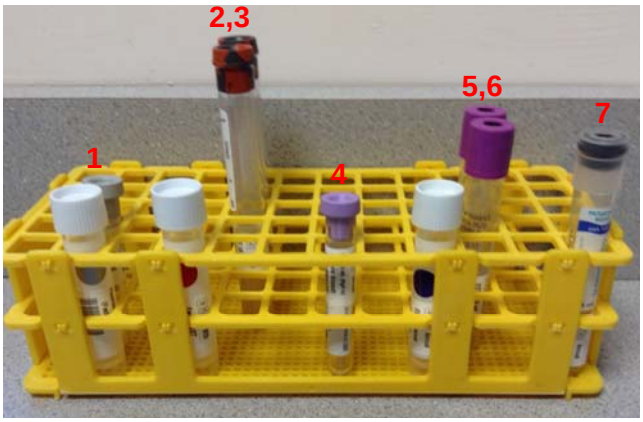
9.7.2.2 Procedures for Urine Specimen Collection at Baseline

The urine sample should be collected as an early morning sample, preferably the first or second void of the day, before the fasting blood samples are collected.

1. Provide participant with a urine collection container pre-labeled with the correct Kit ID label for the visit.
2. Instruct the participant to:
   a. Wash hands before and after voiding.
   b. Cleanse area (vulva or penis) with antiseptic wipes prior to voiding.
   c. Remove the cap from the urine collection container and void into the container.
   d. Carefully seal the cap of the urine container so that it is tight and leak proof.
   e. Notify the staff to collect the sample.
3. Record collection of the urine specimen and the approximate volume on the source document.
4. Promptly after collection (within 15 minutes) refrigerate (4°C) the urine collection cup, until ready to process (maximum of 60 minutes), per the instructions in section 9.9.

9.7.2.3 Procedures for Blood Specimen Collection at Baseline

1. Confirm that the participant has fasted for at least 8 hours and inform them that you will provide them with a cup of water to drink during the OGTT (see section 9.6.1, items 2 and 3)
2. While the participant is fasting, draw blood in the following tubes **in the order shown** (note: if using indwelling venous access, draw blood for discard prior to drawing the study collection tubes).
   - Tube 1 (4 mL Sodium Fluoride): invert 8-10 times, then put in ice bath.
   - Tubes 2 and 3 * (10 mL SST): let sit upright at room temperature for 30 minutes.
   - Tube 4 (3 mL EDTA): gently invert tube for ~30 seconds then refrigerate until ready to ship. *DO NOT CENTRIFUGE.*
   - Tubes 5 and 6 * (10 mL EDTA): gently invert tube for ~30 seconds then place in ice bath.
   - Tube 7 ** (8.5 mL PaxGene DNA **): Invert 3 times by hand, then refrigerate until ready to ship. *DO NOT CENTRIFUGE.*



   - If using indwelling venous access, flush with normal saline.

   * If participant declined specimen repository informed consent for the storage of blood and urine, do not collect tube 3 (2nd SST) and tube 6 (2nd EDTA)
   ** If participant declined specimen repository informed consent for the storage of DNA from blood, do not collect tube 7 (PaxGene DNA)

3. Record the actual time of the blood draw on the source document.
4. As soon as possible after the fasting venipuncture is completed:
   a. Provide the participant with the 75-gram Trutol® (10 ounce) Glucose Tolerance Test beverage.
   b. Instruct the participant that he has 5 minutes to finish drinking the Trutol®.
   c. Record the time of the completion of the Trutol® beverage on the source document.
   d. The time of completion of Trutol® beverage is considered time 0.
5. Immediately after the participant has finished the Trutol® beverage, set a timer (according to local practice) to facilitate on-time collection of the 30 minute and 120 minute specimens.
6. Begin to process fasting (time 0 minutes) tubes, as described in section 9.8.
7. Exactly 30 minutes after the Trutol® beverage is finished, draw blood in the following tubes in the order shown (note: if using indwelling venous access, draw blood for discard prior to drawing the study collection tubes):
   - 4 mL Sodium Fluoride tube; invert 8-10 times, then put in ice bath.
   - 10 mL Serum Separator tube; let sit upright at room temperature for 30 minutes.
   - If using indwelling venous access, flush with normal saline.
8. Record the actual time of the blood draw on the source document.
9. Begin to process the 30-minute tubes, as described in section 9.8.
10. Exactly 120 minutes after the Trutol® beverage is finished, draw blood in the following tubes in the order shown (note: if using indwelling venous access, draw blood for discard prior to drawing the study collection tubes):
    - 4 mL Sodium Fluoride tube; invert 8-10 times then put in ice bath.
    - 10 mL Serum Separator tube; let sit upright at room temperature for 30 minutes.
11. Record the actual time of the blood draw on the source document.
12. Provide the participant with food and drink.
13. Process the 120-minute tubes, as described in section 9.8

9.7.3 Month 3 (M03) Visit Specimen Collection

The urine specimen collected at the M03 visit will be processed and shipped to LCBR, and the blood specimen, indicated by ([L]), will be analyzed at the local laboratory.

The following procedures and tests will be performed during the M03 visit. Urine collection

1. Collection of urine specimen, while fasting, for:
   - Calcium and creatinine measurement
2. Collection of blood, while fasting, for:
   - Serum calcium [L]
   - Serum creatinine [L]

9.7.3.1 Supplies Needed at M03

All supplies are provided by the site, except for the Laboratory Kit, which is provided by LCBR.

- Month 3 visit Kit, provided by LCBR
- Supplies as listed in section 9.4
- Vacutainer tube (sites may use smaller volume tubes if they wish)
  - 10 mL Serum Separator tube (1) for serum calcium and creatinine [L]

9.7.3.2 Procedures for Urine Specimen Collection at M03

The urine sample should be collected as an early morning sample, preferably the first or second void of the day, before the fasting blood samples are collected.

1. Provide participant with a urine collection container pre-labeled with the correct Kit ID label for the visit.
2. Instruct the participant to:
   a. Wash hands before and after voiding.
   b. Cleanse area (vulva or penis) with antiseptic wipes prior to voiding.
   c. Remove the cap from the urine collection container and void into the container.
   d. Carefully seal the cap of the urine container so that it is tight and leak proof.
   e. Notify the staff to collect the sample.
3. Record collection of the urine specimen and the approximate volume on the source document.
4. Promptly after collection (within 15 minutes) refrigerate (4° C) the urine collection cup, until ready to process (maximum of 60 minutes), per the instructions in section 9.9.

9.7.3.3 Procedures for Blood Specimen Collection at M03

1. Confirm that the participant has fasted for at least 8 hours.
2. While the participant is fasting, draw the following tube:
   - 10 mL SST.
3. Record the actual time of the blood draw on the source document.
4. Follow local procedures to send tube to the local laboratory for analyses.
5. Provide the participant with food and drink.

9.7.4 Annual Visits (M12, M24, M36, M48) Specimen Collection

All laboratory specimens collected at these visits will be processed and shipped to LCBR, except those indicated by ([L]) which will be analyzed at the local laboratory.

The following procedures and tests will be performed during the M12, M24, M36, M48 visits.

1. Collection of urine specimen, while fasting, for:
   - Urine calcium and urine creatinine measurement
   - Storage of urine (with and without acid preservative)
2. Collection of blood, while fasting, for:
   - Plasma glucose
   - Serum calcium [L]
   - Serum creatinine [L]
   - Serum insulin
   - HbA1c
   - Plasma 25-hydroxyvitamin D
   - Storage of plasma and serum*
3. 75-gram oral glucose tolerance test, followed by collection of blood for:
   - Plasma glucose and serum insulin, 30 minutes after the glucose load
   - Plasma glucose and serum insulin, 120 minutes after the glucose load

* Collected from participants who provided specimen repository informed consent for the storage of blood and urine.

9.7.4.1 Supplies Needed at Annual Visits

All supplies are provided by the site, except for the Laboratory Kit, provided by LCBR.

- Baseline-Annual visit Kit, provided by LCBR
- Chilled Thermo Scientific 75 gram Trutol® Glucose Tolerance Test beverage. Unless the participant requests otherwise, use the same flavor that was used at the baseline visit.
- Supplies as listed in section 9.4
- Vacutainer tubes (pre-label with barcoded Kit ID labels)
  - 4 mL Sodium Fluoride tubes (3) for plasma glucose at 0', 30' and 120'
  - 10 mL Serum Separator tubes (5) for insulin at 0,' 30' and 120' for safety labs and for storage *
  - 3 mL EDTA tube (1) for HbA1c
  - 10 mL EDTA tube (2) for 25OHD and for storage*
  - Disposable pipettes (7)
  - Ice bucket and ice

* If participant declined specimen repository informed consent for the storage of blood and urine, only 4 SST tubes, and only 1 10mL EDTA tube is needed.

