



D2d Study Manual of Procedures

Section 9. Laboratory Procedures

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9.1 OVERVIEW

Participants enrolled in the D2d study will 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 will serve as the D2d Central Laboratory and will be responsible for performing assays and reporting results. The LCBR will be 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 will be 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 at baseline, 3, 12, 24, 36, and 48 month visits. An oral glucose tolerance test will be 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, to confirm the diagnosis of diabetes or for safety.

This laboratory manual provides study staff with all 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 9.1 Contact Information of this manual 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
208 South Park Dr., Rm.128
Colchester, VT 05446

9.2.2 LCBR Staff Contact Information

Russell Tracy Laboratory Director Tel: 802-656-8961 Fax: 802-656-8965 Email: Russell.Tracy@uvm.edu	Elaine Cornell Laboratory Coordinator Tel: 802-656-8963 Fax: 802-656-8965 Email: Elaine.Cornell@uvm.edu	Rebekah Boyle Project Manager Tel: 802-656-8938 Fax: 802-656-8965 Email: Rebekah.Boyle@uvm.edu
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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 site local laboratory (e.g. transport, use of appropriate forms, arrangements so 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 for tests that are analyzed at the site's local laboratory (e.g. all labs at screening, certain safety labs during the study).

- Four different D2d Laboratory Kits, which are visit-specific:
 - *Baseline - Annual visit* (BAS, M12, M24, M36, M48):
 - Sarstedt 10 mL transfer tubes with different colored screw caps (7)
 - Urine collection container and wipe (1)
 - Sarstedt 10 mL transfer tubes with yellow screw cap (1) and green screw cap (1)
 - Barcode labels for specimens
 - *Month 3 visit* (M03):
 - Urine collection container and wipe (1)
 - Sarstedt 10 mL transfer tube with yellow screw cap (1)
 - Barcode labels for specimen
 - *Semi-annual visit* (M06, M18, M30, M42):
 - Sarstedt 10 mL transfer tubes with different colored screw caps (3) - Note: Visits M18, M30 and M42 will require only 1 transfer tube. Visit M06 will require all 3 transfer tubes.
 - Barcode labels for specimens
 - *Confirmatory visit* (as needed)
 - Sarstedt 10 mL transfer tubes with different colored screw caps (2)
 - Barcode labels for specimens
- 8.5 mL PaxGene DNA tubes
- A kit that contains extra Sarstedt transfer tubes and colored screwed caps, urine collection containers and wipes, and a supply of disposable pipettes.
- Specimen shipping:
 - Ziploc bags with absorbent sleeves
 - 3" x 3" Revco cryoboxes (with 7x7 grid divider)
 - Conical tubes
 - Frozen specimen shipping boxes
 - Refrigerated specimen shipping boxes, with cold packs that should be placed in the freezer upon receipt so they are ready to be used when needed.
 - Labels for shippers, if needed (e.g. dry ice shippers)
 - 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 shipping)
- 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. 401025P). *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 or Greiner Vacuette 454062)
 - 10 mL Serum Separator tubes (BD 367985 or [9 mL] Greiner Vacuette 455010)
 - 3 mL EDTA (BD 367835 or Greiner Vacuette 454020)
 - 10 mL EDTA (BD 366457, or [9 mL] Greiner Vacuette 455036)

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 the results will be 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 will be reported to the sites in real-time. 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 laboratory tests are conducted in the morning after an 8-hour overnight fast.

9.5.3 Specimen Labeling

The LCBR will supply sites with specimen barcode labels to attach to specimen collection source documents, collection tubes, and transfer tubes. Each D2d Laboratory Kit will come with a strip of barcode labels. All labels in a kit have the same barcode and number (the number represents the barcode). *Please note that the number (barcode) on the specimen label is different from the participant D2d study identification number.*

⇒ **Each strip of labels is unique and is to be used only for a single participant at a single visit.**

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



right). *Please do not tape over the barcode labels.* Tape may interfere with the scanning of the transfer tubes at the LCBR.

At the beginning of each visit where specimens are collected, the barcode label will also be placed on the visit's specimen collection source document. The number on the barcode label will be entered into the EDC system on the visit's Central Laboratory e-CRF (see 9.5.4). This barcode links the specimen to the participant and visit, and allows the LCBR to identify the specimen upon receipt and link it to the appropriate participant and visit. A printout of the completed Central Laboratory e-CRF will be included in the sample shipment to LCBR (see 9.5.4).

At the conclusion of the participant's visit, after all of her specimens have been processed, refrigerated, frozen or shipped, any extra labels should be discarded. This action will prevent labels from mistakenly being used for a different participant or visit.

9.5.4 Specimen Collection Source Documentation

Specimen collection 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 specimen collection source document form should be printed from the MOP prior to each visit. After the visit, the information recorded on the document, will be entered into the EDC system on the visit's Local Laboratory or Central Laboratory e-CRF (see next paragraph and MOP section 15). For specimens that will go to the LCBR, the specimen barcode label will be placed on the specimen collection source document.

In the EDC system, there is a Central Laboratory e-CRF and a Local Laboratory e-CRF that need to be completed for each visit when specimens are collected for the LCBR or Local Laboratory respectively. The (Central or Local) 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 0 and 120 minutes will be collected and sent to LCBR

9.6.1 Instructions for the OGTT

1. 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.
2. 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?”

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.

