8. LABORATORY COORDINATING CENTER PROCEDURES

To ensure the integrity of all samples sent to the Laboratory Coordinating Center (LCC) from field centers for analysis or storage, the procedures described here will be used at all times.

Two aliquots of urine for each mother and child will be shipped frozen on dry ice to the LCC.

A maximum of twelve aliquots of blood, plasma or serum per child will be shipped frozen on dry ice to the LCC, one each for the fasting, 30-minute, 1-, and 2-hour plasma glucose determinations from the OGTT, one from each of the fasting, 30-minute, 1-, and 2-hour insulin samples, and one each for lipids, hemoglobin A_{1C}, a storage sample, and DNA (if consented to). The fasting lipids aliquot will also be assayed for hsCRP. In addition, the backup aliquot for each of these will be stored in the field center laboratory and sent in the next shipment to the LCC for storage until they are transferred to the NIDDK Central Repository.

A maximum of eight aliquots of blood, plasma or serum per mother will be shipped frozen on dry ice to the LCC, one each for the fasting and 2-hr plasma glucose determinations from the OGTT and one each of the fasting insulin, lipids, A1c, and DNA (if consented to), and one from the fasting and 2-hr storage samples. In addition, the backup aliquot for each of these will be stored in the field center laboratory and sent in the next shipment to the LCC for storage until they are transferred to the NIDDK Central Repository.

8.1 Receipt of Samples

Upon receipt of a shipment of samples, the LCC Research Technician will note the name of the field center, date of dispatch, tracking number, date and time of receipt on the Receipt Form (see last page of this chapter). The Box ID numbers on the freezer

boxes and on the Shipping Grid pages will be compared and any discrepancies will be noted. Both the field center and the Project Manager will be notified of receipt of the shipment and also of any discrepancies or problems.

8.1.1 Sample Tracking in BC SAMPLE

Sample locations are tracked for the duration of the HAPO Follow-Up Study in BC SAMPLE databases (BC Platforms, headquarters Espoo, Finland) by uploading Excel files created for each freezer box. An Excel Shipping Receipt File with columns labeled Box ID, Row, Column, Sample ID, Shipping Grid ID, Color, Match and Flag will be used to record sample locations in the freezer box. Row and Column will be pre-populated with entries A through J and 1 through 10, respectively, to represent all locations within a freezer box. For each freezer box, the Box ID will be scanned into the first cell of the Box ID column, and all entries for that column will be programmed to auto-fill with the same value. Each of the tubes or cryovials in the freezer box will be scanned into the file in the Sample ID column in the fields corresponding to the appropriate Row and Column values. If there are any tubes or cryovials that are broken, that have leakage, or that have thawed, this will be noted on the Receipt Form and in the Excel file in the Flag column. For cryovial samples, the Excel file will be programmed to list the cryovial color in the corresponding cell of the Color column that matches the sample code and the Research Technician will make sure that the cryovial color matches the color that appears in the Excel file as each sample is scanned in. After each sample in the freezer box has been scanned into the file, each of the sample IDs on the Shipping Grid for the box will then be scanned into the file in the Shipping Grid ID column for each box position. The Excel file will be programmed to perform a cross-check to make sure that the grid matches what is actually in the box and if the values match an entry of ‘Yes’ will appear in the Match column of the Excel file. If there are any discrepancies or missing samples, these will be noted and the information will be forwarded to the Project Manager and the field center. For boxes of urine, backup and DNA samples, the Shipping Receipt File will be directly uploaded into BC SAMPLE for long-term sample tracking. Analysis freezer boxes will be sorted and new Excel files will be created before uploading into BC SAMPLE. Specific instructions are given in Sections 8.5, 8.6, and 8.7.