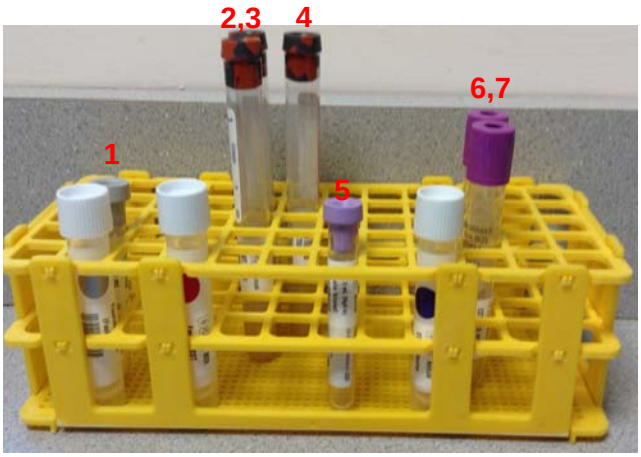
9.7.4.2 Procedures for Urine Specimen Collection at Annual Visits

The urine sample should be collected as an early morning sample, preferably the first or second void of the day, before the fasting blood samples are collected.

1. Provide participant with a urine collection container pre-labeled with the correct Kit ID label for the visit.
2. Instruct the participant to:
   a. Wash hands before and after voiding.
   b. Cleanse area (vulva or penis) with antiseptic wipes prior to voiding.
   c. Remove the cap from the urine collection container and void into the container.
   d. Carefully seal the cap of the urine container so that it is tight and leak proof.
   e. Notify the staff to collect the sample.
3. Record collection of the urine specimen and the approximate volume on the source document.
4. Promptly after collection (within 15 minutes) refrigerate (4° C) the urine collection cup, until ready to process (maximum of 60 minutes), per the instructions in section 9.9.

9.7.4.3 Procedures for Blood Specimen Collection at Annual Visits

1. Confirm that the participant has fasted for at least 8 hours and inform them that you will provide them with a cup of water to drink during the OGTT.
2. While the participant is fasting, draw blood in the following tubes **in the order shown** (note: if using indwelling venous access, draw blood for discard prior to drawing the study collection tubes).
   - Tube 1 (4 mL Sodium Fluoride): invert 8-10 times, then put in ice bath.
   - Tubes 2, and 3 * (10 mL SST): let sit upright at room temperature for 30 minutes.
   - Tube 4 [L] (10 mL SST) send to site's clinical laboratory for serum calcium and serum creatinine analyses
   - Tube 5 (3 mL EDTA): gently invert tube or place on mixer for ~30 seconds then refrigerate until ready to ship. *DO NOT CENTRIFUGE.*
   - Tubes 6 and 7 * (10 mL EDTA): gently invert tube or place on mixer for ~30 seconds, then place in ice bath.
   - If using indwelling venous access, flush with normal saline.



* If participant declined specimen repository informed consent for the storage of blood and urine, do not collect tube 3 (one of the 10 ml SST) and tube 7 (one of the 10 mL EDTA)

3. Record the actual time of the blood draw on the source document.
4. As soon as possible after the fasting venipuncture is completed:
   a. Provide the participant with the 75-gram Trutol® (10 ounce) Glucose Tolerance Test beverage.

b. Instruct the participant that he has 5 minutes to finish drinking the Trutol®.
c. Record the time of the completion of the Trutol® beverage on the source document.
d. The time of completion of Trutol® beverage is considered time 0.

5. Immediately after the participant has finished the Trutol® beverage, set a timer (according to local practice) to facilitate on-time collection of the 30 minute and 120 minute specimens.
6. Begin to process fasting (time 0 minutes) tubes, as described in section 9.8.
7. Exactly 30 minutes after the Trutol® beverage is finished, draw blood in the following tubes in the order shown (note: if using indwelling venous access, draw blood for discard prior to drawing the study collection tubes):
   - 4 mL Sodium Fluoride tube; invert 8-10 times, then put in ice bath.
   - 10 mL Serum Separator tube; let sit upright at room temperature for 30 minutes.
   - If using indwelling venous access, flush with normal saline.
8. Record the actual time of the blood draw on the source document.
9. Begin to process the 30-minute tubes, as described in section 9.8.
10. Exactly 120 minutes after the Trutol® beverage is finished, draw blood in the following tubes in the order shown (note: if using indwelling venous access, draw blood for discard prior to drawing the study collection tubes):
    - 4 mL Sodium Fluoride tube; invert 8-10 times then put in ice bath.
    - 10 mL Serum Separator tube; let sit upright at room temperature for 30 minutes.
11. Record the actual time of the blood draw on the source document.
12. Provide the participant with food and drink.
13. Process the 120-minute tubes, as described in section 9.8

9.7.5 Semi-Annual Visits (M6, M18, M30, M42) Specimen Collection

All laboratory specimens collected at these visits will be processed and shipped to LCBR.

The following procedures and tests will be performed during the M6, M18, M30 and M42 visits.

1. Collection of blood, while fasting, for:
   - Plasma glucose
   - HbA1c
   - Storage of plasma and serum (at **M6** visit only)

9.7.5.1 Supplies Needed at Semi-Annual Visits

All supplies are provided by the site, except for the Laboratory Kit, provided by LCBR.

- Month 6 visit Kit (for the M06 visit only) or Semi-annual visit Kit (for M18, M30, M42) provided by LCBR
- Supplies as listed in section 9.4
- Vacutainer tubes (sites will pre-label tubes with barcoded Kit ID labels, provided by LCBR)
  - 4 mL Sodium Fluoride tube (1) for plasma glucose
  - 10 mL Serum Separator tube (1) for storage at **M6** visit only *
  - 3 mL EDTA tube (1) for HbA1c
  - 10 mL EDTA tube (1) for storage at **M6** visit only *
  - Disposable transfer pipettes (3)
  - Ice bucket and ice

  * If participant declined to provide specimen repository informed consent for the storage of blood and urine, the SST tube and the 10 mL EDTA tube are not needed.

9.7.5.2 Procedures for Blood Specimen Collection at Semi-Annual Visits

1. Confirm that the participant has fasted for at least 8 hours.
2. While the participant is fasting, draw blood in the following tubes **in the order shown**.
   - 4 mL Sodium Fluoride: invert 8-10 times, then put in ice bath.
   - 10 mL SST * (**M06** only): let sit upright at room temperature for 30 minutes.
   - 3 mL EDTA: gently invert tube or place on mixer for ~30 seconds then refrigerate until ready to ship. *DO NOT CENTRIFUGE.*
   - 10 mL EDTA * (**M06** only): gently invert tube or place on mixer for ~30 seconds, then place in ice bath

   * If participant declined to provide specimen repository informed consent for the storage of blood and urine, do not collect the SST tube and the 10 mL EDTA tube.

3. Record the actual time of the blood draw on the source document.
4. Provide the participant with food and drink.
5. Process the tubes, as described in section 9.8.

9.7.6 End of Study Visit Specimen Collection

Laboratory specimens collected at these visits will be processed and shipped to LCBR, except those indicated by ([L]) which will be analyzed at the local laboratory.

The following procedures and tests will be performed during the end of study visit.

1. Collection of urine specimen, while fasting, for:
    a. Calcium and creatinine measurement
2. Collection of blood, while fasting, for:
    a. Plasma glucose
    b. Serum calcium [L]
    c. Serum creatinine [L]
    d. HbA1c

9.7.6.1 Supplies Needed at End of Study Visit

All supplies are provided by the site, except for the Laboratory Kit, provided by LCBR.

- End of Study visit Laboratory Kit, provided by LCBR
- Supplies as listed in section 9.3
- Vacutainer tubes (sites will pre-label tubes with barcoded Kit ID labels, supplied by LCBR)
    - 4 mL Sodium Fluoride tube (1) for plasma glucose
    - 10 mL Serum Separator tube (1) for serum calcium and serum creatinine[L]
    - 3 mL EDTA tube (1) for Hemoglobin
- Disposable transfer pipette (1)
- Ice bucket and ice

9.7.6.2 Procedures for Urine Specimen Collection at End of Study Visit

The urine sample should be collected as an early morning sample, preferably the first or second void of the day, before the fasting blood samples are collected.

1. Provide participant with a urine collection container pre-labeled with the correct Kit ID label for the visit. Instruct the participant to:
    - Wash hands before and after voiding.
    - Cleanse area (vulva or penis) with antiseptic wipes prior to voiding.
    - Remove the cap from the urine collection container and void into the container.
    - Carefully seal the cap of the urine container so that it is tight and leak proof.
    - Notify the staff to collect the sample.
2. Record collection of the urine specimen and the approximate volume on the source document.
3. Promptly after collection (within 15 minutes) refrigerate (4°C) the container with urine, until ready to process, per the instructions in section 9.9.

9.7.6.3 Procedures for Blood Specimen Collection at End of Study Visit

1. Confirm that the participant has fasted for at least 8 hours.
2. Draw blood in the following tubes **in the order shown**.

- 4 mL Sodium Fluoride: invert 8-10 times, then put in ice bath.
- 10 mL SST: Send to site's clinical laboratory for serum calcium and serum creatinine analyses
- 3 mL EDTA: Gently invert tube or place on mixer for ~30 seconds, then refrigerate until ready to ship. *DO NOT CENTRIFUGE.*

3. Record the actual time of the blood draw on the source document.
4. Provide the participant with food and drink.
5. Process the tubes, as described in section 9.8.