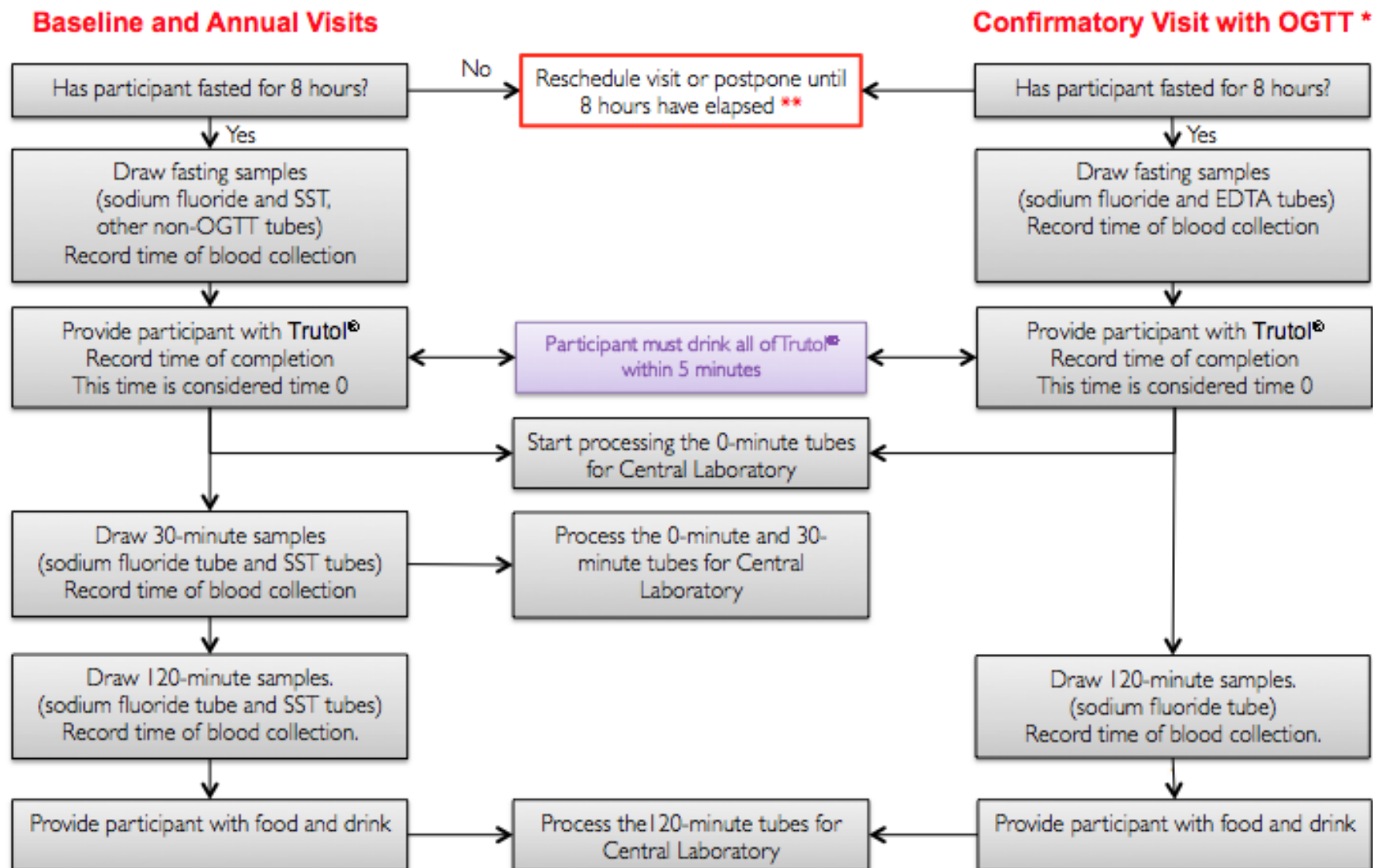
3. Instruct the participant that you will provide them with a cup of water (8 ounces) that they may drink during the OGTT and that they cannot have anything else to eat, drink, smoke or chew during the 2-hour test. Inform the participant that you will provide them with a snack after the last sample is 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.
4. 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.

5. While participant is fasting, draw blood in the following tubes in the order shown:
 - 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.
6. 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.
7. Immediately after the participant has finished the Trutol[®] beverage, set a timer (according to local practice) to facilitate appropriate timed collection of specimens.
8. Begin to process fasting (time 0 minutes) tubes, as described in section 9.8
⇒ *If this is a Confirmatory OGTT*, go to step 11.*
9. 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.
10. Begin to process the 30-minute tubes, as described in section 9.8.
11. 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.
12. Provide the participant with food and drink.
13. Process the 120-minute tubes, as described in section 9.8.

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

Flow diagram for OGTT procedure



*Confirmatory Visit with OGTT – in between scheduled visits to confirm the diagnosis of diabetes, if only the 2hPG was abnormal.

** 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, alkaline phosphatase, total bilirubin).
 - c. Serum calcium.
 - d. Serum creatinine
 - e. Hemoglobin A1c.
 - f. Plasma glucose.

9.7.1.1 Supplies Needed

At screening, all supplies are provided by the site.

- Screening specimen collection source document.
- Urine collection container (1) and wipe, for women of reproductive potential
- Urine point-of-care pregnancy test (1), for women of reproductive potential
- Phlebotomy supplies as listed in section 9.4
- Vacutainer tubes
 - 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 hemoglobin A1c
 - Ice bucket and ice

9.7.1.2 Procedures for Urine Specimen Collection

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

- 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.
- Instruct the participant to:
 1. Wash hands before and after voiding.

2. Cleanse area (vulva or penis) with antiseptic wipes prior to voiding.
 3. Remove the cap from the urine collection container and void into the container.
 4. Carefully seal the cap of the urine container so that it is tight and leak proof.
 5. Notify the staff to collect the sample.
- 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

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.
 - Tube 2 (10 mL SST).
 - Tubes 3 and 4 (EDTA): gently invert tube for ~30 seconds then refrigerate.
 - 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:
 - Hemoglobin A1c
 - Plasma glucose
 - Plasma 25-hydroxyvitamin D
 - Serum insulin
 - 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

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

- Baseline specimen collection source document
- Baseline/Annual visit Laboratory Kit, provided by LCBR
- Chilled Thermo Scientific 75 gram Trutol® Glucose Tolerance Test beverage
- Phlebotomy supplies as listed in section 3
- Vacutainer tubes
 - 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 Hemoglobin A1c
 - 10 mL EDTA tube (2) for 25OHD and for storage*
 - 8.5 mL PaxGene DNA tube (1) **
 - Disposable pipettes (7)
 - 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.

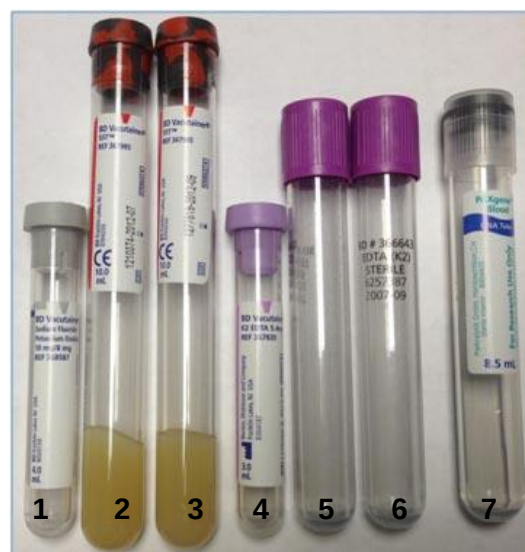
** If participant declined specimen repository informed consent for the storage of DNA from blood, the PaxGene DNA tube is not collected.

