CHAPTER 12

DETERMINATION OF TOTAL CHOLESTEROL WITH THE DT60

12.1 Analyzing Participant Samples

The following procedure can be used to measure total plasma or serum cholesterol with the DT60. The measurements can be made in either fresh or frozen samples.

1. Fingerstick samples. Capillary blood obtained by fingerstick is conveniently collected into a small Microtainer Serum Separator tube (Becton-Dickenson, Cat. No. 5960). This tube is equipped with a scoop to aid collection of the blood and contains a separation gel to isolate serum from cells.
   a. Cleanse puncture site with alcohol wipe and dry with sterile pad.
   b. Puncture the skin with a sterile lancet. Wipe the first drop of blood off with a sterile pad.
   c. Hold the microtainer tube scoop under the puncture and allow the blood to flow down the scoop into the tube. Do not use the scoop to sweep the blood into the tube since this can hemolyze the sample.
   d. When sufficient blood has been drawn, remove the scoop and replace it with the red plug to seal the tube. Allow the blood to clot, then centrifuge briefly to sediment the cells. Centrifugation at about 1000 x g for 90 seconds or so should be sufficient. The cells sediment below the separator gel and the serum remains above the

gel. The sample can be analyzed immediately or stored in the refrigerator for at least 12 hours before analysis. If it is stored, make sure the tube is securely capped to prevent evaporation.

e. At the time of analysis, remove the plug, position the tube horizontally with the opening slightly below the base of the tube and tap the tube to get the drop of sample near the opening. The sample should form a drop at the opening of the tube from which the appropriate aliquot can be taken with the motorized pipet (see below).

2. Venipuncture Samples

a. The serum samples are collected as described in Chapter 13. If an aliquot of the sample is to be stored for analysis with the DT60 at a later date, an aliquot (0.5 ml) is transferred to a 2 ml storage vial, capped and sealed, and stored at -20° C (see Chapter 13).* It is best to use a non-self-defrosting freezer because self-defrosting freezers cycle between -20° and -5° each day. If possible, store the samples at -70°C.

b. At the time of analysis, the samples are removed from the freezer, allowed to thaw and mix for at least 30 minutes before unsealing the vial. It is recommended that the samples be placed on a blood mixing device such as a blood wheel and mixed for 30 minutes. If this is not possible, the samples should be gently agitated by inversion eight to ten times at about five minutes. This

inversion eight to ten times at about five minutes. This is most important, since the sample must be homogeneous before being analyzed.

c. Unseal the tube and analyze (see below).

12.2 Operation of DT60

1. Please refer to Section 2 of the DT60 Analyzer Operators Manual. This manual was provided with the instrument.
2. Turn the DT60 analyzer on. The switch is on the rear of the instrument.
3. The instrument will require a 20-30 minute warm up. During this period the screen displays "wait."
4. When the instrument is ready the "Analyzer Ready" message is displayed.
5. While the instrument is warming up, take the cholesterol test slides from the refrigerator or freezer and allow them to warm to room temperature.
6. Press the PATIENT ID key, enter a participant identifier, then press ENTER.
7. Place a cholesterol test slide in the loading slot, bar code up, notch in first. Push the slide advance level smoothly to move the slide into the instrument. If the slide is inserted properly, the instrument will display the name of the test. After a few seconds, the "apply liquid to slide" message will appear. (If the instrument is unable to read the bar code, it will be necessary to identify the test manually. Press the CHEMISTRY SELECT key repeatedly until the cholesterol test

is displayed. Then press ENTER. At the message "Enter Generation Number," enter the two-digit test generation number shown on the slide package, then ENTER.)

8. Place a clean plastic tip on the motorized pipet. Be sure the red mark is shown in the window at the rear of the pipet. This indicates that the plunger is in the proper position to aspirate the sample.
9. Place the tip into the sample and press the pipet button. the pipet will beep once. Remove the pipet tip from the sample and wait until it beeps again (about 2 seconds). It will have drawn the sample a little further into the tip, which will prevent the sample from being drawn out of the tip when the pipet tip is wiped.
10. Insert the pipet into the sample application port and allow it to seat properly. This positions the pipet above the test slide. Press the pipet button to apply the sample to the slide. Then remove the pipet from the instrument.
11. The instrument will then move the slide into the incubator.
12. When the "Analyzer Ready" message appears, the next slide can be loaded.
13. Enter the next participant identifer.
14. Load the next slide and continue as described above.

12.3 Instrument Calibration

Refer to Section 3 of the Operators Manual. Calibration of the Ektachem DT60 Analyzer is required to maintain the accuracy of instrument.

Calibration should be performed:

1. At least every three months.
2. When a new lot number of Ektachem DT cholesterol slides is used.
3. When the results of a Quality Control (QC) test using Ektachem DT controls are consistently outside of the acceptable range (see below).