9.7.7 Confirmatory Visit Specimen Collection

If any one of the glycemic measures (FPG, HbA1c, 2hPG) meets criteria for diabetes during a scheduled visit, including the end-of-study visit, a confirmatory visit will be completed to repeat the measures.

During the confirmatory visit, one of the following will be required (see protocol section 9.1 to determine which is needed).

1. FPG only
2. HbA1c only
3. FPG, HbA1c and 2hPG (75-gram oral glucose tolerance test)

All laboratory specimens collected at these visits will be processed and shipped to LCBR.

9.7.7.1 Confirmatory Visit – Repeat FPG only

9.7.7.1.1 Supplies Needed for Confirmatory Visit – Repeat FPG

- Unscheduled-Confirmatory visit Kit, provided by LCBR
- Supplies as listed in section 9.4
- Vacutainer tube: (sites will pre-label tube with barcoded Kit ID label, provided by LCBR)
  - 4 mL Sodium Fluoride tube (1) for plasma glucose
- Disposable transfer pipettes (1)
- Ice bucket and ice

9.7.7.1.2 Procedures for Confirmatory Visit – Repeat FPG

1. Confirm that the participant has fasted for at least 8 hours.
2. While the participant is fasting, draw blood in the following tube.
   - 4 mL Sodium Fluoride: invert 8-10 times, then put in ice bath.
3. Record the actual time of the blood draw on the source document.
4. Provide the participant with food and drink.
5. Process the tube, as described in section 9.8.

9.7.7.2 Confirmatory Visit – Repeat HbA1c

9.7.7.2.1 Supplies Needed Confirmatory Visit – Repeat HbA1c

- Unscheduled-Confirmatory visit Kit, provided by LCBR
- Supplies as listed in section 9.4
- Vacutainer tube: (sites will pre-label tube with barcoded Kit ID label, provided by LCBR)
  - 3 ml EDTA tube (1) for HbA1c

9.7.7.2.2 Procedures for Confirmatory Visit – Repeat HbA1c

1. Confirm that the participant has fasted for at least 8 hours.
2. While the participant is fasting, draw blood in the following tube.

- 3 mL EDTA tube: gently invert tube or place on mixer for ~30 seconds, then refrigerate until ready to ship. *DO NOT CENTRIFUGE.*

3. Record the actual time of the blood draw on the source document.
4. Provide the participant with food and drink.
5. Process the tube, as described in section 9.8.

9.7.7.3 Confirmatory Visit – Repeat OGTT (FPG, HbA1c, and 2hPG)

9.7.7.3.1 Supplies Needed for Confirmatory Visit – Repeat OGTT

- Unscheduled-Confirmatory visit Kit, provided by LCBR
- Chilled Thermo Scientific 75 gram Trutol® Glucose Tolerance Test beverage. Unless the participant requests otherwise, use the same flavor that was used for the baseline visit.
- Supplies as listed in section 9.4
- Vacutainer tubes: (sites will pre-label tube with barcoded Kit ID label, provided by LCBR)
  - 4 mL Sodium Fluoride tubes (2) for plasma glucose at 0 and 120'
  - 3 mL EDTA tube (1) for HbA1c
  - Disposable transfer pipettes (2)
  - Ice bucket and ice

9.7.7.3.2 Procedures for Confirmatory Visit – Repeat OGTT

1. Confirm that the participant has fasted for at least 8 hours and inform them that you will provide them with a cup of water to drink during the OGTT.
2. While the participant is fasting, draw blood in the following tubes **in the order shown** (note: if using indwelling venous access, draw blood for discard prior to drawing the study collection tubes).
   - Tube 1 (4 mL Sodium Fluoride): invert 8-10 times, then put in ice bath.
   - Tube 2 (3 mL EDTA): Gently invert tube or place on mixer for ~30 seconds then refrigerate until ready to ship. *DO NOT CENTRIFUGE.*
3. Record the actual time of the blood draw on the source document.
4. As soon as possible after the fasting venipuncture is completed:
   - Provide the participant with the 75-gram Trutol® (10 ounce) Glucose Tolerance Test beverage.
   - Instruct the participant that he has 5 minutes to finish drinking the Trutol®.
   - Record the time of the completion of the Trutol® beverage on the source document.
   - The time of completion of Trutol® beverage is considered time 0.
5. Immediately after the participant has finished the Trutol® beverage, set a timer to facilitate on-time collection of the 120 minute specimen.
6. Begin to process fasting (time 0 minutes) tubes, as described in section 9.8
7. Exactly 120 minutes after the Trutol® beverage is finished, draw blood in the following tubes in the order shown:
   - 4 mL Sodium Fluoride tube; invert 8-10 times then put in ice bath.
8. Record the actual time of the blood draw on the source document.
9. Provide the participant with food and drink.
10. Process the 120-minute tubes, as described in section 9.8.

9.7.8 Unscheduled Visit Specimen Collection

Collection of laboratory samples at an unscheduled visit will occur for any of the following reasons:

1. Serum calcium or creatinine (for creatinine clearance) measured at a scheduled visit was elevated. Per DSMP, an unscheduled visit will occur to repeat the abnormal test.
2. Urine calcium creatinine ratio measured at a scheduled visit was elevated. Per DSMP, an unscheduled visit will occur to repeat the calcium-creatinine ratio.
3. Participant reports outside of study diagnosis of diabetes or the prescribing of a diabetes-specific medication and participant has not started diabetes specific medication. Per protocol, an unscheduled visit will occur to measure FPG and HbA1c.

9.7.8.1 Unscheduled Visit – Repeat Serum Calcium or Creatinine

9.7.8.1.1 Supplies Needed for Unscheduled Visit – Repeat Serum Calcium or Creatinine

- No special Kit is provided as laboratory tests are sent to the local laboratory.
- Supplies as listed in section 9.4
- Vacutainer tube *(sites may use smaller volume tubes, if they wish*)
  - 10 mL Serum Separator tube * (1)

  * *If required by the local laboratory, a green top tube maybe used in place of the serum separator tube. The same tube type used at screening must be used for all subsequent local laboratory calcium and creatinine analyses.*

9.7.8.1.2 Procedures for Blood Specimen Collection for Serum Calcium or Creatinine

1. No fasting is required
2. Draw blood in the following tube:
   - 10 mL SST: let sit upright at room temperature for 30 minutes
3. Record the actual time of the blood draw on the source document.
4. Follow local procedures to send tube to the local laboratory for analyses.

9.7.8.2 Unscheduled Visit – Repeat Urine Calcium Creatinine Ratio

9.7.8.2.1 Supplies Needed for Unscheduled Visit – Repeat Urine Calcium Creatinine Ratio

- Unscheduled-Confirmatory visit Kit, provided by LCBR
- Supplies as listed in section 9.4

9.7.8.2.2 Procedures for Urine Specimen Collection

Please refer to section 9.7.2.2 for urine specimen collection instructions.

9.7.8.3 Unscheduled Visit – Repeat FPG and HbA1c

9.7.8.3.1 Supplies Needed for Unscheduled Visit – Repeat FPG and HbA1c

- Unscheduled-Confirmatory visit Kit, provided by LCBR
- Supplies as listed in section 9.4

- Vacutainer tubes: (sites will pre-label tube with barcoded Kit ID label, provided by LCBR)
  - 4 mL Sodium Fluoride tube (1) for plasma glucose
  - 3 ml EDTA tube (1) for HbA1c
- Disposable transfer pipettes (1)
- Ice bucket and ice

9.7.8.3.2 Procedures for Blood Specimen Collection for FPG and HbA1c

1. Confirm that the participant has fasted for at least 8 hours.
2. While the participant is fasting, draw blood in the following tubes **in the order shown**.
   - 4 mL Sodium Fluoride: invert 8-10 times, then put in ice bath.
   - 3 mL EDTA: gently invert tube or place on mixer for ~30 seconds then refrigerate until ready to ship. *DO NOT CENTRIFUGE.*
3. Record the actual time of the blood draw on the source document.
4. Provide the participant with food and drink.
5. Process the tubes, as described in section 9.8

9.8 BLOOD SPECIMEN PROCESSING AND STORAGE

Processing should be initiated as soon as possible after specimens are obtained, always within 45 minutes following venipuncture. Personal protective equipment, as described in section 9.4 is required for blood processing.

9.8.1 Supplies Need

The following items should be on hand at the clinical site before beginning processing of biospecimens:

- Refrigerated centrifuge (4°C) capable of spinning at a minimum of 2000g
- 10% bleach solution (or approved biohazard disinfectant)
- Freezer (-70°C or colder)
- Refrigerator (4°C) – not being used for human food storage
- Emergency eye wash station
- Biohazard trash can, with large and small biohazard bags (biohazardous waste puncture- proof containers)
- Tube racks
- Sharpie pens
- The appropriate D2d Laboratory Kit for the visit *
- Revco sample cryoboxes 5x5x3″ and dividers (7 x 7 grids) *

* Provided by the LCBR

9.8.2 Centrifugation of Blood Collection Tubes

The following three collection tubes require centrifuging and subsequent processing:

- **4 mL Sodium Fluoride**. Invert 8-10 times, then place in ice bath or refrigerate (4°C) until centrifuged, preferably for less than 15 minutes but not more than 45 minutes.
- **10 mL Serum Separator Tube (SST).** Let sit upright at room temperature for 30 minutes but not more than 90 minutes to allow the serum to clot before centrifugation.
- **10 mL EDTA tube**. Place on mixer for ~30 seconds or gently invert tube for ~30 seconds, then place in ice bath or refrigerate (4°C) until centrifuged, preferably for less than 15 minutes but not more than 45 minutes.