9.7.2.2 Procedures for Urine Specimen Collection

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

9.7.2.3 Procedures for Blood Specimen Collection

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, at time 0 minutes, draw blood in the following tubes in the order shown.
 - 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): Place on mixer for ~30 seconds or gently invert tube for ~30 seconds then refrigerate until ready to ship. DO NOT CENTRIFUGE.
 - Tubes 5 and 6 * (10 mL EDTA): Place on mixer for ~30 seconds or gently invert tube for ~30 seconds, then place in ice bath.
 - Tube 7 ** (8.5 mL PaxGene DNA **): Invert 3 times by hand and then refrigerate until ready to ship. DO NOT CENTRIFUGE.
 - If using indwelling venous access, flush with normal saline.
 - Record the actual time of the blood draw on the source document.



* 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. 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.
4. 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.
5. Begin to process fasting (time 0 minutes) tubes, as described in section 9.8.
6. 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.
 - Record the actual time of the blood draw on the specimen collection source document.
7. Begin to process the 30-minute tubes, as described in section 9.8.
8. 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.
 - Record the actual time of the blood draw on the specimen collection source document.
9. Provide the participant with food and drink.
10. Process the 120-minute tubes, as described in section 9.8

9.7.3 Three Month (M03) Visit Sample Collection

The following procedures and tests will be performed during the M03 visit. All laboratory specimens collected at the M03 visit will be processed and shipped to LCBR, except those indicated by (L) which will be analyzed at the local laboratory.

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

9.7.3.1 Supplies Needed

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

- M03 specimen collection source document
- M03 visit Laboratory Kit, provided by LCBR
- Phlebotomy supplies as listed in section 9.4
- Vacutainer tube
 - 10 mL Serum Separator tube (1) for serum calcium and creatinine ^L

9.7.3.2 Procedures for Urine Specimen Collection

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

9.7.3.3 Procedures for Blood Specimen Collection

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

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

1. Collection of urine specimen, while fasting, for:
 - Urine calcium and urine creatinine measurement
 - Storage of urine (with or without acid preservative)
2. Collection of blood, while fasting, for:
 - Serum calcium L
 - Serum creatinine L
 - Hemoglobin A1c
 - Plasma glucose
 - Plasma 25-hydroxyvitamin D
 - Serum insulin
 - 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

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

- Annual visit specimen collection source document
- Annual visit Laboratory 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.
- Phlebotomy supplies as listed in section 9.3
- Vacutainer tubes
 - 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 Hemoglobin A1c
 - 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

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

9.7.4.3 Procedures for Blood Specimen Collection

- 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.
- 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 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): Place on mixer for ~30 seconds or gently invert tube for ~30 seconds then refrigerate until ready to ship. DO NOT CENTRIFUGE.
 - Tubes 6 and 7* (10 mL EDTA): Place on mixer for ~30 seconds or gently invert tube for ~30 seconds, then place in ice bath.
 - If using indwelling venous access, flush with normal saline.
 - Record the actual time of the blood draw on the source document.

* If participant declined specimen repository informed consent for the storage of blood and urine, do not collect tube 3 (2nd SST) and tube 7 (2nd 10 mL EDTA)

- 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.
- Immediately after the participant has finished the Trutol[®] beverage, set a timer (according to local practice) to facilitate on-time collection of 30 minute and 120 minute specimens.
- Begin to process fasting (time 0 minutes) tubes, as described in section 9.8.
- 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.
 - Record the actual time of the blood draw on the source document.
- Begin to process the 30-minute tubes, as described in section 9.8.
- 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.
 - Record the actual time of the blood draw on the specimen collection source document.
- Provide the participant with food and drink.
- Process the 120-minute tubes, as described in section 9.8.

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

The following procedures and tests will be performed during the M6, M18, M30 and M42 visits. All laboratory specimens collected at these visits will be processed and shipped to LCBR

1. Collection of blood, while fasting, for:
 - Plasma glucose
 - Hemoglobin A1c
 - Storage of plasma and serum (at **M6** visit only)

9.7.5.1 Supplies Needed

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

- Semi-annual specimen collection source document
- Semi-Annual visit Laboratory Kit, provided by LCBR
- Phlebotomy supplies as listed in section 9.3
- Vacutainer tubes
 - 4 mL Sodium Fluoride tubes (1) for plasma glucose
 - 10 mL Serum Separator tubes (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 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

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 * (**M6 only**): let sit upright at room temperature for 30 minutes.
 - 3 mL EDTA: Place on mixer for ~30 seconds or gently invert tube for ~30 seconds then refrigerate until ready to ship. DO NOT CENTRIFUGE.
 - 10 mL EDTA * (**M6 only**): Place on mixer for ~30 seconds or gently invert tube for ~30 seconds, then place in ice bath
 - Record the actual time of the blood draw on the specimen collection source document.

*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 collected.

3. Provide the participant with food and drink.
4. Process the tubes, as described in section 9.8.

9.7.6 Confirmatory Visit Specimen Collection

A confirmatory visit will occur if: (1) at a scheduled visit, **one** out of three glycemic laboratory ([FPG], HbA1c, 2hPG) meets criteria for diabetes or (2) a participant reports she was diagnosed with type 2 diabetes by a physician outside of the study and has not started on a diabetes medication. During the confirmatory visit, one of the following will be required. **See D2d study protocol section 9.1 to determine which of the above is needed.**

- A. Repeat fasting plasma glucose (FPG), see section 9.7.6.1
- B. Repeat fasting HbA1c, see section 9.7.6.2
- C. Repeat FPG, HbA1c, and 2hPG (75-gram oral glucose tolerance test) see section 9.7.6.3

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

9.7.6.1 Confirmatory Visit - Repeat FPG

9.7.6.1.1 Supplies Needed

- Confirmatory visit specimen collection source document (repeat FPG)
- Confirmatory visit Laboratory Kit, provided by LCBR
- Phlebotomy supplies as listed in section 9.3
- Vacutainer tube:
 - 4 mL Sodium Fluoride tube (1) for plasma glucose
- Disposable pipettes (1)
- Ice bucket and ice

9.7.6.1.2 Procedures for Confirmatory 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 specimen collection source document.
4. Provide the participant with food and drink.
5. Process the tube, as described in section 9.8.