8.2 Handling of Urine Samples

Boxes containing analysis and backup urine samples will be stored separately from the other freezer boxes in the -80◦ C Backup freezer. Analysis urine samples will be stored on a separate shelf from the backup urine samples. Shipping Receipt Files for urine samples will be uploaded into BC SAMPLE and assigned to the appropriate freezer, shelf and rack location using the Storage panel of BC SAMPLE. Only the columns labeled Box ID, Row, Column, Sample ID and Flag will be retained in BC SAMPLE; the columns labeled Shipping Grid ID, Color and Match will not. Analysis and Backup urine samples will be sent in separate shipments to the NIDDK Central Repository.

8.3 Handling of Backup Samples

Backup sample freezer boxes will be placed on a separate shelf in the -80◦ C Backup freezer for storage until needed for assay purposes or transfer to the NIDDK Central Repository. Shipping Receipt Files for backup samples will be uploaded into BC SAMPLE and assigned to the appropriate freezer, shelf and rack location using the Storage panel of BC SAMPLE. Only the columns labeled Box ID, Row, Column, Sample ID and Flag will be retained in BC SAMPLE; the columns labeled Shipping Grid ID, Color and Match will not.

8.3.1 Duplicate Samples

In the original HAPO Study, a random 5% of IDs were chosen to have duplicate sample collection. In this follow-up study, the same IDs will have duplicate analysis of their backup samples. As the backup samples are scanned into the Shipping Receipt Excel Files, those IDs that have been preselected for duplicate analysis will be flagged using an Excel macro that will automatically generate a ‘duplicate’ character value in a separate column labeled ‘Duplicate’. Those that are flagged will be placed into separate

NMH Analysis Sample or Clinical Research Unit Analysis “duplicate” boxes and scanned into the relevant Excel file.

8.4 Handling of DNA Samples

DNA sample freezer boxes will be stored in the -80◦ C Pre-Analytic freezer in the LCC laboratory. Shipping Receipt Files for DNA samples will be uploaded into BC SAMPLE and assigned to the appropriate freezer, shelf and rack location using the Storage panel of BC SAMPLE. Only the columns labeled Box ID, Row, Column, Sample ID and Flag will be retained in BC SAMPLE; the columns labeled Shipping Grid ID, Color and Match will not.

DNA sample freezer boxes will be transferred periodically to the Center for Genetic Medicine (CGM) at the Northwestern University Feinberg School of Medicine for DNA preparation. Once the DNA has been prepared, the samples will be returned to the LCC Research Technician who will scan them into Backup Freezer DNA Sample Excel Files. The freezer boxes will be stored in the -80◦ C Backup freezer in the freezer farm for temporary storage until transfer to the NIDDK Central Repository. The Excel files for the prepared DNA samples will be uploaded into BC SAMPLE and assigned to the appropriate freezer, shelf and rack location using the Storage panel of BC SAMPLE.

8.5 Handling of the Analysis Aliquot of the Storage Samples

Serum samples for storage are collected at the fasting draw for children and at the fasting and 2-hour draw for the mothers. Two aliquots are made of each, one analysis and one backup. The analysis aliquots of the storage samples will be pulled from the Analysis boxes and placed into separate Storage Analysis Sample freezer boxes, and then placed on a separate shelf in the -80◦ C Backup freezer for storage until eventual transfer to the NIDDK Central Repository. Sample locations will be recorded in an Excel Storage Analysis Sample File with column headings of Box ID, Row, Column, Sample ID and Sample Type. Row and Column entries will be pre-populated with values of A

through J and 1 through 10, respectively. The samples should be scanned in on the line corresponding to their position in the freezer box. The corresponding Sample Type cell will be programmed to auto-fill based on Sample ID. When the box is full and the Excel file is complete, it will be uploaded into BC SAMPLE and mapped to the correct Freezer, Shelf and Rack position using the Storage panel of BC SAMPLE. The backup aliquots of the storage samples will be handled in the same way as the backup aliquots of other types of samples (Section 8.3).