Prior to centrifuging samples, ensure the centrifuge is balanced. The Sodium Fluoride, SST and 10 mL EDTA tubes are centrifuged using a refrigerated centrifuge at a minimum of 2,000g for 15 minutes, or 3,000g for 10 minutes (for a total of 30,000 g-minutes) Once centrifugation is complete, tubes are carefully placed on ice and are ready to aliquot. The time of centrifuging is recorded in the source documents.

⇒ **Please note:** The 3mL EDTA tube (used for HbA1c) and the PaxGene tube are **not centrifuged** and should be stored in the refrigerator until ready to package for shipment. They must not be

frozen. These tubes are shown below after blood collection (left picture) and after being placed in conical tubes for shipment (right picture).

9.8.3 Aliquoting of Serum and Plasma

After centrifugation, plasma or serum will be transferred via a disposable transfer pipette into the pre-labeled 10 mL Sarstedt color-coded transfer tubes, provided by LCBR.

1. Ensure each Sarstedt 10mL transfer tube is pre-labeled with the participant's correct Kit ID barcode label.
2. It is of utmost importance that the **correct sample type and time point** are aliquoted into the **correct Sarstedt transfer tube.** The Sarstedt transfer tube labels clearly indicate the sample type and sample time point. In addition, the tubes are color-coded (see below) to minimize errors during aliquoting.
3. Aliquot while the blood collection tubes and Sarstedt color-coded transfer tubes are on ice (unless otherwise noted).
4. **Ensure that a new disposable transfer pipette is used for each collection tube type at each time point.**
5. When aliquoting serum and plasma from the centrifuged blood collection tube, be careful not to disturb the top of the next cell layer with the pipette tip, as this will result in platelet, white cell and red cell contamination.
6. If a blood collection tube is accidentally mixed during pipetting such that plasma is contaminated with red cells, the collection tube may be re-centrifuged.

Once the samples are aliquoted, the filled transfer tubes should be immediately frozen (within 15 minutes) in an upright position at -70^{o}C. Filled transfer tubes can be placed in a sample cryobox, provided by LCBR (arranged as shown in the photo in section 9.10), and stored in the -70^{o}C freezer until time to ship to LCBR. One of the Kit ID barcode labels assigned to that participant's visit should be placed on the lid of the sample box to indicate sample sets in the box.

After samples have been aliquoted and placed in the freezer, the 10 mL EDTA, SST and Fluoride blood collection tubes can be discarded in the biohazard waste (puncture-proof containers).

See next pages for a detailed aliquoting plan for the 4 mL Sodium Fluoride, 10 mL SST and 10 mL EDTA blood collection tubes.

9.8.3.1 Aliquoting Plan for 4 mL Sodium Fluoride Tube

After centrifugation, plasma (approximately 2 mL) will be transferred via a disposable transfer pipette from the 4 mL Sodium Fluoide blood collection tube into the appropriate 10 mL pre-labeled Sarstedt color-code transfer tubes, as follows. Use a new transfer pipette for each time-point.

Aliquoting Plan – 4 mL Sodium Fluoride Tube					
Visit	**Time point**	**Transfer tube label**	**Transfer tube 3-digit ID**	**Transfer tube color code**	**Transfer tube volume**
Baseline, M12, M24, M36, M48	0 min (fasting)	Fluoride plasma (fasting) Transfer tube.	901	silver	2 mL
	30 min OGTT	Fluoride plasma (30 min OGTT) Transfer tube.	904	black	2 mL
	120 min OGTT	Fluoride plasma (120 min OGTT) Transfer tube.	906	white	2 mL
M06	0 min (fasting)	Fluoride plasma (fasting) Transfer tube.	913	silver	2 mL
M18, M30, M42,	0 min (fasting)	Fluoride plasma (fasting) Transfer tube.	917	silver	2 mL
UNCO	0 min (fasting)	Fluoride plasma (fasting) Transfer tube.	919	silver	2 mL
	120 min OGTT	Fluoride plasma (120 min OGTT) Transfer tube.	920	white	2 mL
EOS	0 min (fasting)	Fluoride plasma (fasting) Transfer tube.	922	silver	2 mL

9.8.3.2 Aliquoting Plan for 10 mL Serum Separator Tube (SST)

After centrifugation, serum (approximately 4 mL) will be transferred via a disposable transfer pipette from the 10 mL SST tube into the appropriate pre-labeled 10 mL Sarstedt color-coded transfer tube, as follows. Use a new transfer pipette for each time-point.

Aliquoting Plan – 10 mL Serum Separator Tubes (SST)					
Visit	**Time point**	**Transfer tube label**	**Transfer tube 3-digit ID**	**Transfer tube color code**	**Transfer tube volume**
Baseline, M12, M24, M36, M48.	0 min (fasting)	SST (fasting) Transfer tube.	902	red	~4-9 mL *
	30 min OGTT	SST (30 min OGTT) Transfer tube.	905	pink	5 mL
	120 min OGTT	SST (120 min OGTT) Transfer tube.	907	orange	5 mL
M06	0 min (fasting)	SST (fasting) Transfer tube.	914	red	5 mL

***** If a participant provided specimen repository informed consent, at the baseline and annual blood draws, two SST tubes are collected at the fasting time-point (time 0-minutes). Serum from both SST tubes will be aliquoted and pooled into a single transfer tube with a red top.

9.8.3.3 Aliquoting Plan for 10 mL EDTA Tube

After centrifugation, plasma (approximately 4 mL) will be transferred via a disposable transfer pipette from the 10 mL EDTA blood collection tube into the appropriate pre-labeled 10 mL Sarstedt color-coded transfer tube, as follows.

Aliquoting Plan – 10 mL EDTA Tubes					
Visit	**Time point**	**Transfer tube label**	**Transfer tube 3-digit ID**	**Transfer tube color code**	**Transfer tube volume**
Baseline, M12, M24, M36, M48.	0 min (fasting)	EDTA plasma (fasting) Transfer tube.	903	violet	~4-9 mL*
M6	0 min (fasting)	EDTA plasma (fasting) Transfer tube.	915	violet	5 mL

* If the participant provided specimen repository informed consent, at the baseline and annual blood draws, two 10-mL EDTA tubes are collected at the fasting time-point (time 0-minutes). Plasma from both EDTA tubes will be aliquoted and pooled into a single pre-labeled transfer tube with a violet top.

9.8.4 Special Circumstances & Troubleshooting

Low sample volume

Any partially-filled transfer tube (less than the specified volume) should be marked with a sharpie pen, **"P"** on the label to indicate so. On the source document and on the Central Laboratory e-CRF, indicate which transfer tube has a low sample volume (e.g. plasma from fasting EDTA tube) and the reason for the low volume.

Blood specimens (Sodium Fluoride, EDTA) cannot be centrifuged and processed within 45 minutes of collection

If centrifugation cannot be performed within 45 minutes of collection, maintain the Sodium Fluoride and EDTA tubes on ice until centrifugation and try to process specimens as soon as possible after that time. **Note time of collection and centrifugation** on the source document and on the Central Laboratory e-CRF.

Serum and plasma cannot be frozen within 15 minutes of aliquoting

If specimens cannot be placed immediately at -70°C, they may be temporarily (less than 2 hours)* stored on dry ice until transferred to -70°C. *Placing the specimens at -20°C should be avoided (due to the self-defrost cycling common in most -20°C freezers).* If unable to place samples at -70°C immediately, make note of when the specimens were placed at -70°C and the reason why it was delayed in the source document and on the Central Laboratory e-CRF.

Failure of -70°C freezer failure and back-up freezer space not readily available.

Samples may be stored on dry ice provided there is sufficient dry ice to keep samples thoroughly frozen. Do not allow the transfer tubes to be in direct contact with the dry ice for a prolonged period of time. The tubes need to be either in a rack with lab mat protecting them from direct contact with the dry ice, or in their sample boxes.

9.9 URINE SPECIMEN PROCESSING AND STORAGE

9.9.1 Supplies Need

The following items should be on hand at the clinical site before beginning processing of biospecimens:

- 10% bleach solution (or approved biohazard disinfectant)
- Freezer (-70°C or colder)
- Emergency eye wash station
- Biohazard trash can, with large and small biohazard bags (biohazardous waste puncture- proof containers)
- Tube racks
- Sharpie pens
- The appropriate D2d Laboratory Kit for the visit *
- Revco sample boxes 5x5x3″ and dividers (7 x 7 grids) *

* Provided by the LCBR

9.9.2 Aliquoting of Urine

The urine collection container should be marked with the participant's assigned Kit ID for that visit (or other identification to clearly match the sample to the correct Kit ID) and stored in the refrigerator until ready to aliquot the sample. The urine sample must be aliquoted within 60 minutes of collection.

