10. DLW PROCEDURES AT THE SITES

10.1 Introduction

10.1.1 Overview

The scientific inferences in CALERIE depend critically on having a reliable measure of energy expenditure. This is important at baseline so that the correct calorie restriction can be prescribed for a participant as s/he initiates the intervention. It is also a crucial component of the calculations to quantify "adherence" to the intervention during the study. Doubly-labeled water (DLW) procedures are therefore applied in CALERIE to derive this measure of total energy expenditure (TEE).

The CALERIE protocol also prescribes a formal test-retest reliability study of the DLW process. That is, duplicate sets of urine samples from the same subject at the same protocol time point are forwarded to the DLW lab for analysis. The specimens are labeled in such a way that DLW lab personnel are masked to the participant ID, treatment arm, and protocol time point to which the specimens refer. They are also masked as to whether it is an original or a retest urine sample set. A link is created at the Coordinating Center to link the identity of the test and retest specimens to the true CALERIE ID number. Statistical analyses are ultimately performed to determine what is the reliability of the DLW process, and if there is a significant difference between the test and retest specimens.

In this section we describe how DLW studies are conducted in CALERIE, and how to integrate the reliability study into this process.

10.1.2 Baseline vs. "Post-Randomization" DLW Studies

As described in the protocol, the pair of DLW determinations performed at baseline is not included in the test-retest study. This is to avoid delaying the lab in deriving the baseline TEE values so that the correct energy prescription can be derived. Otherwise, the follow-up DLW studies for CR participants at Months 6, 12, 18 and 24, and for controls at Months 12 and 24 are eligible for this study. They are collectively referred to as the "post-randomization DLW studies." We therefore distinguish between baseline and post-randomization DLW procedures throughout this chapter.

10.1.3 Definition of a "Urine Sample Set"

A "urine sample set", or equivalently, a "DLW study" consists of a set of urine samples collected over the course of a 14-day DLW period. They include two separate urine samples collected at the following time points:

- Pre-dosing (PD): PDa and PDb
- Day 0: D0a and D0b
- Day 7: D7a and D7b
- Day 14: D14a and D14b

for a total of 8 individual urine samples. On Day 0, the samples should be collected no less than 4 hours and no more than 7 hours after the DLW dose was administered, with the preferred times being 4.5 and 6.0 hours after the DLW dose.

10.1.4 Sufficient Urine Volume for the Baseline DLW Studies

Sufficient urine volume must be collected from every participant for each baseline DLW study so that there is sufficient volume for: (1) the lab set, and (2) a backup set. Thus, at every urine collection time point, a minimum of 2×1.5 ml = 3 ml of urine must be collected. This urine sample is aliquoted into two urine vials – one for the lab set, and one for the backup set.

During the baseline period, the two DLW studies are performed back-to-back. The DLW lab uses the D14a and D14b urine samples from the first DLW study as the PDa and PDb urine samples for the second DLW study. Thus, for the first baseline DLW study, 8 urine vials are created as described above. For the second DLW study, however, only 6 new urine vials are created corresponding to the pairs of urine samples on days 0, 7 and 14. Thus, a total of 14 urine vials are created for the pair of baseline DLW studies.

10.1.5 Sufficient Urine Volume for a Post-Randomization DLW Study

It is not possible to select a participant for the reliability study at the moment s/he is actually performing any DLW study. The retest samples are therefore selected retrospectively from the set of eligible post-randomization urine samples at any point in calendar time as described below. This implies that sufficient urine volume must be collected from every participant during every post-randomization DLW study so that there is sufficient volume for: (1) the test set, (2) a backup set, and (3) a retest set if the DLW study is selected for the reliability study. Thus, as described below, at every urine collection time point for a post-randomization DLW study, a minimum of 3×1.5 ml = 4.5 ml of urine must be collected. This urine sample is aliquoted into three urine vials – one for the test set, one for the backup set, and one for a retest set. Over the duration of a 14-day DLW study, therefore, a total of 3×8=24 urine vials are created for any post-randomization DLW study.

10.2 DLW Study Materials

10.2.1 Label Sheets for Baseline DLW Studies

Labels are affixed to each of the urine vials to be sent to the DLW lab. Labels are pre-printed on an 8½" × 11" sheet of adhesive labels. One label sheet is specifically generated for the pair of DLW studies conducted on each participant at baseline. There are 36 labels per sheet, and an example is provided in Appendix A. The purpose of each label in the sheet is described below.

Because the DLW lab is not masked to the baseline studies, each label is pre-printed with the true CALERIE ID number for that participant. Moreover, each label corresponding to the first baseline DLW study is pre-printed with the suffix, "-1", and similarly each label corresponding to the second baseline DLW study is pre-printed with the suffix, "-2". Thus, for example, for CALERIE ID No. 03-0025, the DLW ID numbers are 03-0025-1 and 03-0025-2 for the first and second baseline DLW studies, respectively. In preparation for the baseline clinic visit, the DLW Tech prints a label sheet for the pair of DLW studies using a template provided by the CC.

10.2.2 Individual Labels on the Baseline Label Sheets

The 48 labels are used as follows. (Please refer to the example in Appendix A).