The calibration procedure is as follows:

1. Turn on the DT60 and allow it to warm up for at least 30 minutes. The "Analyzer Ready" message should be displayed.
2. Allow the calibrators and diluents to equilibrate at room temperature.
   a. 15 minutes for refrigerated calibrators and diluents.
   b. 30 minutes for frozen calibrators and diluents.
3. Warm the slides to room temperature approximately 15 minutes.
4. Reconstitute the calibrators according to the instructions on the box.
   a. Tap the top of each bottle so that any lyophilized material

      adhering to the inside of the rubber stopper will drop down into the bottle.
   b. Add exactly 3.0 ml of the appropriate diluent to each vial of lyophilized calibrator. Cap the bottle.
   c. Gently swirl the bottles periodically while allowing the calibrators to equilibrate for 30 minutes. Invert the vial several times to wash any residual lypophilized sample from the rubber stopper into the sample.

5. The calibration procedure is performed while the analyzer is in the calibration mode.
   a. Press the CAL key. The word CAL will appear in the upper right hand of the display.
   b. Enter the calibrator kit number printed on the carton containing the calibrators, then press the ENTER key.
   c. Enter the generation number, then press the ENTER key.
6. Put a test slide into the instrument.
7. Enter calibrator bottle number, then press the ENTER key. (Run the bottles in sequence. The display will prompt you which bottle numbers to use).
8. Aspirate the calibrator bottle number, then press the ENTER key. (Run the bottles in sequence. The display will prompt you which bottle numbers to use.)
9. Carefully position pipet in the sample application port and apply the sample to the slide as described above.
10. Change the pipet tip and repeat steps 6-9 for each of the calibrators.
11. Examine the test results. The printout should read as follows:

    *1 Rep #1

    The number after the * is the bottle number and the number after the # is a successful replicate completed. If the slide is not processed successfully, a zero is reported instead of a valid replicate number. If this occurs, run another slide for the same calibrator, same test (the same bottle number will be indicated on the display screen). When the slide is

processed successfully, the instrument will prompt you to go on to the next calibrator.

12. Exit the calibrator mode by pressing the CAL key. This will put you back into the run mode.
13. Date and save the calibration printout.
14. Analyze the quality control samples to confirm that calibration has been successful. The instrument is now ready to analyze samples.

12.4 Quality Control Analyses

Refer to Section 4 in the Operator's Manual.

1. Analyze quality control pools in duplicate. All of the centers should use the two Kodak Ektachem DT lyophilized controls and analyze them once each day.
2. Reconstitute the lyophilized controls in the same manner as the calibrators (see above).
3. If additional frozen control materials are analyzed (CDC pools or locally prepared pools), allow them to equilibrate at room temperature for 30 minutes. During this period the samples should be gently mixed on a blood mixing wheel or inverted and mixed manually at intervals as above.
4. Analyze the control fluids. Substitute the identification number on the bottle of control fluid for the participant ID. (The instrument is in the run mode, not the calibrate mode, for these analyses.)
5. Establish control limits for the instrument. Temporary control limits are to be calculated after ten runs, and

permanent limits after 20 runs. The proper operation of the quality control system requires the availability of control pools that can be analyzed in each run. It will therefore be necessary for each center to purchase a sufficient quantity of the two Ektachem DT60 lyophilized control pools. If possible, sufficient control material should be purchased to allow the same two pools to be used for at least six months, and preferably for an entire year (within the limits of the expiration dates for the pools). However, it will not be necessary and may not be feasible for all six clinics to use the same pools (see below).

6. The control limits are calculated as follows.

   a. After each run, record the individual results for the duplicate analysis of each pool. For each pool, calculate the daily mean by averaging the two values for the pool.

$$\bar{x} = \frac{\text{value 1 + value 2}}{2}$$

   b. For each pool calculate the daily range as the difference between duplicates.

$$R = \text{value 1 - value 2}$$

   c. After the requisite number of runs, calculate the overall mean for the pool, $\bar{\bar{x}}$, from the daily means.

$$\bar{\bar{x}} = \frac{\text{sum of daily means}}{\text{number of runs}} = \frac{\Sigma \bar{x}}{n}$$

   For temporary limits n = 10; for permanent limits n = 20.

d. Calculate the standard deviation of the run means,

$$S_x = \sqrt{\frac{(\bar{x} - \bar{\bar{x}})^2}{n - 1}}$$

where $\bar{x}$ is the daily mean and $\bar{\bar{x}}$ is the overall mean.

e. Calculate the average range,

$$\bar{R} = \frac{\Sigma R}{n} \quad .$$

Again n = 10 for temporary limits; n = 20 for permanent limits.

f. Control limits for the daily means are calculated as follows:

Upper control limit = $\bar{\bar{x}}$ + 3 std dev.