1. Ensure each Sarstedt 10 mL transfer tube is pre-labeled with the participant's correct Kit ID barcode label.
2. Mix urine by swirling the closed container. Pipette 9 mL of urine from the urine collection container into the 10 mL transfer tube(s) as shown in the table below; place transfer tube on ice.
3. **DO NOT OVERFILL the urine aliquots**. There needs to be adequate space for the urine to expand when frozen without displacing the transfer tube cap.
4. Freeze the filled transfer tubes at -70°C.
5. Discard remaining urine.

9.9.2.1 Aliquoting Plan for Urine

Aliquoting Plan – Urine					
Visit	Sample	Transfer tube label	Transfer tube 3-digit ID	Transfer tube Color code	Transfer tube volume
M03	Urine	Urine (plain)	912	Yellow	9 mL
Baseline, M12,M24, M36,M48	Urine	Urine (plain)	908	Yellow	9 mL
	Urine *	Urine (with Acid)	909	Green	9 mL
UNCO	Urine	Urine (plain)	925	Yellow	9 mL
EOS	Urine	Urine (plain)	923	Yellow	9 mL

* If the participant provided specimen repository informed consent, at the baseline and annual visits draws, pipette 9 mL of urine from the urine container into the 10 mL transfer tube labeled “Urine (with Acid),” which is pre-loaded with boric acid, and color coded green. If there is less than 9 mL of urine available to add to the transfer tube containing the boric acid powder, skip making the aliquot.

9.10 PROCESSING COMPLETION AND SPECIMEN STORAGE

Sarstedt 10 mL transfer tubes (with plasma, serum or urine) are placed into 5x5x3" sample cryoboxes, with 7x7 grids (to prevent shifting) for freezer storage and shipping. Please note the following and see figure below:

- Each sample cryobox can hold tubes from multiple participant-visits.
- Sample boxes should be divided into two types:
  - *Baseline-Annual visit box*: Transfer tubes from multiple participants' baseline or annual visits will be shipped together in the same sample box.
  - *Other visits*: Transfer tubes from all other visits (M03, M06, M18, M30, M42, UNCO, and EOS) will be shipped together in the same sample box.
- In the Baseline-Annual visit box, each column should have tubes from <u>just one</u> participant (either serum, plasma or urine). There should be some empty spaces between tubes from different participants, as illustrated in the photo below.
- Please leave a row (or column) along one edge of the box empty as this allows a better fit when placing the lid on the box. Be sure to rubber band the box lid closed!
- For each participant whose samples are in the box, affix a corresponding Kit ID barcode label to the cover of the box.

This box has samples from 3 participants and one row is empty

This box cover has a Kit ID bar code label for each of the 3 participant-visits in the box.

9.11 SHIPPING BLOOD AND URINE SAMPLES

9.11.1 Timing of Shipping

⇒ **Samples are shipped to LCBR on Mondays, Tuesdays, or Wednesdays ONLY**.
⇒ **Refrigerated samples (3 ml EDTA and PaxGene tubes) collected on Wednesdays should always be shipped on Wednesday**.
⇒ **Refrigerated samples (3 ml EDTA and PaxGene tubes) collected on Thursdays and Fridays must be shipped on the next Monday.**
⇒ Please do not schedule participant visits on a Thursday or Friday if the next Monday is a holiday.

Multiple participant samples can be batched shipped together to reduce shipping costs.

Shipping schedule – Refrigerated EDTA 3 mL for HgA1c and Paxgene DNA Tubes

Day blood collected	Day to ship EDTA and Paxgene tubes
Monday	Monday
Tuesday	Wednesday
Wednesday	Wednesday
Thursday	Next Monday
Friday	Next Monday

Frozen samples are shipped to LCBR once a week. The sites and the LCBR will establish a frozen sample shipping schedule. Each site will be assigned a set day of the week to ship frozen specimens.

9.11.2 Supplies Needed for Shipment of Samples to LCBR

Sample Shipping Checklist:

- Sample boxes *
- Frozen and Refrigerated Specimen Shippers (Styrofoam Mailing Containers with outer cardboard sleeves) *
- Rubber bands
- Specimen storage/shipping bags*
- Absorbent material (e.g., special absorbent material strips * and lab mat)
- Packaging tape
- Dry ice (~10 lbs per mailing container) for frozen shipments
- Frozen ice packs (2 per container) for refrigerated shipments *
- 50 mL conical tubes *
- Labels: FedEx address labels *
- Biological substances category B UN3373 labels *
- Dry Ice Labels (class 9, UN1845) for frozen shipments *
- Pre-labeled transfer tubes packed in the sample boxes with the Kit ID labels on the top

- Completed Central Laboratory e-CRFs printed from EDC for all samples to be shipped

* Provided by the LCBR

9.11.3 Frozen Shipments to LCBR

The following shipping instructions comply with the International Air Transport Association's Dangerous Goods Regulations-Packaging Instructions 650 and 904.

1. Line Styrofoam mailer(s) with absorbent material (e.g. lab mat).
2. Place approximately 5 lbs. of the dry ice on the bottom of the mailer.

3. Place another layer of absorbent material (e.g. lab mat) as shown below on top of the dry ice so it will be in between the dry ice and the sample boxes containing the samples.
4. Collect the sample boxes containing samples to be shipped and check the Kit ID numbers against the Central Laboratory e-CRF for that shipment.
5. If not previously done, place a rubber band around each sample box, and then place each box in the provided specimen storage bag, with an absorbent material strip. Ensure the specimen bag is securely closed. The rubber band is important for aiding in the prevention of a transfer tube spill; the specimen storage bag and absorbent strip are for compliance with IATA regulations.
6. Carefully place the bagged boxes in the mailer.

7. Another layer of absorbent material (e.g. lab mat) is placed on top of the sample boxes.
8. The remaining dry ice is placed on top of the last layer of absorbent material (e.g. lab mat) before placing the lid on the Styrofoam mailer.

9. Printouts of the Central Laboratory e-CRFs for all samples included in the Styrofoam mailer are placed together in one Ziploc bag then placed on top of the Styrofoam lid before the outer sleeve is securely taped closed.
10. Affix shipping labels (Fed Ex label, Biological Substances Category B UN3373 label, and Dry Ice Class 9 UN1845 label) to the outside of shipping container.

11. Enter the Fed Ex tracking number of the shipment into the Central Laboratory e-CRF for each sample in the box. Entering this information will generate an e-mail notification to the LCBR that the specimens have been shipped.

9.11.4 Refrigerated Shipments to LCBR

⇒ Refrigerated samples (<u>3 mL EDTA for HbA1c and PaxGene DNA tubes</u>) must arrive at the LCBR within 5 days of being drawn.

1. Line Styrofoam mailer(s) with absorbent material (e.g. lab mat).
2. Place two ice packs on the bottom of the mailer.
3. Remove the EDTA 3 mL and PaxGene tubes (if present) from their refrigerated storage locations. Check the Kit ID numbers against the Central Laboratory e-CRFs for that shipment.
4. Place each tube in separate 50 mL conical tubes to serve as a secondary container during shipping.
5. Place a thick rubber band around the tubes and seal in a provided specimen bag along with the provided absorbent strips. Ensure the specimen bag is securely sealed. The rubber band aids in the prevention of a transfer tube spill; the specimen bag (and absorbent strip) are for compliance with commercial carrier specifications.

6. Another layer of absorbent material (e.g. lab mat) is placed on top of the specimen bags before placing the lid on the Styrofoam mailer. Ensure the lab mat sufficiently protects the bagged samples from direct contact with the frozen cold packs to avoid samples accidentally freezing in transit.
7. The printouts of the Central Laboratory e-CRF for all samples are placed together in one Ziploc bag and placed on the top of the Styrofoam lid before the outer sleeve is securely taped closed.
8. Affix shipping labels (Fed Ex label, Biological Substance Category B UN3373 label) to outside of shipping container.

9. Place the entire box in the refrigerator if pickup is not immediate.
10. Enter the Fed Ex tracking number for the shipment into the Central Laboratory e-CRF for each sample in the box. Entering this information will generate an e-mail notification to the LCBR that the specimens have been shipped.