1. There are 8 labels corresponding to the 8 urine collection cups used to collect the urine samples from the participants during DLW Baseline Study #1. They are located in rows 1-8 under the column labeled "DLW #1 Urine Collection".
2. There are 8 labels corresponding to the 8 urine vials sent to the DLW lab for analysis for DLW Baseline Study #1. They are located in rows 1-8 under the column labeled "DLW #1 Lab Labels".

3. There are 8 labels corresponding to the backup set of 8 urine vials for DLW Baseline study #1 which are stored at the site. They are located in rows 1-8 under the column labeled “DLW #1 Back-up Labels”.

   These labels are preprinted with true CALERIE ID number as well as the sample type (i.e., “Collection”, “Lab”, “Back-up”), and with the urine collection time point (i.e., PDa, PDb, …, D14a, D14b).

4. One label is affixed to the case report form (CRF) page corresponding to DLW Baseline Study #1 as described below. It is located in row 9 under the column labeled “DLW #1 Lab Labels”. Its purpose is to inform the CC (through the CRF) that a Baseline DLW Study #1 has been performed.

   When the CRF page is received at the CC, the true CALERIE ID number and protocol time point written in the header at the top the CRF page are entered into the CALERIE database. Additionally, the CALERIE ID number pre-printed on this label is entered into the CALERIE database. A computer check is made to make sure that the two ID numbers match .

5. One label is affixed to the entry on the DLW Tracking Log maintained at the CALERIE site. This label is located in row 10 under the column labeled “DLW #1 Lab Labels”. As discussed below, this log provides primary documentation at the site that a urine sample set has been shipped to the DLW lab.

6. Finally, one label is held in reserve in case back-up urine vial(s) must be shipped to the DLW lab. It is located in row 10 under the column labeled “DLW #1 Back-up Labels”. If a backup sample is shipped to the DLW lab, then this label is affixed to the DLW Tracking Log as described below for primary documentation. (There is no need to complete a CRF page for a back-up sample.)

This brings the total number of labels for Baseline DLW Study #1 to 27 labels.

There is a corresponding set of labels for Baseline DLW Study #2. They are located under the columns labeled “DLW #2 Urine Collection”, “DLW #2 Lab Labels” and “DLW #2 Back-up Labels”. Their purpose is completely analogous to that described above except that they refer to the Baseline DLW Study #2 performed with that participant. Note that there are no labels for the pre-dose urine samples. As described above, the DLW lab will use the D14a and D14b urine samples from the first DLW study as the PDa and PDb urine samples for the second DLW study. This brings the total for the DLW #2 study to 21, for a grand total of 48 labels. Extra labels are also provided for use at the site in Rows 12 and 13.

10.2.3 DLW ID Numbers for Post-Randomization Studies

Prior to starting the study, the CC statistician generates a master list of identification numbers to be used for all post-randomization DLW urine sample sets. These “DLW ID numbers” are similar in format to the true CALERIE ID number used to identify participants screened and enrolled into the study, i.e., a number of the form **ab-wxyz-3**.

The first two digits of the DLW ID number identify the CALERIE site at which the urine set is collected. This part is not encrypted so that the DLW lab can contact the site directly if there are problems or questions with the shipment, the urine vials, the DLW analyses, etc.

The next 4 digits of the DLW ID number are drawn at random from the numbers 1000 to 9999. Unlike the CALERIE ID number, however, there is no particular ordering to these numbers – they are drawn in random order. Moreover, there is no correspondence between the true CALERIE ID number and the DLW ID number. They are completely independent numbering

systems. This is to ensure that the DLW lab is masked to the identity of the CALERIE participant, the protocol time point, and whether it is an original or retest urine sample set.

The suffix "-3" in the DLW ID number is a placeholder for the CALERIE database so that the same number of digits is used for all baseline and post-randomization ID numbers. It also signifies that this is a post-randomization DLW study.

Moreover, because any post-randomization is eligible for the reliability study, two DLW ID numbers are used for each post-randomization DLW study. The first DLW ID number is used for the "test" urine sample set sent to the lab for the first analysis. The second DLW ID number is used for the "retest" urine sample set. It is used to identify the retest sample set if this study is selected for the reliability study. Because both numbers are drawn at random independently of each other, there is no correspondence between them. The lab is therefore masked to the true CALERIE ID number, the protocol time point of the study, and whether the sample is a test or retest sample.

Thus, for example, the pair of DLW ID numbers for the test and retest samples for a participant at site 02 might be 02-8120-3 and 02-7614-3.

10.2.4 Label Sheets for Post-Randomization DLW Studies

The CC prepares label sheets for all post-randomization DLW studies performed in CALERIE. A sufficient supply of post-randomization DLW label sheets is provided to the Study Coordinator at each site at the beginning of Phase 2. Additional supplies are requested from the CC as required during the study.

Label sheets are stored in a file folder in a secure but accessible location at the site. When a new post-randomization DLW study is performed with a participant, the DLW tech removes the next label sheet from the folder and it is used for the study as described below. Once selected, it is stored in an accessible location (e.g., the inner sleeve of the participant's binder or a file folder) so that the individual labels can be retrieved as needed during the DLW study. The used label sheets should also be kept in a secure but accessible location (e.g., the site's tracking log book, or in a secondary log book) in case the backup or retest tracking sheet labels are needed.

10.2.5 Individual Labels on the Post-Randomization Label Sheets

Each label sheet consists of 38 labels corresponding to a complete urine sample set for a post-randomization DLW study. The labels are used as follows. (Please refer to the example in Appendix B).