Lower control limit = $\bar{\bar{x}}$ - 3 std dev.

rounded to the nearest whole number.

Warning limits for the daily means are calculated as follows:

Upper warning limit = $\bar{\bar{x}}$ + 2 std dev.

Lower control limit = $\bar{\bar{x}}$ - 2 std dev.

rounded to the nearest whole number.

g. Control and warning limits for the daily ranges are calculated as follows:

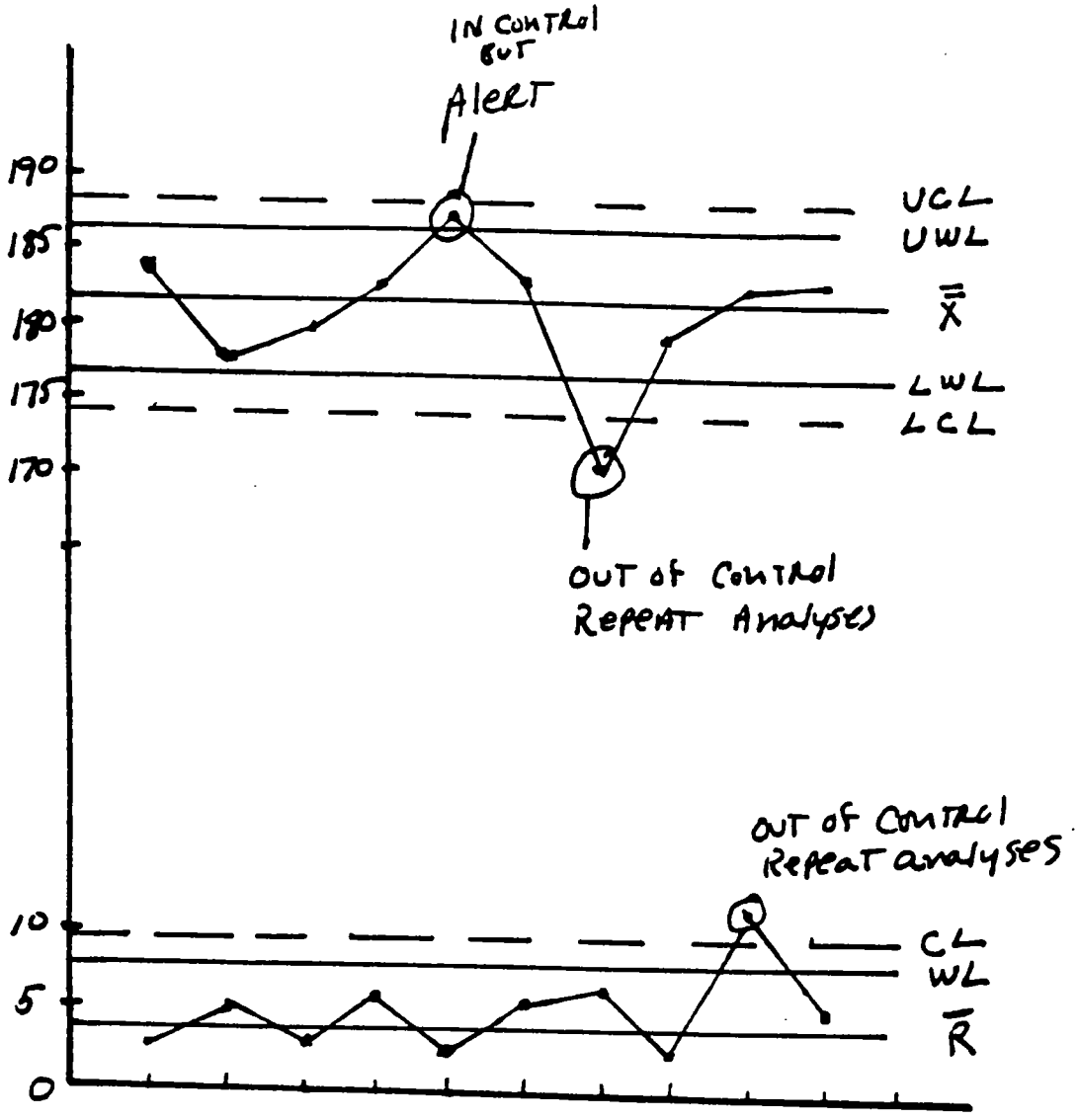
Range control limit = 3.27 $\bar{R}$

Range warning limit = 2.46 $\bar{R}$

There is no lower limit for the range chart since there is no negative range.