9.7.6.2 Confirmatory Visit - Repeat HbA1c

9.7.6.2.1 Supplies Needed

- Confirmatory visit specimen collection source document (HbA1c)
- Confirmatory visit Laboratory Kit - provided by LCBR
- Phlebotomy supplies as listed in section 9.3
- Vacutainer tube:
 - 3 ml EDTA tube (1) for Hemoglobin A1c

9.7.6.1.2 Procedures for Confirmatory 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: Place on mixer for ~30 seconds or gently invert tube for ~30 seconds then refrigerate until ready to ship. DO NOT CENTRIFUGE.
- 3. Record the actual time of the blood draw on the specimen collection source document.
- 4. Provide the participant with food and drink.
- 5. Process the tube, as described in section 9.8.

9.7.6.3 Confirmatory Visit - Repeat FPG, HbA1c, and 2hPG (75-gram OGTT)

9.7.6.3.1 Supplies Needed

- Confirmatory visit specimen collection source document (OGTT)
- Confirmatory visit Laboratory Kit - HbA1c, FPG, 2hPG, 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.
- Phlebotomy supplies as listed in section 9.3
- Vacutainer tubes:
 - 4 mL Sodium Fluoride tubes (2) for plasma glucose at 0 and 120'
 - 3 mL EDTA tube (1) for Hemoglobin A1c
 - Disposable pipettes (2)
 - Ice bucket and ice

9.7.6.3.2 Procedures for Confirmatory FPG, HbA1c and 2hPG

- 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.
- 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 put in ice bath.
 - Tube 2 (3 mL EDTA): Place on mixer for ~30 seconds or gently invert tube for ~30 seconds then refrigerate until ready to ship. DO NOT CENTRIFUGE.
- Record the actual time of the blood draw on the source document.
- 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.
 - Immediately after the participant has finished the Trutol[®] beverage, set a timer (according to local practice) to facilitate on-time collection of the 120 minute specimen.
- Begin to process fasting (time 0 minutes) tubes, as described in section 9.8
- 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.
 - Record the actual time of the blood draw on the source document.
- Provide the participant with food and drink.
- Process the 120-minute 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:

- Personal protective equipment (e.g. non-permeable lab coats and gloves, latex gloves, face shields).
- Refrigerated centrifuge (4°C) capable of spinning at a minimum of 2000g
- 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 Freezer Boxes 5x5x3" and dividers (7 x 7 grids)*

* Provided by the LCBR

9.8.2 Preparation

Prior to the start of the visit, a tube rack should be prepared with all of the Sarstedt transfer tubes (which have different colored screw caps) arranged in the appropriate working order (i.e. set up in a way that corresponds to the blood collection vacutainer tubes). All of the Sarstedt transfer tubes should be labeled with the visit-specific barcode labels.

9.8.3 Centrifugation

The following three collection tubes require centrifuging and subsequent processing:

- **4mL Sodium Fluoride.** Invert 8-10 times, then put 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 put in ice bath or refrigerate (4°C) until centrifuged, preferably for less than 15 minutes but not more than 45 minutes.

Note: The 3mL EDTA tube (used for hemoglobin A1c) 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.

- 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 on the D2d specimen collection Form.

9.8.4 Aliquoting of Blood

After centrifugation, plasma or serum in small amounts (i.e. 2 mL, 4 mL) will be transferred via a disposable transfer pipette into 10 mL Sarstedt color-coded transfer tubes.

- Color-coding of the transfer tubes is predetermined by the LBCR and used to identify sample type (e.g., EDTA plasma vs. serum) and procedure time-point (i.e., 0-minute, 30-minute, 120-minute).
- Aliquoting must be done while the blood collection tubes and transfer tubes are on ice (unless otherwise noted).
- Below is an example of a tube rack where blood drawn tubes are placed next to the respective color-coded transfer tubes.



- Use a new disposable pipette for each cell type at each time point.
- 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.
- If a tube is accidentally mixed during pipetting such that plasma is contaminated with red cells, the collection tube may be re-centrifuged.

4mL Sodium Fluoride: After centrifugation, plasma (approximately 2 mL) will be transferred via a disposable transfer pipette into a 10 mL color-coded transfer tubes, as follows.

- Plasma from the fasting (*time 0-minutes*) Sodium Fluoride tube goes in the silver-capped transfer tube.

- Plasma from the **30-minute** Sodium Fluoride tube goes in the **black**-capped transfer tube.
- Plasma from the **120-minute** Sodium Fluoride tube goes in the **white**-capped transfer tube.

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 a 10 mL Sarstedt color-coded transfer tube, as follows:

- Serum from the fasting (**time 0-minutes**) SST tube goes in the **red**-capped transfer tube. **Please Note:** If the participant provided specimen repository informed consent, at the baseline and annual blood draws, two SST tubes are collected at fasting (time 0-minutes). Serum from both SST tubes will be aliquotted and pooled into one **red**-capped transfer tube.
- Serum from the **30-minute** SST tube goes in the **pink**-capped transfer tube.
- Serum from the **120-minute** SST tube goes in the **orange**-capped transfer tube.

10 mL EDTA tube: After centrifugation, plasma (approximately 4 mL) will be transferred via a disposable transfer pipette into a **violet**-capped 10 mL transfer tube. **Please Note:** If the participant provided specimen repository informed consent, at the baseline and annual blood draws, two 10-mL EDTA tubes are collected at fasting (time 0-minutes). Plasma from both EDTA tubes will be aliquotted and pooled into one **violet**-capped transfer tube.

Once the samples are aliquotted, transfer tubes will be placed in a cryobox provided by LCBR (arranged as shown in the photo in section 9.10). One of the specimen barcode labels used for the visit should be placed on the lid of the cryobox, to identify what samples are in the box. The cryobox with the samples should be immediately frozen (within 15 minutes) in an upright position at -70°C.

After samples have been aliquotted 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).