1. There are 8 labels corresponding to the 8 urine collection cups used to collect the urine samples from the participants. They are located in rows 1-8 under the column labeled "Urine Collection Labels".
2. There are 8 labels corresponding to the set of 8 urine vials sent to the DLW lab for the "test" analysis of this DLW Study. They are located in rows 1-8 under the column labeled "Test Sample Labels".
3. There are 8 labels corresponding to the backup set of 8 urine vials for this DLW study which are stored at the site. They are located in rows 1-8 under the column labeled "Back-up Sample Labels".

   These labels are preprinted with the DLW ID Number corresponding to the test sample, in this example, 02-8102-3. They also include the sample type (i.e., "Lab" vs. "Back-up") and the urine collection time point (i.e., PDa, PDb, …, D14a, D14b).

4. There are 8 labels corresponding to the "retest" set of 8 urine vials for this DLW study which are also stored at the site. They are located in rows 1-8 under the column labeled "Retest Sample Labels".

   These labels are preprinted with the DLW ID Number corresponding to the retest sample, in this example, 02-7614-3. They also include the sample type (i.e., "Lab") and the urine collection time point (i.e., PDa, PDb, …, D14a, D14b). Note that back-up samples for the retest samples are not being stored in CALERIE.

5. Two labels are affixed to the CRF page corresponding to this DLW study. They are located in row 9 under the columns labeled "Test Sample Labels" and "Retest Sample Labels". These labels identify the DLW ID numbers used for test and retest samples, respectively, for this participant in this DLW study.

   When the CRF page is received at the CC, the true CALERIE ID number and protocol time point (as written on the CRF page) are entered into the CALERIE database. Then, the DLW ID number pre-printed on the test label is entered into the CALERIE database. This provides the link between the true CALERIE ID number, the protocol time point and the test DLW ID number. The DLW ID number for the retest sample is also entered. This provides the link between the true CALERIE ID number, the protocol time point and the DLW ID number for the retest sample. It also provides the link between the two DLW ID numbers corresponding to the test and retest samples sent to the DLW lab.

6. One label is affixed to the entry on the DLW Tracking Log maintained at the CALERIE site. This label is located in row 10 under the column labeled "Test Sample Labels". As discussed below, this log provides primary documentation at the site that a urine sample set has been shipped to the DLW lab.

7. One label is held in reserve in case back-up samples must be shipped to the DLW lab. It is located in rows 10 under the column labeled "Back-up Sample Labels". If a set of backup samples is shipped to the DLW lab, then this label is affixed to the DLW Tracking Log as described below as primary documentation.

8. Finally, if this DLW study is chosen for the reliability study, then two labels are used for the shipment of retest urine vials to the DLW lab. They are located in rows 11-12 under the column labeled "Retest Sample Labels". The label in row 11 is affixed to the DLW Retest Tracking Form as described below, while the label in row 12 is affixed to the DLW Tracking Log as described above as primary documentation.

Extra labels are also provided for use at the site in Row 13.

10.2.6 DLW Tracking Log

A log is kept at the CALERIE site providing a tracking mechanism for all DLW urine sample sets sent to the lab for analysis. Please refer to the example in Appendix C.

For each urine sample set sent to the DLW lab, the Research Assistant creates an entry on the tracking log consisting of the following information:

- the true CALERIE ID number
- the participant's initials (as secondary identification)
- the protocol time point (i.e., BL1, BL2, Month 6, 12, 18 or 24) of the DLW study
- the date of the DLW dosing (Day "0")
- whether it is a baseline study; or, for post-randomization studies, whether it is a test or retest urine sample set

- the number of urine vials sent to the DLW lab
- the shipping date to the DLW lab.

The log also contains a space where the “DLW Tracking Log” label corresponding to this particular urine sample set is affixed as described above.

For post-randomization DLW studies, this entry therefore serves as a cross-reference between the true CALERIE ID number, the protocol time point, and the DLW ID number. If the DLW lab seeks clarification on any particular urine sample set, it communicates the shipping date and DLW ID number to the CALERIE site. The site staff member consults this log, and determines the true CALERIE ID number and protocol time point corresponding to this urine sample set. S/He then consults the participant’s records and answers the lab's question (without unmasking the lab).

A similar entry is made for any backup samples shipped to the DLW lab. In this case, the label held in reserve for the backup sample (row 10 under the column labeled “DLW #1 Back-up Labels”) is affixed to the DLW Tracking Log.

10.2.7 DLW Case Report Form for Baseline and Test Samples

For each baseline and “test” urine sample set shipped to the DLW lab for analysis, the Research Assistant completes a DLW CRF and forwards it to the CC. (A CRF page is <u>not</u> needed when a back-up urine sample is shipped to the lab.) This information documents that a DLW study has been performed. It also alerts the CC that it should expect an electronic record from the DLW lab so that the results from all DLW studies are accounted for. The following information is recorded on the CRF:

- the true CALERIE ID number
- the protocol time point of the DLW study (i.e., BL1, BL2, Month 6, 12, 18 or 24)
- date and time of the DLW dosing
- DLW lot number and bottle number on the DLW dose mixture bottle
- exact weight of the DLW mixture (to the nearest 0.01 gm)
- dates and times of the PD urine samples as well as those of the Day 0, 7 and 14 urine samples. These dates are used at the CC to determine whether the DLW study is eligible for a retest study as described below. It also serves as the basis for masking the dates and times for the retest sample as described below.