9.11.5 Mailing Address for Shipping Frozen and Refrigerated Specimens to LCBR

University of Vermont-Pathology
Laboratory for Clinical Biochemistry Research
Colchester Research Facility
Attention: D2d Study – Room 128
208 South Park Drive,
Colchester, VT 05446

(802) 656-8963 Phone
(802) 656-8965 Fax

9.12 APPENDICES

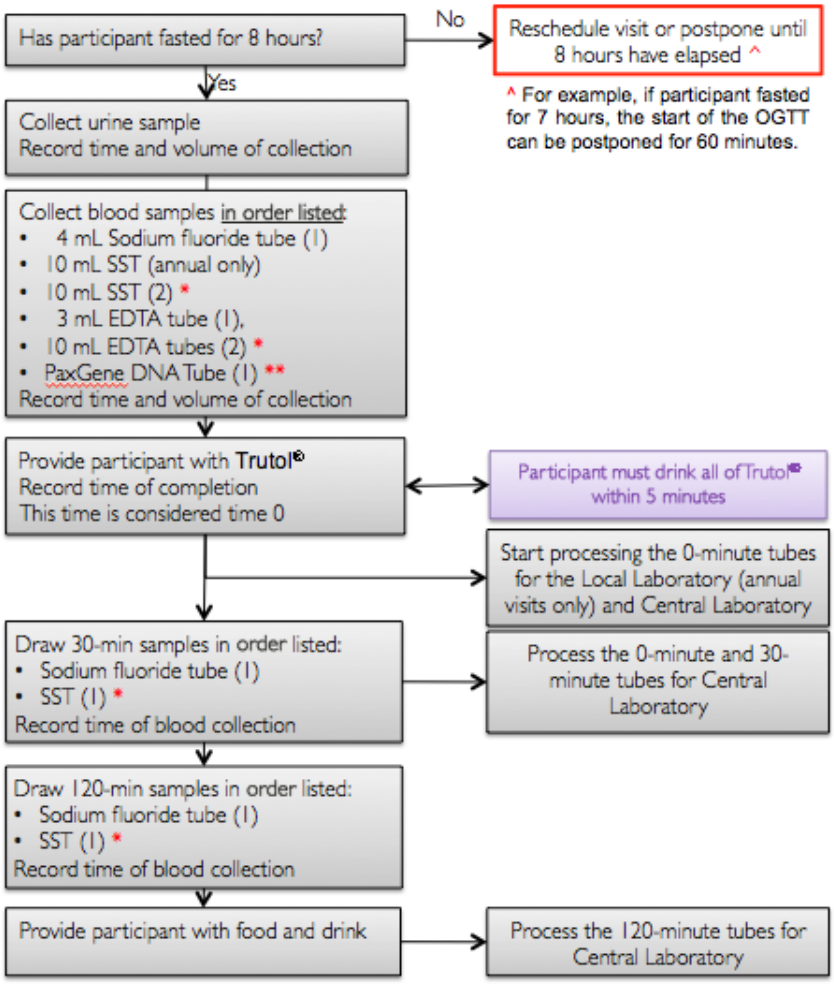
Appendix 1 D2d Site Blood Specimen Processing

D2d Site Blood Specimen Processing						
Collection Tube	Test	Mix vs. Clot	Temporary Storage	Refrigerated Centrifuge	10 mL Transfer Tubes	Storage and Shipment
4mL Sodium Fluoride	Glucose (0 min, 30 min, 120 min)	Invert 8-10 times	Ice bath or refrigerate (4°C) until centrifuged (Preferably <15 min but not more than 45 min)	At 2,000 x g for 15 min. or 3,000 x g for 10 min. P	Transfer plasma as follows: Time 0-min.→ silver color coded tube Time 30-min. → black color coded tube Time 120-min. → white color coded tube	Freeze upright at -70° Ship weekly (M-W) to LCBR
10 mL SST	Insulin (0 min, 30 min, 120 min) and Repository (0 min)	Sit upright at room temp., at least 30 min to clot, max 90 min	Sit upright at room temperature, at least 30 min to clot, max 90 min	At 2,000 x g for 15 min. or 3,000 x g for 10 min.	Transfer serum as follows: Time 0-min.→ red color coded tube Time 30-min. → pink color coded tube Time 120-min. → orange color coded tube *If 2nd SST was collected at 0-min. for repository, serum from both 0-min SSTs is transferred into one red color coded tube.*	Freeze upright at -70° Ship weekly (M-W) to LCBR
3mL EDTA	HbA1c	Put on mixer for 30 sec. or gently invert x 30 sec.	Refrigerate (4°C)	Do Not Centrifuge	Do not transfer contents. Whole blood is stored and shipped in vacutainer EDTA tube	Refrigerate upright Ship twice weekly (M-W) to LCBR
10 mL EDTA	25OHD and Repository	Put on mixer for 30 sec. or gently invert x 30 sec.	Ice bath or refrigerate (4°C) until centrifuged (Preferably <15 min but not more than 45 min)	At 2,000 x g for 15 min. or 3,000 x g for 10 min.	Transfer plasma to violet color coded tube. *If 2nd 10 mL EDTA tube was collected at 0-min. for repository, plasma from both EDTA tubes is transferred into one violet color coded tube.*	Freeze upright at -70° Ship weekly (M-W) to LCBR
PaxGene	DNA	Invert 3 times	Refrigerate (4°C)	Do Not Centrifuge	Do not transfer contents. Whole blood is stored and shipped in the draw tube.	Refrigerate upright Ship twice weekly (M-W) to LCBR
D2d Site Urine Specimen Processing						
Collection Receptacle	Test	Mix vs. Clot	Temporary Storage	Refrigerated Centrifuge	10 mL Sarstedt Tube	Storage and Shipment
Urine Collection Cup	Urine calcium creatinine ratio and Repository	Not required	Refrigerate (4°C)	Do Not Centrifuge	Transfer 9 mL of urine: -Into yellow color coded tube -Into green color coded tube (if *participant consented to specimen repository)* at Baseline and annual visits only.	Freeze upright at -70° Ship weekly (M-W) to LCBR

Appendix 2 D2d Study – Baseline and Annual Specimen Collection Summary (LCBR and Local Lab)

Tests	Supplies needed
1. Urine specimen, while fasting, for: calcium, creatinine, and Repository * 2. Collection of blood, while fasting, for: • Plasma glucose • Calcium and Creatinine (Annual only) • Plasma 25-hydroxyvitamin D • Serum insulin • Hemoglobin A1c • Repository of plasma and serum * • Whole blood for DNA [baseline only] ** 3. 75-gram oral glucose tolerance test: • 30 minute plasma glucose and serum insulin • 120 minute plasma glucose and serum insulin	• Baseline-Annual visit Kit • Chilled Thermo Scientific 75 gram Trutol beverage • Phlebotomy supplies • Disposable pipettes (7) • Ice bucket and ice • Vacutainer tubes o 4 mL Sodium Fluoride tubes (3) o 10 mL Serum Separator tubes (5) * o 3 mL EDTA tube (1) o 10 mL EDTA tube (2) * o 8.5 mL PaxGene DNA tube (1) **

Procedure Summary at the baseline and annual visits



* If participant declined specimen repository informed consent for the storage of blood and urine, only three SST tubes, and only one 10mL EDTA tube is needed.

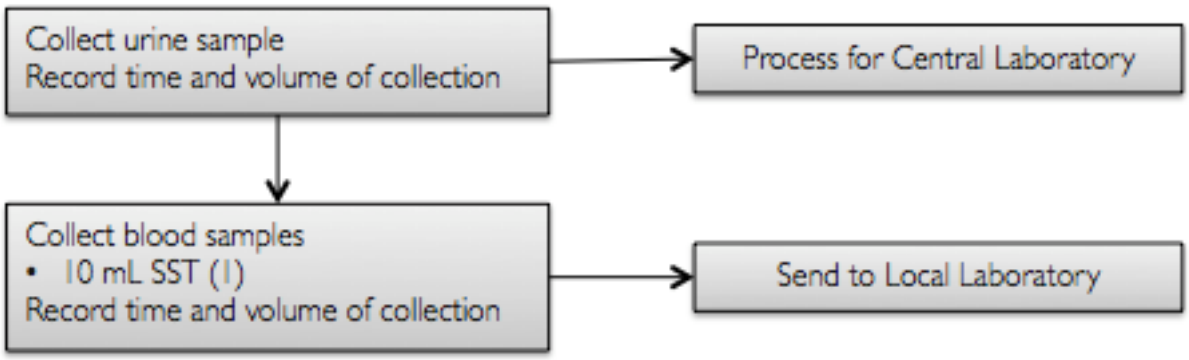
** If participant declined specimen repository informed consent for the storage of DNA from blood, the PaxGene DNA tube is not collected.

Note: If indwelling venous access is used, flush with saline after the fasting and 30 minutes blood draws. Draw blood for discard (per your institutions policy) prior to the drawing the samples.