9.8.5 Special Circumstances

Low sample volume: Any partially-filled transfer tube (less than the specified volume) should be marked with a sharpie pen, dot on the cap. Indicate which tube has a low sample volume (e.g. plasma from fasting EDTA tube) and the reason for the low volume on the specimen collection source document and in the comment field in the Central Laboratory e-CRF.

Blood specimens (Sodium Fluoride, EDTA) cannot be centrifuged and processed within 30 minutes of collection: If centrifugation cannot be performed within 30 minutes of collection, try to process specimens as soon as possible after that time. **Note time of collection and centrifugation** on the specimen collection source document and in the comment field in the Central Laboratory e-CRF. Maintain the Sodium Fluoride and EDTA tubes on ice until centrifugation.

Serum and plasma cannot be frozen within 15 minutes of aliquoting: If specimens cannot be placed immediately at -70°C or below, they may be temporarily (less than 2 hours) stored on dry ice or at -20°C until transfer to -70°C. Placing at -20°C should be a rare occasion. If unable to place at -70°C or below immediately, make note of when the specimens were placed at -70°C and the reason why it was delayed in the specimen collection source document and in the comment field in the Central Laboratory e-CRF.

9.9 URINE SPECIMEN PROCESSING

The D2d Baseline/Annual Visit Laboratory Kit includes the following for the urine processing:

- Sarstedt 10mL transfer tubes with yellow screw cap
- Sarstedt 10mL transfer tubes (pre-loaded with boric acid preservative) with green screw cap

The 3 Month Visit Laboratory Kit includes the following for the urine processing:

- Sarstedt 10mL transfer tubes with yellow screw cap

9.9.1 Aliquotting of Urine

The urine collection container should be clearly marked with participant identification. The container with urine should be removed from the refrigerator when you are ready to aliquot the sample.

1. Ensure each Sarstedt 10mL transfer tube is labeled with a bar code label from the visit Laboratory Kit.
2. Pipette one 9 mL sample of urine from urine container into the 10 mL yellow-capped tube and place on ice.
3. If participant provided specimen repository informed consent, pipette another 9 mL sample of urine from urine container into the 10 mL green-capped tube (pre-loaded with boric acid powder) and place on ice (baseline and annual visits only).
4. Freeze the samples at -70° C or on dry ice within 10 minutes of aliquot preparation.
5. Discard remaining urine.

Aliquot Summary:

Container #	Sample	Cap Color	Tube Size (mL)	Volume (mL)
1	Urine	<u>Yellow</u>	10	9
2	Urine + Acid	<u>Green</u>	10	9

NOTE: 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.

9.10 PROCESSING COMPLETION AND SPECIMEN STORAGE

Transfer tubes (10 mL with plasma or serum; 10 mL with urine) are placed into a 3" cryobox, with 7 x7 grids (to prevent shifting during shipping) for freezer storage and shipping. Please note the following:

- Each cryobox can hold tubes from multiple participants.
- Each row should be for tubes from just one participant (either serum/plasma or urine). Between each participant's row of serum and plasma there should be some empty grids as illustrated in the photo below.
- Please leave a row along the edge of the box empty. The grid may shift during transport and the tubes will fall out.
- For each participant whose samples are in the box, attach a bar code label to the cover of the box.



This box has samples from 3 participants. To illustrate, 9 samples from one participant are outlined in a black box.



The box cover has the bar code label for each of the 3 participants whose samples are 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.
 - Samples collected on Wednesdays should always be shipped on Wednesday.
 - Samples 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.

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

9.11.2 Supplies Needed

Sample Shipping Checklist:

- Frozen and Refrigerated Specimen Shippers (Styrofoam Mailing Containers with outer cardboard sleeves)*
- Rubber bands
- Ziploc plastic bags with absorbent sleeves
- Absorbent material (i.e. paper towels, newspaper)
- Packaging tape
- Dry ice (~10lbs per mailing container) for frozen shipments
- Frozen ice packs (2 per refrigerated container)*
- 50mL conical tubes*
- Labels: FedEx address labels*
- Dry Ice Labels (class 9, UN1845) for frozen shipments*
- Participant samples packed in Revco freezer boxes with, labels on outside
- 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

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

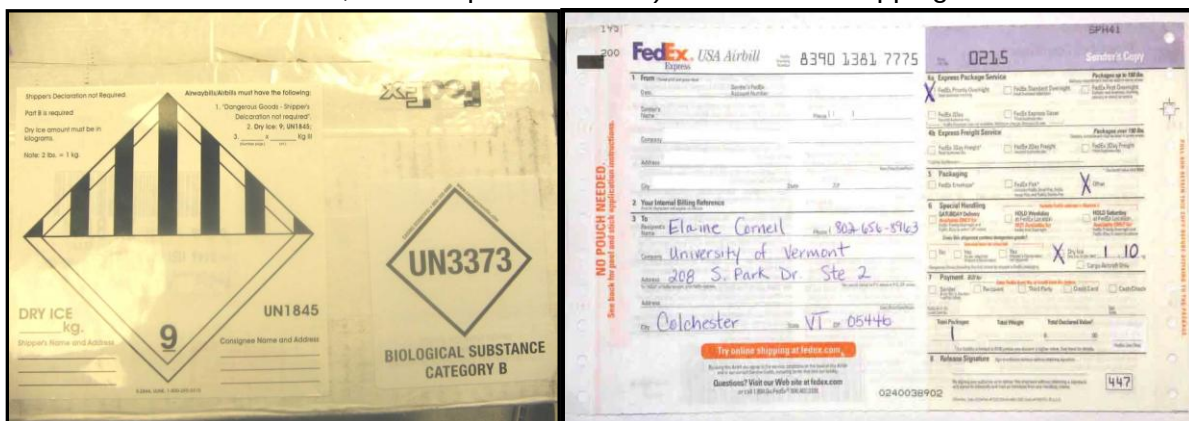


3. Place another layer of absorbent material (e.g. paper towels) on top of the dry ice so it will be inbetween the dry ice and the freezer boxes containing the samples.

4. Collect the Revco freezer boxes containing samples to be shipped and check the sample ID numbers against the D2d Specimen Forms for that shipment.
5. Place a rubber band around each Revco cardboard freezer box containing samples, and then place each box in a Ziploc plastic bag, with an absorbent sleeve. The rubber band is important for aiding in the prevention of a transfer tube spill; the Ziploc bag and absorbent sleeve is for compliance with commercial carrier specifications.
6. Carefully place the bagged boxes in the mailer.