The CRF also contains a space where the “CRF Page” label(s) corresponding to this particular urine sample set is affixed as described above.

10.3 Roles and Responsibilities

Staff at the CALERIE sites with specific responsibility for overseeing and conducting the DLW studies include the DLW Tech, a Research Assistant, the Study Coordinator, and the Study Manager. These individuals have the following roles and responsibilities.

10.3.1 DLW Tech

- Stores the doubly-labeled water at the site.
- Prints a label sheet for the pair of baseline DLW studies using the template provided by the CC.
- Selects the next label sheet for a post-randomization DLW study.

- Prepares the dose of doubly-labeled water for a new DLW study.
- Administers the dose to the participant.
- Collects the 8 urine samples during a DLW study.
- Transfers urine from the urine collection bottle to properly labeled 2 ml urine vials.
- Stores the urine vials in a freezer at the CALERIE site.
- Creates a shipping spreadsheet summarizing the urine vials being sent to the DLW lab.
- Checks the integrity of the sample vials and other materials included in any shipment to the DLW lab.
- Arranges for shipment of the frozen urine vials to the DLW lab.
- Retrieves the "retest" urine sample vials from the freezer if chosen for the reliability study, and similarly arranges for their shipment to the DLW lab.
- Responds to any problems and questions concerning the DLW studies raised by internal staff at the CALERIE site, the DLW lab or the CC.

10.3.2 Research Assistant

- Completes the CRF form for each DLW study using information provided by the DLW tech.
- Completes and updates the DLW Tracking Log for each DLW study.
- Checks and verifies information entered in the shipping spreadsheet.
- Initials and dates the sample shipping spreadsheet.

10.3.3 Study Coordinator

- Stores the label sheets prepared by the CC for post-randomization studies in a secure but accessible location at the site.
- Informs the DLW tech of upcoming DLW studies for the CALERIE participants.

10.3.4 Study Manager

- Checks and verifies information recorded on the CRF before it is sent to the CC.

10.4Storing Doubly-Labeled Water at the Sites

- Upon receipt of the doubly-labeled water mixtures, the Study Manager verifies the accuracy of the shipment and notifies Dr. William Wong by email (wwong@bcm.tmc.edu) of the shipment.
- The Study Manager turns over the doubly-labeled water mixtures to the DLW Tech so that they can be stored in a secure refrigerator.
- The refrigerator should be restricted to storage of the doubly-labeled water mixtures and non-radioactive study materials.
- No biological samples or laboratory chemicals should be stored in the same refrigerator.

10.5 Conducting a DLW Study

10.5.1 DLW Dose Preparation

The Study Coordinator notifies the DLW tech of a scheduled DLW study. Then, the DLW tech performs the following procedures.

- Prior to the arrival of the study subject, pull out a bottle of DLW dose mixture from the dose refrigerator.
- Record the DLW lot number and bottle number on the DLW dose mixture bottle on the CRF page.
- Obtain the participant's weight as measured on the official clinic scale from the Study Coordinator. It may be the clinic weight recorded at the beginning of the clinic visit <u>provided</u> it occurs on the same day as the DLW dosing. If dosing occurs on a different day, then a new weight must be obtained on the <u>same</u> day as the DLW dosing using the official clinic scale.
- Based on the participant's official clinic weight, calculate the weight of DLW needed by multiplying the body weight of the study subject in kilograms by 1.75 grams / kilogram body weight, i.e.,

  Amt DLW needed (gm) = 1.75 × subject weight (kg).

- **<u>Don't guess or perform mental arithmetic! Use a calculator to perform the multiplication. Confirm the DLW weight by re-performing the multiplication on the calculator.</u>**
- Record the amount on the DLW CRF.
- Put a wide-mouth, clean drinking container on a digital scale with accuracy up to at least two decimal places. Record the weight of the drinking container with cap and tare the scale to zero.
- While the cap of the bottle containing the DLW dose mixture is still closed, whirl the DLW mixture in the bottle several times to make sure the mixture is uniformly mixed.
- Carefully, pour an approximate amount of DLW dose mixture needed into the drinking container. **Be careful not to spill the dose mixture on the side of the drinking container or onto the digital scale or weighing pan**.
- **It is best to pour a little more (but no greater than 5 gm more) of the DLW mixture into the drinking container. Do not use less than the calculated amount.**
- **If spillage occurs, dry out the spillage completely especially if the spillage occurs on the digital scale.**
  - Use a new drinking container to weigh out the dose mixture for the study subject. **<u>Repeat the steps outlined above</u>.**
  - Add the DLW dose mixture that is already poured into the first drinking container to the new drinking container.
  - **However, do <u>not</u> return any DLW dose mixture that is already poured out back into the DLW dose mixture bottle.**
  - **Save the leftover DLW mixture in a separate bottle and label it "LEFTOVER DLW MIXTURE".**
- Cap and return the DLW dose mixture bottle to the dose refrigerator.
- Record the exact weight of the DLW mixture to the nearest 0.01 gm on the CRF. Note that If the container and the cap have already been tared to zero above, this weight will represent the weight of the DLW dose mixture in the container.