8.6 Handling of Samples for Analysis at Northwestern Memorial Hospital (NMH)

Samples to be analyzed in the Clinical Chemistry Laboratory (CCL) of Northwestern Memorial Hospital (NMH) (glucose, lipids/hsCRP, A1c) will be pulled from the Analysis freezer boxes and placed into separate NMH Analysis Sample freezer boxes. There will be a total of 10 analysis cryovials for each mother/child pair if all samples are collected. They should all be placed into the next available row in the NMH Analysis Sample freezer box, e.g., placed into the slots A1-A10. If any of the cryovials for the pair is missing, there will be an empty slot(s) in that row. When the analysis cryovials for the next pair are added to the NMH Analysis Sample freezer box, they should be placed into the next row, e.g., B1-B10. Sample locations will be recorded in an Excel NMH Analysis Sample File with column headings of Box ID, Row, Column, Sample ID and Sample Type. Row and Column entries will be pre-populated with values of A through J and 1 through 10, respectively. For each box position, the Sample ID should be scanned into the NMH Analysis Sample File on the line corresponding to the correct Row and Column entries. The Sample Type cells will be programmed to automatically generate the appropriate sample type based on the Sample ID. When the box is full and the Box ID, Row, Column, Sample ID and Sample Type columns are complete, the Excel file will be uploaded into BC SAMPLE and mapped to the correct Freezer, Shelf and Rack position using the Storage panel of BC SAMPLE. The BC SAMPLE database will contain three additional columns labeled Lab ID, Date Samples Sent to Lab and Date Data Retrieved. These will be filled in later in order to track processing of all samples in the lab. The NMH Analysis Sample freezer boxes will be stored in the -80◦ C Pre-Analytic freezer and one box per weekday will be taken to the hospital lab for analysis.

8.6.1 Sample Retrieval

During the morning of the day before the samples from an NMH Analysis Sample freezer box are transferred to NMH, the Research Technician will remove the box from the freezer and allow the samples to thaw at room temperature. Once the samples are thawed, they will be centrifuged and an aliquot of each (500 uL, if possible) will be transferred into NMH analysis tubes and the NMH ID label will be attached. The ID will be scanned into the Lab ID column in BC SAMPLE on the line that matches that sample's HAPO ID. The NMH tubes will be placed on NMH racks and placed in the Cold Room for storage until transfer the next morning to NMH. The tubes should remain in the vertical position. When samples are taken to the hospital lab, the date should be recorded in BC SAMPLE in the Date Samples Sent to Lab column.

The residual from each sample will be returned to its original location in the NMH Analysis Sample freezer box and will be transferred to the -80◦ C Backup freezer for longterm storage. When an NMH Analysis Sample freezer box is transferred to the -80◦ C Backup freezer, its associated Excel file will be uploaded into BC SAMPLE and mapped to the correct Freezer, Shelf and Rack position using the Storage panel of BC SAMPLE.

8.6.2 Preparation for Transfer to Northwestern Memorial Hospital

In the morning, the NMH racks will be carried to NMH and deposited at the Clinical Chemistry Laboratory.

8.6.3 Preparation of Requisition Forms

On the NMH Requisition Form, the first name will be 'HAPO'. The last name will be the HAPO Follow-Up Study vial code, e.g. '600' for fasting child glucose. The date of birth

will be ‘99/99/1960’ for the mother and ‘99/99/2000’ for the child. Gender will be ‘female’ for all.

8.6.4 Sample Analysis

All tests will be performed on Beckman-Coulter SYNCHRON LX analyzers using standard methodologies.

8.6.4.1 Glucose Analysis

Glucose will be measured on plasma samples. The estimation of glucose concentration will be carried out by an oxygen rate method employing a Beckman-Coulter oxygen electrode. A precise volume of sample (10 uL) is injected in a reaction cup containing a glucose oxidase solution. The ratio used is one part sample to 76 parts reagent. The peak rate of oxygen consumption is directly proportional to the concentration of glucose in the sample.