7. Another layer of absorbent material (e.g. paper towels) is placed on top of the sample freezer boxes.
8. The remaining dry ice is placed on top of the last layer of absorbent material.
9. The top of the Styrofoam insert is sealed on with tape, and then enclosed in the outer cardboard sleeve.
10. Printouts of the Central Laboratory e-CRFs for all samples included in the Styrofoam mailer, are stapled together and placed on the top of the Styrofoam insert before the outer sleeve is taped closed. These are used as a packing slip.
11. Affix shipping labels (Fed Ex label, Biological Specimen Category B UN3373 label, Dry Ice Class 9 UN1845 label, and Keep Frozen label) to outside of shipping container.



12. Place the entire box in the refrigerator if pickup is not immediate. (Frozen samples should not be on dry ice for > 24 hours).
13. Enter the Fed Ex tracking number of each sample in the box, into Central Laboratory e-CRF. Entering this information will generate an e-mail notifying the LCBR that the specimens have been shipped.

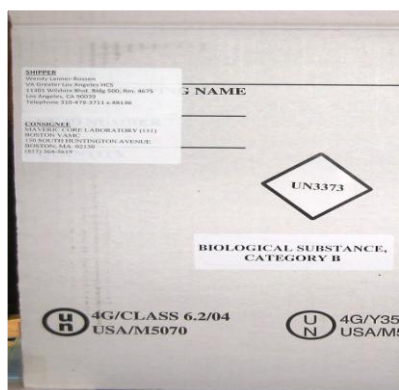
9.11.4 Refrigerated Shipments to LCBR (EDTA tube for HbA1c and PaxGene DNA tube)

⇒ Refrigerated samples must arrive at the LCBR within 5 days of being drawn.

1. Line Styrofoam mailer(s) with absorbent material (e.g. paper towels).
2. Place two ice packs on the bottom of the mailer.
3. Remove the EDTA and PaxGene tubes (if present) from their refrigerated storage locations. Check the sample ID numbers against the Specimen Forms 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 Ziploc bag with absorbent sleeve. The rubber band aids in the prevention of a transfer tube spill; the Ziploc bag and absorbent material are for compliance with commercial carrier specifications.



6. Another layer of absorbent material (e.g. paper towels) is placed on top of the sample bags.
7. The top of the Styrofoam insert is sealed on with tape, and then enclosed in the outer cardboard sleeve.
8. The printouts of the Central Laboratory e-CRFs for all samples are stapled together and are placed on the top of the Styrofoam insert before the outer sleeve is securely taped closed.
9. Affix shipping labels (Fed Ex label, Biological Specimen Category B UN3373 label, to outside of shipping container.



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

9.11.5 Mailing Address

University of Vermont
Department of Pathology
Colchester Research Facility, Room T205
Attn: Elaine Cornell – D2d study
208 South Park Drive, Suite 2
Colchester, VT 05446

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

9.12 APPENDICES

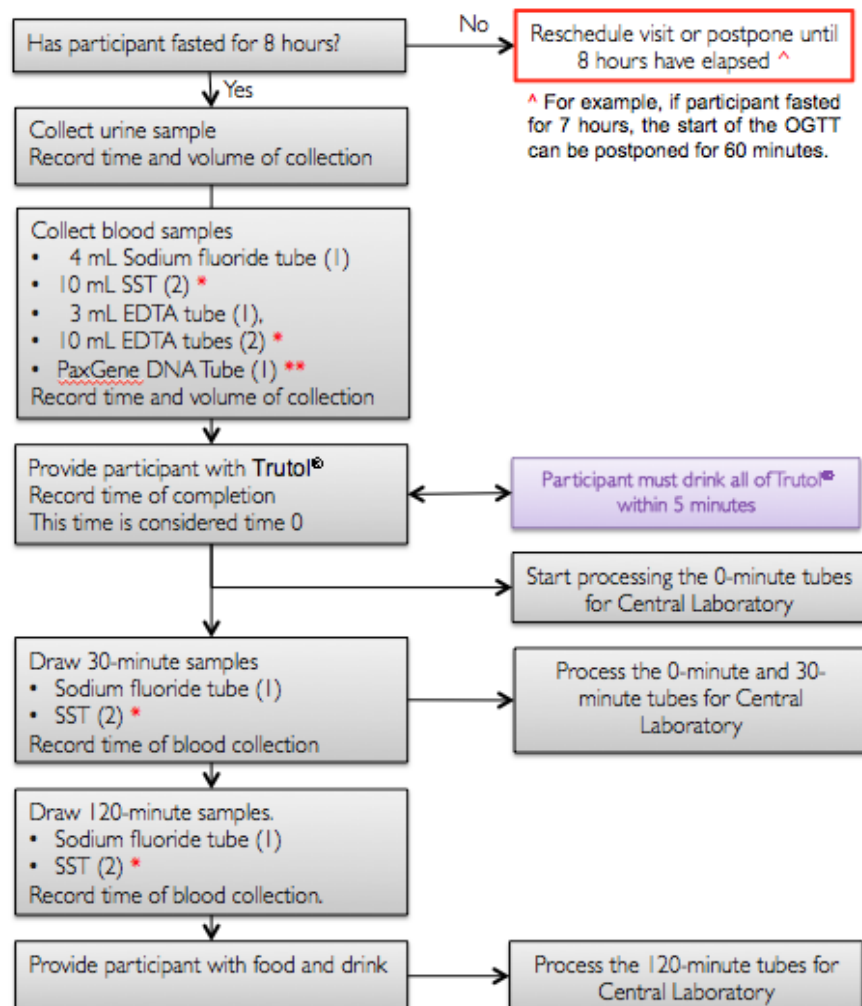
Appendix 9.12.1 D2d Central Laboratory Blood Specimen Processing