10.5.2 DLW Dose Administration

To administer the DLW dose, the DLW tech performs the following procedures**.**

- **First, make sure to collect the two <u>pre-dose</u> urine samples from the study subject using the procedures described below**.
- Then, ask the subject to drink the DLW water. **It is critical that the entire solution is consumed without any loss.**
- Record the date and time of dose administration on the CRF.
- Rinse the DLW drinking container with about 20 ml of drinking water. It is best to keep the drinking water in a plastic squeeze bottle in order to control the rinsing
- Add the drinking water to the container, replace and tighten the lid or cap, and shake the water inside to make sure that any DLW that adheres to the wall of the container or the bottom of the lid or cap is captured by the rinse water.
- Ask the subject to drink the entire amount of the rinse water.
- Repeat the rinsing procedure one more time.

10.5.3 Urine Sample Collection

To collect the sample at a specific urine collection time point, the DLW tech performs the following procedures**.**

- **All sample collection devices must be clean and dry in order to avoid contamination of the samples.**
- At each urine collection time point, prepare the appropriate urine collection bottle or hat, and prepare the appropriate number of 2-ml sample vials with O-ring screw caps. As described above, there are two vials for baseline studies (i.e., one for the lab set, and one for the back-up set), and three vials for any post-randomization study (i.e., one for the lab set, one for the back-up set, and one for the retest set).
- Go to the row of the label sheet corresponding to this urine collection time point, i.e., PD(a), PD(b), …, Day 14(a), Day 14(b). Peel off the labels from the label sheet and affix to the urine collection bottle or hat and the urine vials.
- **Do not collect the first void in the morning!**
- **Do not reuse any of the sample collection devices such as urine cups and transfer pipettes!**
- Instruct the study participants to void into a DRY urine collection bottle or hat and not to contaminate the urine sample with washing water.
- Transfer approximately 1.5 ml of urine (3/4 full) from the urine collection bottle or hat into the corresponding pre-labeled sample vials using a transfer pipette.
- Once the sample has been transferred, cap the sample vials, make sure the caps are properly tightened, and verify the accuracy of the labels on the sample vials.
- Discard the excess urine in the hat and dispose the hat.
- Store the samples at -80^{o}C until ready for shipment to the Central DLW Laboratory.
- Record the date and time of sample collection on the CRF.
- Check the accuracy of the data recorded on the CRF to make sure all the entries are legible and that none of the information is missing.

  The Study Manager verifies the accuracy of the information on the CRF and the identities of the vials stored in the freezer. S/He then signs and dates the CRF.

10.5.4 Urine Sample Storage

To store the urine sample set at the CALERIE site, the DLW tech performs the following procedures**.**

- The urine vials for each DLW study must be arranged in sequence in the freezer according to the CALERIE ID number and protocol time point. Urine vials for test samples (if not already sent), back-up samples and retest samples (for post randomization studies) must be distinguished, so that the appropriate vials can be easily and correctly retrieved for any shipment to the DLW lab.
- All DLW urine samples must be stored at -80°C and placed in the appropriate boxes as soon as possible after they are collected. The 5 x 5 sample storage boxes (Nunc® Cryotube Polyethylene-coated Chipboard Storage Box) containing the backup and retest samples (for post-randomization studies) must be kept in steel sliding racks within the freezer compartment for security and for ease of retrieval.
- Place one set of the sorted "test" samples in a Styrofoam sample box provided by the Central DLW Laboratory and store in the -80°C freezer until shipment.
- Place the other set(s) of the sorted samples in the 5x5 sample storage box
- **For baseline studies**: place the backup sets for both baseline studies for a single participant in a single box. Place the backup samples for the first baseline study in sequence in the first two columns, with four samples per column. Leave an empty column to separate the studies, and place the backup samples for the second baseline study in sequence in the last two columns.
- **For post-randomization studies**: place the backup set and the retest set for each DLW study for a single participant in a single box. Place the backup samples in sequence in the first two columns, with four samples per column. Leave an empty column to separate the sets, and place the retest samples in sequence in the last two columns. **Do not place samples from different participants or from different post-randomization time points in the same box**.
- Label the outside of the box on one side of the lid with the CALERIE ID number, the protocol time point, the masked "test" DLW ID number, and the masked "retest" DLW ID number (for post-randomization studies) using a permanent (e.g. Sharpie) marker.
- Place the storage box in the next available slot in the steel rack and slide into the -80°C freezer, and record its location (including the same information on the outside of the box) on the freezer "map" kept in the logbook at the site.

The storage boxes that will fit perfectly in the steel racks are Fisher Cat. No. 12-565-183, NNI No. 378247; these racks have 8 slots that each measure 3"x6"x6", and one can fit 6 boxes per slot. However, other sites may have something different, and we may want to leave the specific brand/style of storage box up to the sites.