Appendix 3 D2d Study – Month 3 (M03) Visit Specimen Collection Summary (LCBR and Local Lab)

Tests	Supplies needed
1. Urine specimen for: calcium, creatinine 2. Collection of blood, while fasting, for • Serum calcium and creatinine (Local Laboratory)	• Month 3 visit Kist (M03) • Phlebotomy supplies • Vacutainer tube o 10 mL Serum Separator tube (1)

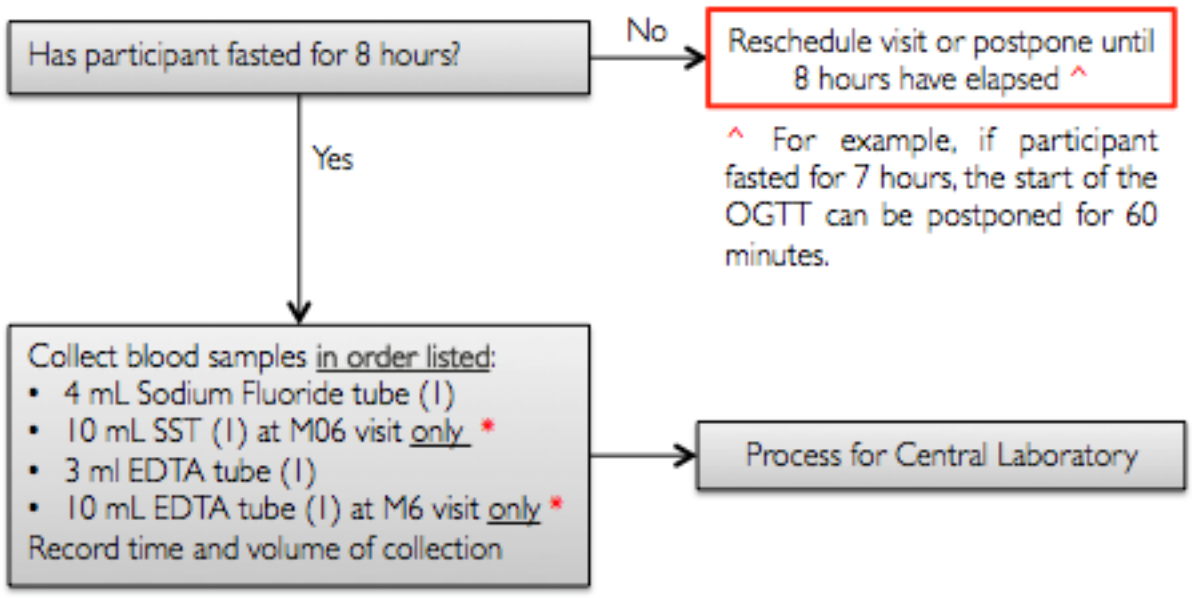
Procedure Summary at the month 3 visit



Appendix 4 D2d Study – Semi-Annual Visits (M06, M18, M30, M42) Specimen Collection Summary (LCBR)

Tests	Supplies needed
1. Collection of blood, while fasting, for: • Hemoglobin A1c • Plasma glucose • Repository of plasma and serum (M06 only)	• M06 or Semi-Annual visit Kit • Phlebotomy supplies • Disposable pipettes (3) * • Ice bucket and ice • Vacutainer tubes o 4 mL Sodium Fluoride tubes (1) o 10 mL Serum Separator tubes (1) for storage (**M06** visit only) * o 3 mL EDTA tube (1) o 10 mL EDTA tube (1) for storage (**M06** visit only) *
* If participant declined to provide specimen repository informed consent for the storage of blood and urine, the SST tube and the 10 mL EDTA tube are not needed along with 2 of the disposable pipettes.	

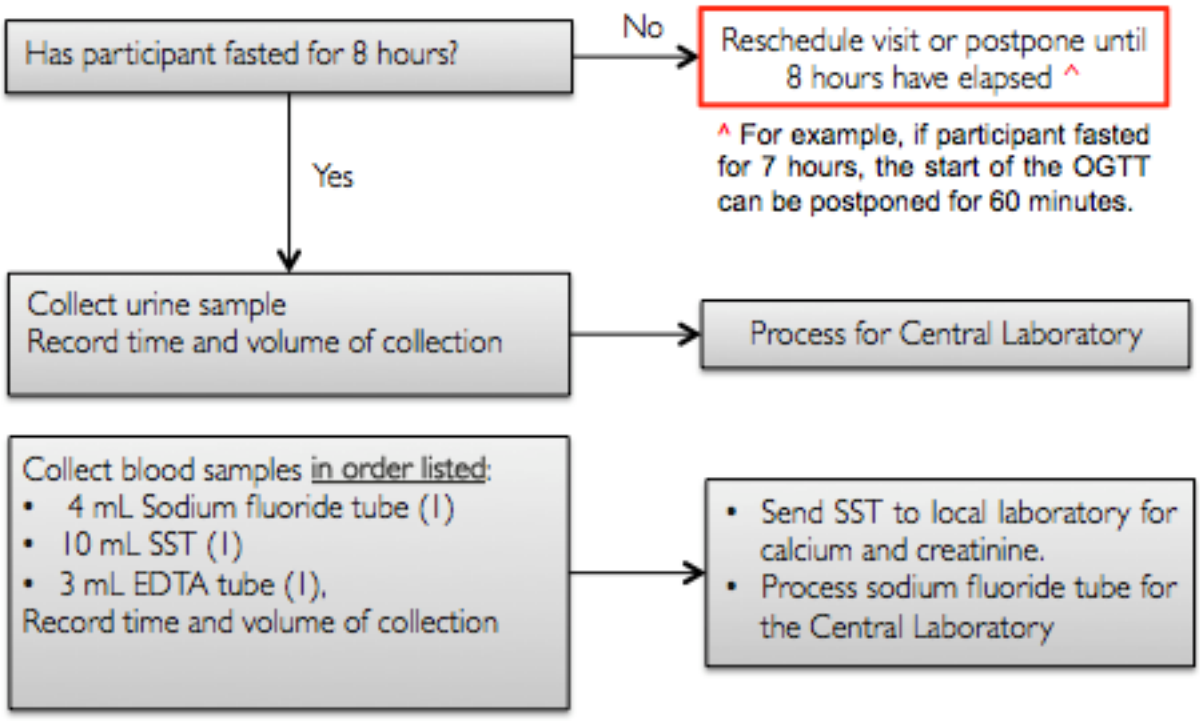
Procedure Summary at semi-annual visits



Appendix 5 D2d Study – End of Study Collection Summary (LCBR and Local Lab)

Tests	Supplies needed
1. Urine specimen, while fasting for: calcium, creatinine 2. Collection of blood, while fasting, for: • Hemoglobin A1c • Plasma glucose • Serum calcium and creatinine (Local Laboratory)	• End of Study visit Kit • Phlebotomy supplies • Disposable pipettes (1) • Ice bucket and ice • Vacutainer tubes o 4 mL Sodium Fluoride tube (1) o 10 mL Serum Separator tube (1) o 3 mL EDTA tube (1)

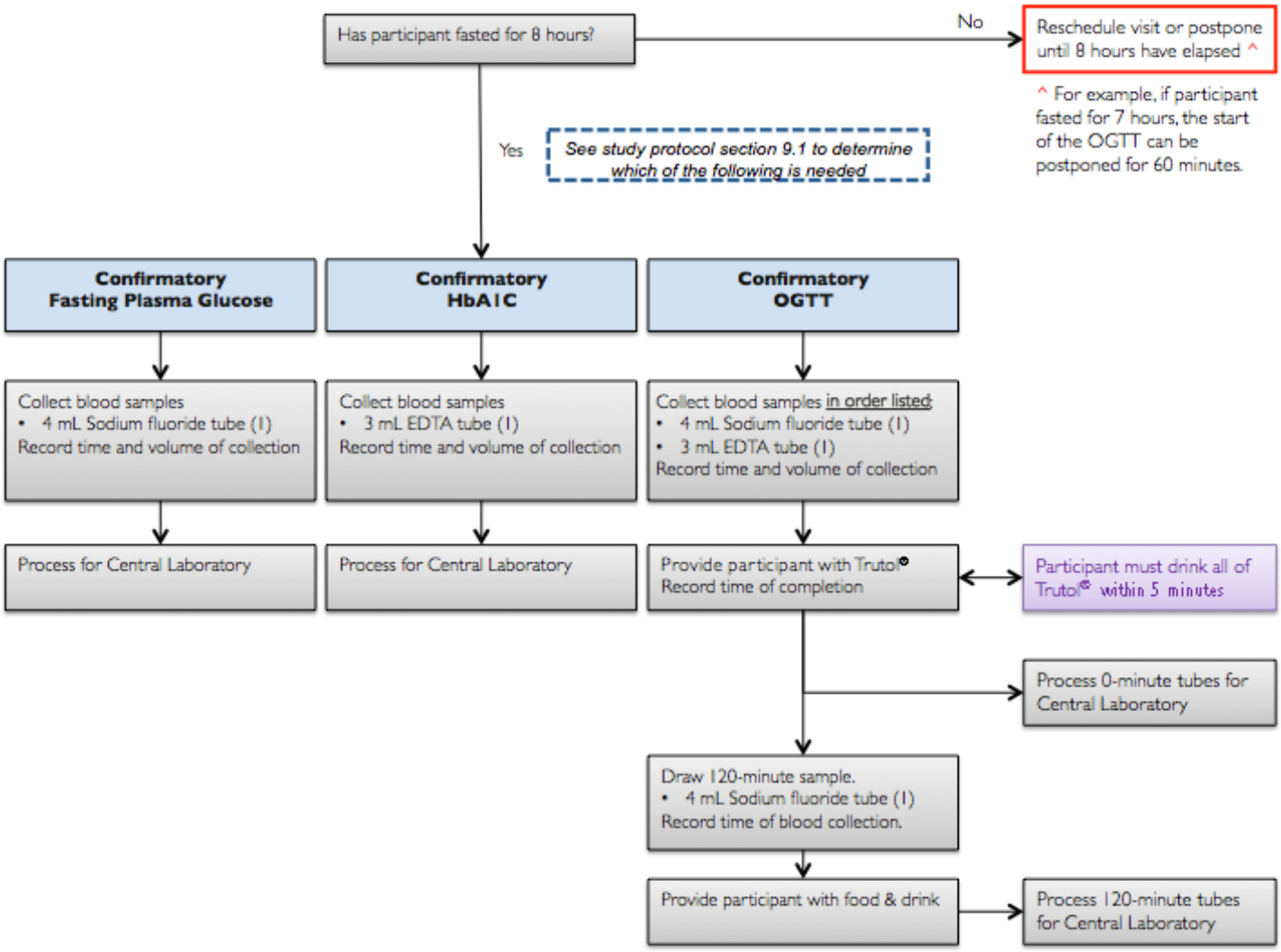
Procedure Summary at the end of study visit