D2d Central Laboratory (LCBR) 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 <u>no more than 45 min</u>)	At 2,000 x g for 15 min. or 3,000 x g for 10 min.	Transfer plasma as follows: Time 0-min. → <u>silver</u> capped tube Time 30-min. → <u>black</u> capped tube Time 120-min. → <u>white</u> capped 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. → <u>red</u> capped tube Time 30-min. → <u>pink</u> capped tube Time 120-min. → <u>orange</u> capped tube <i>If 2nd SST was collected at 0-min. for repository, serum from both 0-min SSTs is transferred into one <u>red</u> capped tube.</i>	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 <u>no more than 45 min</u>)	At 2,000 x g for 15 min. or 3,000 x g for 10 min.	Transfer plasma to a <u>violet</u> capped tube. <i>If 2nd 10 mL EDTA tube was collected at 0-min. for repository, plasma from both EDTA tubes is transferred into one <u>violet</u> capped tube.</i>	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.	Refrigerate upright Ship twice weekly (M-W) to LCBR
D2d Central Laboratory (LCBR) Urine Specimen Processing						
Collection Tube	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 <u>yellow</u> capped tube -Into <u>green</u> capped tube (if participant consented to specimen repository) at BAS and annual visits only.	Freeze upright at -70° Ship weekly (M-W) to LCBR

Appendix 9.12.2 D2d Study - Baseline and Annual Specimen Collection Summary

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

Procedure Summary at the baseline and annual visit



* 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.

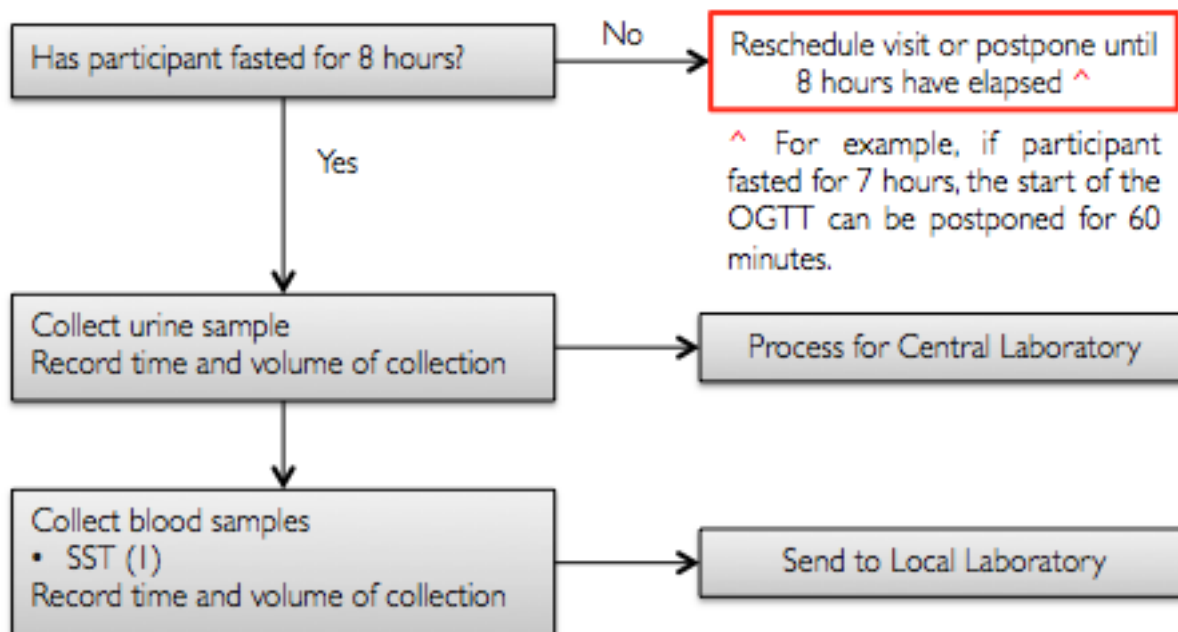
** If participant declined specimen repository informed consent for the storage of DNA from blood, the PaxGene DNA tube is not collected.

Note: If indwelling venous access is used, flush with saline after the fasting and 30 minutes blood draws, and before drawing the 30 and 120 minute samples, Draw blood for discard (per your institutions policy).

Appendix 9.12.3 D2d Study - Month 3 Specimen Collection Summary

Tests	Supplies needed
- Collection of urine specimen, while fasting for: calcium, creatinine - Collection of blood, while fasting, for Local Laboratory measurement of: - Serum calcium and serum creatinine	- M03 visit Laboratory Kit - Phlebotomy supplies - Vacutainer tube 10 mL Serum Separator tube (1)

Procedure Summary at the month 3 visit



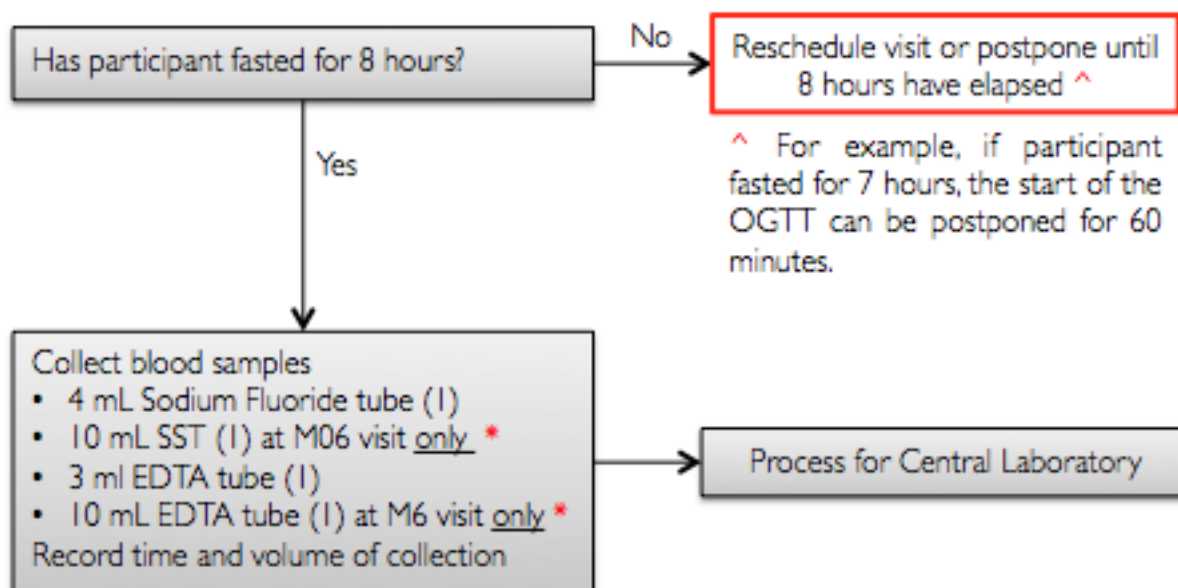
Appendix 9.12.4 D2d Study - Semi-Annual Visits (M06, M18, M30, M42) Specimen Collection Summary

Tests	Supplies needed
1. Collection of blood, while fasting, for: • Plasma glucose • Hemoglobin A1c • Repository of plasma and serum *	• Semi-Annual visit Laboratory kit • Phlebotomy supplies • Disposable pipettes (3) * • Ice bucket and ice • Vacutainer tubes ○ 4 mL Sodium Fluoride tubes (1) ○ 10 mL Serum Separator tubes (1) for storage at M6 visit <u>only</u> * ○ 3 mL EDTA tube (1) ○ 10 mL EDTA tube (1) for storage at M6 visit <u>only</u> *

* Blood is only collected for repository at the month 6 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.