8.6.4.2 Lipid Analysis

Lipids (total cholesterol, HDL cholesterol, triglycerides) will be measured on a serum sample. Cholesterol concentration is measured with CHOL reagent in a timed-endpoint method. Cholesterol esterase (CE) hydrolyzes cholesterol esters to free cholesterol and fatty acids. Free cholesterol is oxidized to cholestene-3-one and hydrogen peroxide by cholesterol oxidase. Peroxidase catalyzes the reaction of hydrogen peroxide with 4-aminoantipyrine and phenol to produce a colored quinoneimine product.

The SYNCHRON System automatically proportions the appropriate sample and reagent volumes into the cuvette. The ratio used is one part sample to 100 parts reagent. The system monitors the change in absorbance at 520 nanometers. This change in absorbance is directly proportional to the concentration of cholesterol in the sample and is used by the System to calculate and express the cholesterol concentration.

8.6.4.3 hsCRP Analysis

For children participating in the HAPO Follow-Up Study, the lipid sample will also be assayed for hsCRP. High sensitivity C-reactive protein (hsCRP) will be measured using high sensitivity CRPH reagent on the serum sample by rate turbidimetry using the SYNCHRON System. The CRPH reagent is based on the highly sensitive Near Infrared Particle Immunoassay rate methodology. An anti-CRP antibody-coated particle binds to CRP in the serum sample resulting in the formation of insoluble aggregates causing turbidity.

The SYNCHRON System automatically proportions the appropriate sample and reagent volumes into a cuvette. The ratio used is one part sample to 26 parts reagent. The system monitors the change in absorbance at 940 nanometers. This change in absorbance is proportional to the concentration of C-reactive protein in the sample and is used by the System to calculate and express CRP concentration based upon a single-point adjusted, pre-determined calibration curve.

8.6.4.4 Hemoglobin A1c Analysis

Hemoglobin A1c (hbA1c) is measured using the hemoglobin A1c reagent kit in conjunction with SYNCHRON Systems HbA1c calibrators and hemolyzing reagent and provides a quantitative determination of HbA1c concentration as a percentage of total hemoglobin in human whole blood. The hemolyzing reagent is brought to room temperature prior to use. Hemolyzing reagent (1000 uL) is pipetted into a test tube, 10 uL of whole blood sample is added and gently mixed to ensure a uniform distribution of erythrocytes. Complete hemolysis is indicated by a color change from red to brown-green (approximately 1-2 minutes). The sample is then analyzed. The hemolysate is stable for 4 hours at room temperature.

The SYNCHRON System utilizes two unique cartridges, Hb and A1c, to determine hbA1c concentration as a percentage of total hemoglobin. Hemoglobin reagent is used to measure the hbA1c concentration by a turbidimetric immunoinhibition method. In the

reaction, hbA1c antibodies combine with hbA1c from the sample to form soluble antigen-antibody complexes. Polyhaptens from the reagent then bind with the excess antibodies and the resulting agglutinated complex is measured volumetrically. The SYNCHRON System proportions the appropriate sample and reagent volumes into the cuvette. The ratio used is one part sample to 31.6 parts reagent. The system monitors the change in absorbance at 340 nanometers. This change in absorbance is inversely proportional to the concentration of hbA1c in the sample and is used by the SYNCHRON System to calculate and express hbA1c concentration as a percentage of total hemoglobin.

8.6.5 Quality Control

The Clinical Chemistry Laboratory (CCL) utilizes robust quality control and quality assurance protocols which have been accredited by the College of American Pathologists (CAP) that ensures the accuracy of the reported results. The CAP Laboratory Accreditation Program is an internationally recognized program designed to go well beyond regulatory compliance to ensure that laboratories achieve the highest standards of excellence in patient care. The CCL also participates in a CAP Proficiency Program that allows clinical laboratories to regularly (monthly) evaluate their performance and ensure the accuracy of the patient results they provide. They will forward these reports to the DCC on a monthly basis.