Appendix 6 D2d Study – Confirmatory Visit Specimen Collection Summary (LCBR)

Tests	Supplies needed
Collection of blood, while fasting, for **one** of the following (refer to protocol section 9.1): • Plasma glucose, • Hemoglobin A1c [HbA1c], • Plasma glucose, Hemoglobin A1c and 120-minitue post load plasma glucose (after 75-gram oral glucose tolerance test [OGTT])	• UNCO visit Kit • Chilled Thermo Scientific 75 gram Trutol beverage (required for OGTT) • Phlebotomy supplies • Disposable pipettes (up to 2) • Ice bucket • Vacutainer tube(s) o 3 mL EDTA (1) for HbA1c o 4 mL Sodium Fluoride tubes (up to 2) [1 tube if plasma glucose alone; 2 tubes for OGTT]

Procedure Summary at confirmatory visit



Appendix 7 Venipuncture Procedure

ALWAYS WEAR LATEX (OR NITRILE) GLOVES AND LAB COAT

1. Arrange blood collection tubes in order of draw on the table top or in the tube rack within easy reach. Assemble butterfly apparatus and Vacutainer holders, gauze, and alcohol prep prior to tourniquet application.
2. Apply tourniquet.
3. Examine participant's arms for the best site for venipuncture. Release tourniquet
4. Cleanse venipuncture site by wiping with alcohol prep pad in a circular motion from center to periphery. Allow area to dry.
5. Reapply tourniquet. **NOTE 1**: If possible, it is best to release the tourniquet as soon as possible after flow has been established. Tightened tourniquet should be on no longer than 2 minutes. Loosen or remove tourniquet, then reapply if necessary. In our experience, however, especially with sick and/or elderly subjects, this may result in flow stopping, and the trauma of a second venipuncture. Therefore, this is a "judgment call" based upon the phlebotomist's experience and skill.
6. Grasp the participant's arm firmly, using your thumb to draw the skin taut. This anchors the vein. The thumb should be 1 or 2 inches below the venipuncture site. With the needle bevel upward, enter the vein in a smooth continuous motion. Make sure the participant's arm is in a flat or downward position while maintaining the tube below the site when the needle is in the vein. It may be helpful to have the participant make a fist with the opposite hand and place it under the elbow for support. Grasp the flange of the Vacutainer holder and gently push the tube forward until the butt end of the needle punctures the stopper, exposing the full lumen of the needle.
7. **NOTE 2**: Attention should be paid to minimizing turbulence whenever possible. Small steps, such as slanting the needle in the Vacutainer to have the blood run down the side of the tube instead of shooting all the way to the bottom, may result in significant improvement.
8. Note the blood flow into the first collection tube. If blood is flowing freely, the butterfly needle can be taped to the participant's arm for the duration of the draw. If the flow rate is very slow, the needle may not be positioned correctly. Try moving the needle slightly without causing discomfort to the participant.
9. Keep a constant, slight forward pressure (in the direction of the needle) on the end of the tube. This prevents release of the shutoff valve and stopping of blood flow. Do not vary pressure nor reintroduce pressure after completion of the draw.
10. Fill each Vacutainer tube as completely as possible; i.e., until the vacuum is exhausted and blood flow ceases. If a Vacutainer tube fills only partially, remove the tube and attach another without removing the needle from vein.
11. When the blood flow ceases, remove the tube from the Vacutainer holder. The shutoff valve re-covers the point, stopping blood flow until the next tube is inserted (if necessary).
12. Release tourniquet, if still applied.
13. To remove the needle, lightly place clean gauze over venipuncture site. DO NOT have the patient bend his/her arm as this can cause a hematoma. Remove the needle quickly and immediately apply pressure to the site with a gauze pad. Have the participant hold the gauze pad firmly for one to two minutes to prevent a hematoma. Discard needle into puncture-proof sharps container.

Appendix 8 Troubleshooting

Note: Follow all clinical site policies and procedures; the following is meant to be a guide and does not supersede any clinical site policies.

Working with People who are Apprehensive about Blood Draws: Do not under any circumstance force the participant to have blood drawn. It may help to explain to the participant that the blood drawing is designed to be as painless as possible. It may be helpful to have the participant relax in the bed so the phlebotomist can check the veins in the participant's arms, without actually drawing blood. If the participant has "good veins" the phlebotomist can reassuringly say, "Oh, you have good veins; there should be no problem."

Bandaging the Arm: If the patient continues to bleed apply pressure to the site with a gauze pad. Keep the arm elevated and straight until the bleeding stops. A gauze bandage can be tightly wrapped around the arm over the pad, and left on for at least 15 minutes.

Procedures for Difficult Draw: If a blood sample is not forthcoming, the following manipulations may be helpful.

- If there is a sucking sound, turn needle slightly or lift the holder in an effort to move the bevel edge away from the wall of the vein. If no blood appears, move needle slightly in hope of entering vein. Do not probe. If not successful, release tourniquet and remove needle. A second attempt can be made on the other arm.
- Loosen the tourniquet. It may have been applied too tightly, thereby stopping the blood flow. Reapply the tourniquet loosely. If the tourniquet is a Velcro type, quickly release and press back together. Be sure, however, that the tourniquet remains on for no longer than two minutes at a time.
- Reassure the participant that the inability to obtain a clean venipuncture is not any sign of a medical problem on their part.
- If venipuncture is unsuccessful or requires multiple sticks, this should be noted in the source documents.

When a Participant Feels Faint or Looks Faint:

- If the person is seated, he can sit with his head between his knees if necessary, or allow him to lie down.
- Provide him with a basin if he feels nauseous.
- Have him remain supine or seated until the color returns and he feels better.
- Place a cold washcloth on the back of his neck.
- If he continues to feel sick, contact a medical staff member who will advise you on further action.

Not all tubes are collected (blood flow ceases, difficult venipuncture): Always fill collection tubes in the order specified. Make notations of difficulties on the source document and the Central Laboratory e-CRF. If the participant is willing, another attempt should be made to complete the draw, collecting only those tubes that were not filled in the first venipuncture.

Vacutainer tube does not fill: First, try another tube of the same type. Partially filled plasma EDTA or fluoride tubes are not acceptable if less than 1/2 full. Partial tubes for serum are okay, but will result in a reduced number of aliquots. If a tube is not completely filled, clearly note so in the source documents and the Central Laboratory e-CRF as this can affect the ability to perform future assays or assay results.

Appendix 9 Procedures for Sites without -70°C Freezer and/or Refrigerated Centrifuge

1. If you do not have a refrigerated centrifuge:

Spin samples in room temperature centrifuge as described in section 9.8.3 and 9.12.1 and immediately place samples on ice post centrifugation. Then proceed to the aliquoting steps described in 9.8.4 and 9.12.1.

2. If you do not have a -70°C freezer:

A. Ensure 10 lbs. of dry ice and a Styrofoam shipping container are present prior to the participant visit.
B. After aliquoting is complete, place filled transfer tubes upright in a rack.
C. Place approximately 5 lbs. of dry ice in the bottom of the shipping container and place a lab mat on top of the dry ice.
D. Place the rack with the tubes upright in the box on top of the lab mat.
E. Do not allow the tubes to have direct contact with the dry ice.
F. Place another lab mat over the tubes in the rack and cover the lab mat with the remaining dry ice, and place lid on box.
G. Leave the transfer tubes on dry ice for at least one hour to freeze (they may stay on the dry ice until the afternoon, if you will not prepare them for shipping until the afternoon).
H. Inspect to make sure tubes are thoroughly frozen. Once frozen, tubes will be transferred to their sample box(es) in preparation for shipping (as described in section 9.10).
I. If shipping is not occurring on the day of collection it must occur on the next day and the sample box(es) containing the frozen tubes may remain on dry ice till shipping. It is critical to frequently check there is adequate dry ice to ensure the samples remain frozen. Additional dry ice will likely need to be added to the box at the end of the day and will need to be added on the day of shipping. Any accidental thawing of frozen samples must be documented on the Central Laboratory e-CRFs.

⇒ Specimens may only be shipped on Monday-Wednesday. ***Therefore, if you do not have a -70°C Freezer participants must be seen on Sunday-Wednesday.*** Participant visits should be scheduled to limit shipping frozen shipments to once a week.