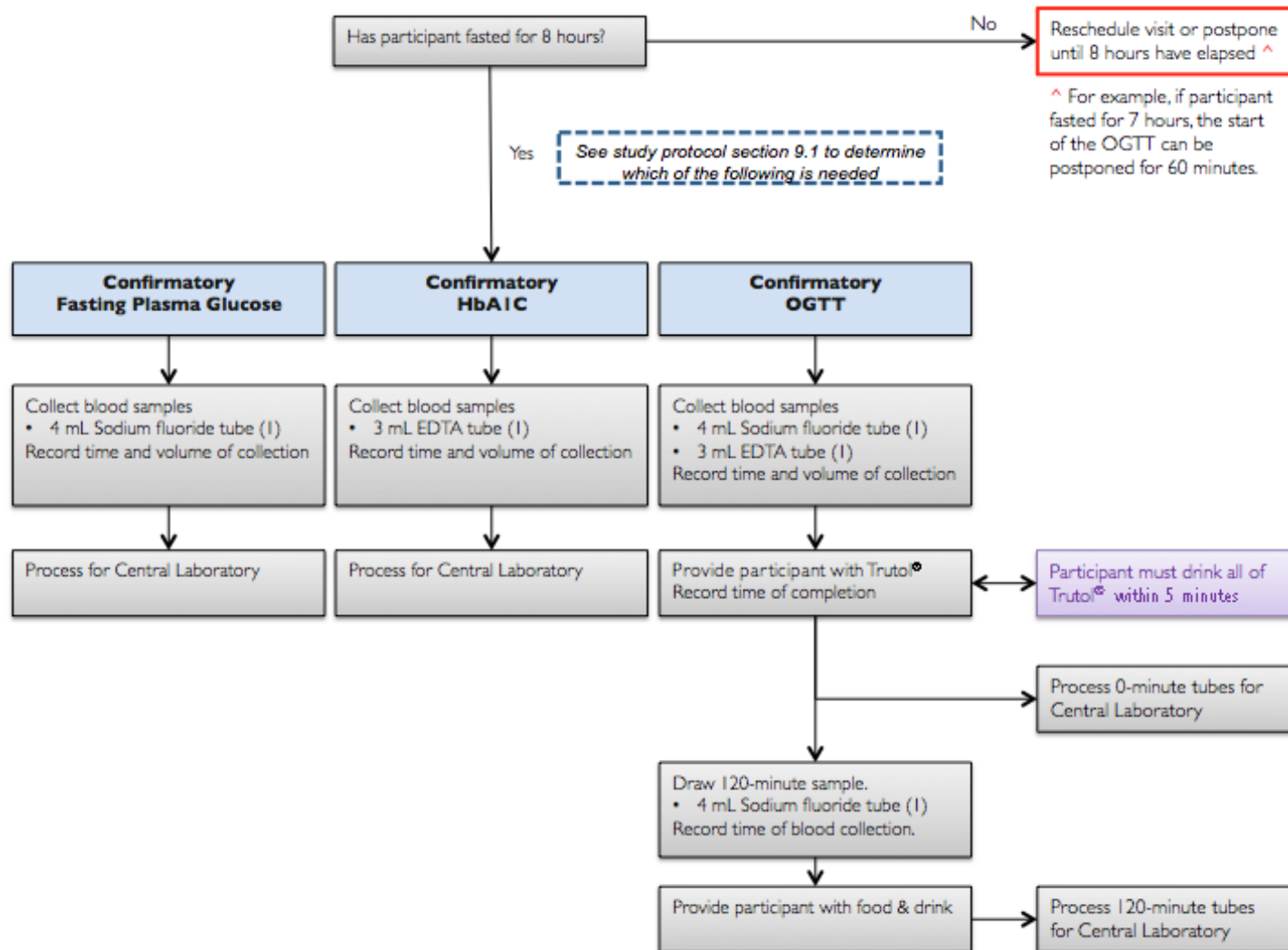
Procedure Summary at semi-annual visits



Appendix 9.12.5 D2d Study - Confirmatory Specimen Collection Summary

Tests	Supplies needed
Collection of blood, while fasting, for one of the following (refer to protocol section 9.1): 1. Fasting plasma glucose (FPG) 2. Fasting HbA1c 3. Fasting plasma glucose, HbA1c and 75-gram oral glucose tolerance test: • 120 minute plasma glucose and serum insulin	• Confirmatory visit kit • Chilled Thermo Scientific 75 gram Trutol beverage* • Phlebotomy supplies • Vacutainer tube(s) ○ 3 MI EDTA (1) (for HbA1c) ** ○ 4 mL Sodium Fluoride tubes*** • Disposable pipettes (0-3) • Ice bucket and ice * Required for OGTT ** Required for HbA1c and OGTT *** 1 Sodium Fluoride tube required for FPG, 2 Sodium Fluoride tubes required for OGTT

Procedure Summary at confirmatory visit



Appendix 9.12.6 Venipuncture Procedure

ALWAYS WEAR LATEX 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 9.12.7 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 on the Phlebotomy Form.

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 the person with a basin if he/she feels nauseous.
- Have the person remain supine or seated until the color returns and he/she feels better.
- Place a cold washcloth on the back of the person's neck.
- If the person 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, etc.): Always fill collection tubes in the order specified. Make notations of difficulties on the specimen collection 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 on specimen collection source document and the Central Laboratory e-CRF as this can affect the ability to perform future assays or assay results.