The CCL is certified by the Clinical Laboratory Improvement Amendments (CLIA), a program mandated by the Centers for Medicare and Medicaid Services whose objective is to ensure quality laboratory testing in the United States.

8.6.6 Transfer of Results from Northwestern Memorial Hospital

Lab values generated at Northwestern Memorial Hospital are routinely deposited into a Cerner production domain. Everyday at 5am, the Enterprise Data Warehouse (EDW) pulls all data from Cerner that were updated the previous day. Lab values will be retrieved directly from the EDW by the Data Coordinating Center. All values will undergo initial checks to ensure appropriate values for each sample type. The Data Coordinating

Center will then record the appropriate date in the Date Data Retrieved Column of BC SAMPLE and the lab data will be merged with all other data.

8.7 Handling of Insulin Samples for Analysis at the Clinical Research Unit

Samples for insulin analysis will be placed into separate Insulin Analysis Sample freezer boxes in sets of 87 samples per box. Sample locations will be recorded in a Clinical Research Unit (CRU) Analysis Sample File with column headings of Box ID, Row, Column, Sample ID, Sample Type and Value. Row and Column entries will be pre-populated with values of A through I and 1 through 9, respectively. The samples will be scanned into the Sample ID column of the CRU Analysis Sample File, one file per box, in the line that matches the appropriate pre-populated Row and Column positions. Entries for Sample Type will be automatically generated as described for the NMH Analysis Sample Files. When a box is full and the Excel file is complete, it should be uploaded into BC SAMPLE.

When insulin samples are taken to the Clinical Research Unit the relevant Excel files will be transmitted to the Clinical Research Unit. The date should be recorded in BC SAMPLE in the column labeled Date Samples Sent to Lab. Once the samples have been analyzed, the Excel files will be returned to the Laboratory and Data Coordinating Centers with the results recorded in the Value column. The date of transfer should be recorded in the Date Data Retrieved column of BC SAMPLE and the data will be merged with all other data by the Data Coordinating Center.

8.7.1 Method of Analysis

Serum insulin will be measured by radioimmunoassay (RIA) using a Human Insulin Specific RIA kit (HI-14K) from Millipore, 290 Concord Road, Billerica, MA 01821, USA. The instrumentation used to count the radioactivity will be a Cobra II Perkin Elmer Auto-Gamma Counter.

In the Millipore insulin radioimmunoassay a fixed concentration of labeled tracer antigen (^{125}Iodine human insulin) is incubated with a constant dilution of antiserum (human insulin antiserum) such that the antigen binding sites on the antibody are limited. When unlabeled antigen is added to this system there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. The amount of radiolabeled tracer bound to the antibody will decrease as the concentration of unlabeled antigen increases. This is measured after separating antibody-bound from free tracer by double antibody/PEG technique and counting the radioactivity in the precipitate fraction. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated.

This assay will be carried out strictly according to the manufacturer's instructions and the Clinical Research Unit's Standard Operating Procedures.

8.7.2 Sample Preparation

Serum samples for insulin will be stored frozen in the Laboratory Coordinating Center and transferred on dry ice biweekly to the Clinical Research Unit Core Laboratory in boxes of 87 samples per Insulin Analysis Sample freezer box. While at the CRU Core Lab they will be stored in a monitored -80◦ C freezer until thawed for assay. The CRU will be provided both an electronic and hard copy spreadsheet containing the identification numbers of the samples in each box.

Insulin samples will be removed from the -80◦ C freezer and thawed at room temperature on the day of assay. They will be gently mixed, notation of any hemolysis will be made and the tubes centrifuged at 4◦ C at 3000 rpm for 10 minutes. Samples will then be kept on ice and then returned to the -80◦ C freezer immediately after completion of the assay set-up pipetting.