10.5.5 Shipping Spreadsheet

Prior to shipping urine sample sets to the DLW lab, the DLW tech creates a new Excel spreadsheet providing a complete accounting of all urine samples being forwarded to the DLW lab. The spreadsheet is created from a template provided by the DLW lab. **Because the lab performs a complete verification of all urine vials sent, there is an entry in the spreadsheet for each urine vial included in the shipment.** Thus, there are up to 8 rows for any DLW study corresponding to the 8 time points at which urine is collected. The columns of the spreadsheet consist of the following fields:

- Urine vial ID number. For example, this would be 03-0025-1-PDa through 03-0025-1-D14b for a baseline study; or, 02-8120-3-PDa through 02-8120-3-D14b for a post-randomization study.
- Participant's weight (kg)
- Participant's height (cm)
- DLW lot number and bottle number on the DLW dose mixture bottle
- Exact weight of the DLW mixture (to the nearest 0.01 gm)
- Date (mm/dd/yyyy format) and time (24-hr format) the dose was administered
- Date (mm/dd/yyyy format) and time (24-hr format) the urine sample was collected

The spreadsheet is saved in a folder called the "DLW Shipping Spreadsheets" on a server at the CALERIE site. The file is named according to the following convention: "DLW Shipping Spreadsheet – mm-dd-yyyy.XLS" (without the quotation marks), where mm-dd-yyyy is the date when the shipment is shipped to the DLW lab.

10.5.6 Urine Sample Set Shipment to the DLW Lab

To ship DLW samples to the central lab, the DLW Tech performs the following procedures.

- **Samples for the two baseline DLW studies should be shipped to the Central DLW Laboratory as soon as both DLW studies for that participant are complete**.
- For the post-randomization DLW studies, the samples should be shipped to the Central DLW Laboratory as soon as three DLW studies (from three different participants) are complete.
- Only the "Lab" set of urine vials are shipped to the Central DLW Laboratory. The "Backup" set of samples are stored at the study site.
- No samples are shipped during holidays.
- Print the shipping Excel spreadsheet corresponding to this shipment
- Prior to shipment, the DLW Tech checks the integrity of the sample vials to make sure that the caps are properly secured, that the vials are properly labeled, and that the samples match the list on the sample shipping spreadsheet. If all is correct, the Research Assistant initials and dates the sample shipping spreadsheet.
- To conserve dry ice, pack the samples 1-2 hr prior to the scheduled pickup by Federal Express.
- When samples are ready for shipment, obtain the weight of an empty Styrofoam shipping container.
- Put a layer of dry ice at the bottom of the Styrofoam shipping container.
- Secure the lid and the body of the sample Styrofoam box containing the urine vials with couple of rubber bands and place the box on top of the dry ice (both the sample Styrofoam boxes and shipping containers are provided by the Central DLW Laboratory prior to the commencement of the DLW protocol).
- Add more dry ice on top and around the sample Styrofoam box(es).
- Reweigh the shipping container with the dry ice and sample box(es) to get an approximate weight of the dry ice. The information is needed in order to make shipment with dry ice.

- To minimize movement inside the shipping container, use some crumbled paper to take up free spaces above and around the sample Styrofoam box(es).
- Put the hard copy of the Excel shipping spreadsheet in an envelope and place it on top of the crumbled paper.
- Tape shut the lid of the shipment container with shipping tape.
- Put the shipping container inside the cardboard box that comes with the shipping container.
- Tape the cardboard box shut with shipping tape.
- Paste one of pre-printed and pre-paid Federal Express mailing labels on top of the package (the pre-printed and pre-paid Federal Express mailing labels are provided by the Central DLW Laboratory).
- Put the weight of the dry ice on a "Dry Ice" shipping label and paste it on top of the package (the pre-printed "Dry Ice" shipping labels are provided by the Central DLW Laboratory).
- It is important to put the completed "Dry Ice" shipping label on the package or Federal Express would not pick it up for delivery.
- Take the package to the Federal Express pickup location.
- Samples are shipped by express courier for overnight morning delivery and with sufficient dry ice to keep the samples frozen overnight.
- Packages are shipped on Monday, Tuesday or Wednesday in order to allow sufficient time for the study site and the Central DLW Laboratory to track the shipment in case we run into a delivery delay.
- **In addition to the hard copy included in the shipping box, the Excel spreadsheet described above is attached to an e-mail message sent to Dr. William Wong and Ms. Lucinda Clarke at the following email addresses**:

  William Wong:
  Email: wwong@bcm.tmc.edu
  Tel: 713-798-7168
  Fax: 713-798-7194

  Lucinda Clarke:
  Email: lucindac@bcm.tmc.edu
  Tel: 713-798-7122
- The Fedex tracking number is included in the body of the email message.
- Samples are shipped to the following address using the provided prepaid Federal Express labels.

  Lucinda Clarke
  USDA/ARS Children's Nutrition Research Center
  1100 Bates Avenue
  Houston, Texas 77030
  Tel: 713-798-7122

10.6 Reliability Study Procedures

10.6.1 Eligibility Criteria

DLW urine sample sets must satisfy the following eligibility criteria:

- Only post-randomization DLW studies (at Months 6, 12, 18 and 24 for CR participants, and at Months 12 and 24 for controls) are eligible for this study. Thus, only studies whose DLW ID number contains the suffix "-3" are selected.
- Only DLW urine samples with a sufficient amount of urine, i.e., at least 1.5 ml each at all 8 urine collection time points, are included. This is determined by staff at the site.
- Must adhere to the standard Day 0, 7, 14 protocol to avoid unmasking the DLW lab. This is determined by checking the dates of the urine collection time points as recorded on the CRF. Those failing this criterion are screened out by the CC.

10.6.2 DLW Retest Tracking Form

For each DLW study selected for the reliability study, the CC generates from the database a two-page tracking form. It consists of the following fields:

- the true CALERIE ID number and protocol time point for the selected DLW study
- the "test" DLW ID number as affixed to the CRF page and entered into the database
- the "retest" DLW ID number as affixed to the CRF page and entered into the database.
- The masked date and time of the DLW dosing as described below.
- The masked dates and times of the urine collection as described below.
- The masked weight of the participant as described below.

The Site Management group at the DCRI forwards this report to the sites, and in this way notifies the site that this DLW study has been selected for the reliability study.

10.6.3 Procedures

The following procedures are performed by the statistician at the DCRI to select the samples for the reliability study.

1. At periodic intervals in calendar time, the CALERIE database is temporarily "locked." The exact time and date when this occurs is recorded by the DM group at the CC for future reference.
2. Every CRF entered into the master database at the CC is "stamped" with the time and date when it was entered. Thus, by manipulating the two dates, the CC statistician identifies DLW data forms that have been newly entered into the CALERIE database since the previous data lock.
3. Samples are selected when approximately 120 new post-randomization DLW studies have accumulated since the previous samples were selected. It is expected that the first batch of 120 post-randomization samples will accrue approximately 15 months after the first participant has been enrolled into the study. Thereafter, 120 new post-randomization DLW studies are expected to accrue every 4-6 months in calendar time.
4. S/He compiles a complete list of all post-randomization test DLW studies whose DLW CRF page has been entered into the CALERIE database over the intervening period. The list includes the CALERIE ID number and the protocol time point of each DLW study.

5. Any ineligible DLW studies according to the criteria above are removed from the list.
6. An 8% sample of the remaining eligible DLW studies is selected at random from the list of eligible samples. The selection is stratified by site so that approximately 8% of the eligible samples at each site is selected.

Then, the following procedures are performed by the CC statistician to mask the identity of the retest samples.

7. The date of the DLW dosing is offset by a number of days drawn at random from a range from 14 days prior to 14 days after the true date of the DLW dosing.
8. The time of the DLW dosing is offset by a number of minutes drawn at random from 60 minutes before to 60 minutes after the true time of the DLW dosing.
9. The dates and times of the urine collection time points are calculated from this "DLW dosing" date and time so that **the elapsed times relative to the DLW dosing are exactly the same as that observed in the true DLW study**.
10. The participant's weight at the DLW dosing is offset by an amount drawn at random from 1.0 kg less to 1.0 kg more that the participant's true weight at the DLW dosing. It is rounded to 0.1 kg.
11. The DLW Retest Tracking Form containing this information is prepared by the CC Site Management group at the DCRI. It is forwarded to DLW Tech at the CALERIE site as described above.

The following procedures are performed by the DLW tech:

12. Go to the freezer, and locate the container of urine samples corresponding to this CALERIE ID at this protocol time point.
13. Verify the test and retest DLW ID numbers as written on the container against the numbers printed on the DLW Retest Tracking Form from the CC. This is the site check that the right samples are being sent to the DLW lab.
14. Verify that there is sufficient quantity of urine in all the "retest" urine vials to perform a DLW study. If the quantity is inadequate, this is entered onto the DLW Retest Tracking Form, and it is FAXed back to the CC. The CC then selects another eligible post-randomization study at random from the same site and starts anew.
15. Otherwise, retrieve the retest urine sample set and ship it to the DLW lab as described above.
16. Make an entry in the DLW Tracking log. **The date of the DLW dosing is the masked date provided by the CC in the DLW Retest Tracking form**.
17. Affix the "DLW Tracking Log" label (i.e., Row 12 under "Retest Sample Labels") to the DLW Tracking Log as described above.
18. Make entries in the Excel spreadsheet for each retest urine vial. **The participant's weight, the date and time of the DLW dosing, and the dates and times of the urine collections are the masked versions provided by the CC in the DLW Retest Tracking form**.
19. Affix the "Retest Tracking Form" label (i.e., Row 11 under "Retest Sample Labels") to the DLW Retest Tracking Form and include it in the next batch of CRFs sent to the DCRI. The CC enters this form into the database, and a computer check is made that the DLW ID number printed on the DLW Retest Tracking Form at the CC matches the DLW ID number on the label affixed at the site.

10.6.4 Fields to be Analyzed in the Reliability Study

The follow data fields are included in the formal DLW reliability study. The exact statistical analyses will be outlined in a separate Statistical Analysis Plan.

1. Total Energy Expenditure (TEE)
   - Mean of 14 days using regression method
2. Dilution spaces & ratio
   - Nd
   - No
   - Nd/No
3. Elimination Rates
   - Kd
   - Ko
4. rCO_2

Appendix A:

Sample Label Sheet for a Pair of DLW Studies

Performed at Baseline

Row	Identifier	DLW #1 Urine Collection	DLW #1 Lab Labels	DLW #1 Back-up Labels	DLW #2 Urine Collection	DLW #2 Lab Labels	DLW #2 Back-up Labels
1	PD(a)	03-0025-1 Collection PDa	03-0025-1 Lab PDa	03-0025-1 Backup PDa			
2	PD(b)	03-0025-1 Collection PDb	03-0025-1 Lab PDb	03-0025-1 Backup PDb			
3	Day 0(a)	03-0025-1 Collection D0a	03-0025-1 Lab D0a	03-0025-1 Backup D0a	03-0025-2 Collection D0a	03-0025-2 Lab D0a	03-0025-2 Backup D0a
4	Day 0(b)	03-0025-1 Collection D0b	03-0025-1 Lab D0b	03-0025-1 Backup D0b	03-0025-2 Collection D0b	03-0025-2 Lab D0b	03-0025-2 Backup D0b
5	Day 7(a)	03-0025-1 Collection D7a	03-0025-1 Lab D7a	03-0025-1 Backup D7a	03-0025-2 Collection D7a	03-0025-2 Lab D7a	03-0025-2 Backup D7a
6	Day 7(b)	03-0025-1 Collection D7b	03-0025-1 Lab D7b	03-0025-1 Backup D7b	03-0025-2 Collection D7b	03-0025-2 Lab D7b	03-0025-2 Backup D7b
7	Day 14(a)	03-0025-1 Collection D14a	03-0025-1 Lab D14a	03-0025-1 Backup D14a	03-0025-2 Collection D14a	03-0025-2 Lab D14a	03-0025-2 Backup D14a
8	Day 14(b)	03-0025-1 Collection D14b	03-0025-1 Lab D14b	03-0025-1 Backup D14b	03-0025-2 Collection D14b	03-0025-2 Lab D14b	03-0025-2 Backup D14b
9	CRF Page		03-0025-1 Lab CRF Page			03-0025-2 Lab CRF Page	
10	DLW Tracking Log		03-0025-1 Lab Site Log	03-0025-1 Backup Site Log		03-0025-2 Lab Site Log	03-0025-2 Backup Site Log
11							
12	Extra Labels		03-0025-1	03-0025-1		03-0025-2	03-0025-2
13	Extra Labels		03-0025-1	03-0025-1		03-0025-2	03-0025-2

Appendix B:

Sample Label Sheet for a Post-Randomization

DLW Study

Row	Identifier	Urine Collection Labels		Test Sample Labels	Back-up Sample Labels		Retest Sample Labels
1	PD(a)	02-8120-3 Collection PDa		02-8120-3 Lab PDa	02-8120-3 Backup PDa		02-7614-3 Lab PDa
2	PD(b)	02-8120-3 Collection PDb		02-8120-3 Lab PDb	02-8120-3 Backup PDb		02-7614-3 Lab PDb
3	Day 0(a)	02-8120-3 Collection D0a		02-8120-3 Lab D0a	02-8120-3 Backup D0a		02-7614-3 Lab D0a
4	Day 0(b)	02-8120-3 Collection D0b		02-8120-3 Lab D0b	02-8120-3 Backup D0b		02-7614-3 Lab D0b
5	Day 7(a)	02-8120-3 Collection D7a		02-8120-3 Lab D7a	02-8120-3 Backup D7a		02-7614-3 Lab D7a
6	Day 7(b)	02-8120-3 Collection D7b		02-8120-3 Lab D7b	02-8120-3 Backup D7b		02-7614-3 Lab D7b
7	Day 14(a)	02-8120-3 Collection D14a		02-8120-3 Lab D14a	02-8120-3 Backup D14a		02-7614-3 Lab D14a
8	Day 14(b)	02-8120-3 Collection D14b		02-8120-3 Lab D14b	02-8120-3 Backup D14b		02-7614-3 Lab D14b
9	CRF Page			02-8120-3 Lab CRF Page			02-7614-3 Lab CRF Page
10	DLW Tracking Log			02-8120-3 Lab Site Log	02-8120-3 Backup Site Log		
11	Retest Tracking Form						02-7614-3 Lab CRF Page
12	DLW Tracking Log						02-7614-3 Lab Site Log
13	Extra Labels	02-8120-3		02-8120-3	02-8120-3		02-7614-3

Appendix C:

DLW Tracking Log

CALERIE ID No.	Partici-pant's Ini-tials	Protocol Time Point[1]	Date of DLW Dosing (mm/dd/yyyy)	(B)aseline, (T)est) or (R)etest	No. Urine Vials Shipped	Shipping Date (mm/dd/yyyy)	Affix "Tracking Log" Label here

1. BL1, BL2, M6, M12, M18, M24

Appendix D:
Baseline DLW
Studies Submission
Form

DLW Baseline studies submission form (Site_DLW_Shipping_Spreadsheet-MM-DD-YYYY.xls)

Site	Subject ID	Study Sequence	Sample Collection Sequence	Sample Date (MM/DD/YYYY)	Sample Time (24 hr)	Baseline Y/N	Body Weight (kg)	Height (cm)	DOB (MM/DD/YYYY)	Dose/Bottle ID	Dose amount (g)	RQ or FQ	Dosing time (24h)	Dosing Date (MM/DD/YYYY)	Gender	Ethnicity
		1	PDa			Y										
			PDb			Y										
			D1a			N										
			D1b			N										
			D7a			N										
			D7b			N										
			D14a			N										
			D14b			N										

Site	Subject ID	Study Sequence	Sample Collection Sequence	Sample Date (MM/DD/YYYY)	Sample Time (24 hr)	Baseline Y/N	Body Weight (kg)	Height (cm)	DOB (MM/DD/YYYY)	Dose/Bottle ID	Dose amount (g)	RQ or FQ	Dosing time (24h)	Dosing Date (MM/DD/YYYY)	Gender	Ethnicity
		2	PDa			Y										
			PDb			Y										
			D1a			N										
			D1b			N										
			D7a			N										
			D7b			N										
			D14a			N										
			D14b			N										