8.7.3 Sample Analysis

All samples will be assayed in duplicate. Samples coded 601 and 701 (fasting samples) will be assayed undiluted and will have a reportable insulin range of 2-200 µU/mL. All samples coded 651, 611, and 621 (post ingestion of Trutol) will be assayed at a dilution of 1:2 to allow a reportable insulin range of 4-400 µU/mL. This dilution will minimize the need for repeat assay by expanding the reporting range of the stimulated samples. Standards at levels 100 and 200 µU/mL will also be diluted 1:2 for each assay to confirm dilution linearity.

If the assay results of a sample show a coefficient of variation greater than 10% then that sample will be re thawed and repeated in another assay. If a sample has an insulin result greater than the range of the assay then it will be repeated at a greater dilution. Previous studies have indicated no difference in insulin levels with as many as three thaws of a sample.

8.7.4 Quality Control

Insulin quality controls are provided with the Millipore kits at three levels, low (1.16-2.40 µU/mL), mid-range (~10 µU/mL) and high (~40 µU/mL). For each assay both controls will be assayed twice in duplicate, first, immediately after the standard curve and again at the end of the assay. Each lot lasts approximately 18 months. When new lot numbers are released they will be run alongside the old lot for at least five assay runs.

Assay performance will be assessed for acceptance using the parameters of B0 binding, slope and intercept of log-logit plot, ED_{80} - ED_{50} - ED_{20} points on the standard curve, and quality controls prior to release of data. If both levels of quality controls lie outside the established range (fixed mean ± 2SD) then the assay will be repeated.

8.7.5 Reporting of Insulin Results

The insulin results will be reported electronically on the spreadsheet provided by the Laboratory Coordinating Center.

Hard copy data of all assay related worksheets, calculations and results will be maintained in the Clinical Research Unit Core Laboratory for the duration of the study.

8.8 Reporting Results to Field Centers

When samples for glucose, A1c, lipids and child hsCRP are assayed, the results will be provided to the field centers on a weekly basis for reporting back to the mothers. When the laboratory results are provided to the centers, those with a fasting plasma glucose $\geq$ 126 mg/dl (7 mmol/l) and/or a 2-hour plasma glucose $\geq$ 200 mg/dl (11.1 mmol/l) who are not known to have diabetes will be flagged as having a level diagnostic of diabetes. Other values that will be flagged as alert values will be LDL cholesterol $\geq$ 160 mg/dl (4.14 mmol/L), triglycerides $\geq$ 500 mg/dl (5.65 mmol/L), and A1c > 8.0%. Field centers will decide locally what specific values require urgent notification of the participant.

8.9 Transfer of Samples to NIDDK Central Repository

The backup aliquots of all urine and blood samples, the analysis aliquots of urine and storage samples, and DNA samples will be transferred to the NIDDK Central Repository for those participants who have given consent for this. Before samples are transferred, a cross-check will be made to assure that consent was given.

At the time of each shipment to the NIDDK Central Repository, each freezer box in the shipment will be removed from the -80◦ C Backup freezer BC SAMPLE file and transferred to the -80◦ C NIDDK freezer BC SAMPLE file. The rack ID in the -80◦ C NIDDK file will be the shipping date.

HAPO Follow-Up Study Sample Shipment – RECEIPT FORM

1. Field Center: _____

2. Date of Dispatch:_____

3. Tracking/Way Bill Number: _____

4. Date of Receipt:_____ Time of Receipt:_____

5. Box ID Numbers: _____

   Any discrepancies? ☐ Yes ☐ No

6. Condition of cryovials: ☐ OK ☐ Broken/leakage ☐ Thawed

   IDs of boxes with problematic cryovials:_____

7. Cryovial barcodes cross-checked with Shipping Grids ☐ Yes ☐ No

   Any discrepancies? ☐ Yes ☐ No

   Missing sample? ☐ Yes ☐ No

   Details:_____

8. Field Center/Project Manager notified by:_____