7. Evaluation of Quality Control Data. Construct a graph for each pool which illustrates the overall mean, warning limits control limits, and the average range and its warning control limits. For each pool plot the daily mean and range after each day's analyses. The analyses can be accepted if the

Example:



Run Number

or

Date of Analysis

daily mean falls between the upper and lower warning limits and the range fall at or below the warning limit. The analyses are suspect if the control limits are exceeded for either the mean or the range. Such results would not normally be accepted. Corrective steps would be taken and the patient analyses would be repeated in a new run. If a single daily mean or range falls between the warning and control limit, the results can be accepted but the operator should be alerted and there may be a problem. Bear in mind that statistically, the results will exceed the control limits 1% of the time and will exceed the warning limits 5% of the time even if everything is working properly. If the warning limits are exceeded for two or more successive runs, it is probable that the system is not working properly and corrective action should be taken. (Examples might include checking to see that the motorized pipet is working properly, that test slides have been stored properly, instrument calibration hasn't changed, the instrument is clean, etc.)

8. After acquiring the two serum control pools that will be used for screening, each center should send a vial of each of the two pools to the Central Laboratory. These pools will be analyzed with the standardized laboratory method. In this way, the screening cholesterol analyses can be linked to the Central Laboratory standardized cholesterol method. Contact the Director of the Central Laboratory before sending the control serum supplies.

12.5 Equipment Maintenance

Refer to Section 5 of the Operator's Manual. The Ektachem DT60 Analyzer requires care to keep it operating reliably.

1. Daily cleaning:
   a. Empty the slide disposal box.
   b. Wipe the DT Pipette with an absorbant clean cloth to avoid any
      serum build-up.
2. Weekly cleaning, depending on how often the analyzer is used: Using a cotton swab dipped in 70% isopropyl alcohol clean the following:
   a. The Pipette locator.
   b. Visible slide track.
   c. Bar code reader.
   d. The drop detector surfaces.
3. Clean the Fiber Optic Reflectance System (FORS) head as needed since routine cleaning is not recommended.
4. Changing the battery.
   a. When the battery is low in the Ektachem DT Pipette, it will signal with four short beeps and a flashing LED. The Pipette uses a standard 9-volt alkaline battery.
   b. Changing the battery is explained in Section 5.4 of the Operator's Manual. If the battery is not replaced when indicated, the volume measurement can be wrong leading to errors in the test results.

5. Loading the printer paper.

Refer to Section 5.5 of the Operator's Manual. Paper changing is indicated by the appearance of red strips on each side of the printer age.

a. Remove the paper core from the printer cradle and cut it.

b. Remove the remaining paper by firmly pulling it away from the analyzer.

c. Insert new paper.

(1) Cut both corners from the leading edge of the new roll of paper.

(2) Load the paper into the printer cradle keeping the paper in a position so that it feeds from the bottom of the roll. This is illustrated in Section 5.5.2 of the Operator's Manual.

d. Feed the paper through the slot in the back of the print head. When the paper has been engaged by the pressure roller you will feel resistance.

e. Press the print key to feed the paper through the print head.

f. Replace the printer cover.

g. Check printer operation by pressing SHIFT key, and then SERVICE MODE key, Option 4. This causes the printer to print the entire character set.

h. After the character set has been printed, press the SHIFT and SERVICE MODE key to exit the service mode.

i. The instrument is now ready to be used.

12.6 Criteria for acceptable correspondence of cholesterol measurements made with the DT60 Analyzer and the CDC Standardized method used at the Clinical Centers and the Central Lipid Laboratory.

12.6.1 Background

During the DISC Feasibility Study, four of the DISC Clinical Centers analyzed total cholesterol in the same SV01 and SV02 samples that were sent to the Central Laboratory for lipid lipoprotein and apolipoprotein analysis. This comparison was made in 556 samples during a 6 month period. The samples had a mean cholesterol level of about 200 mg/dl. During this period, the overall bias of the DT60 analyses was approximately 4.4% (3.5%) (mean (SD)).*

This reflects the average bias of a single sample analyzed once with the DT60 and once with the laboratory method. Based on these findings, the following criteria were established to judge acceptable correspondence of DT60 cholesterol values with Central Lipid Laboratory analyses of the same samples.

On average, the DT60 analysis should agree with the laboratory analysis within 4.4%. Statistically, individual values can be expected to differ from the lab values as shown in the following table.

% of total samples analyzed	acceptable difference (% of lab value)
67%	+4.4% ± 3.5%
28%	+4.4% ± 7.0%
4%	+4.4% ± 10.5%
1%	+4.4% ± >10.5%

*Note: At the level of about 200 mg/dl, the bias of the laboratory method was -1.6% with respect to CDC reference values. Thus, the actual mean bias of the DT60 analyses would have been 4.4% minus 1.6%, or about 2.8% with respect to CDC reference values during the period of the study.

Thus, based on our past experience with the DT60 in the DISC study, the agreement between DT60 and Laboratory cholesterol analyses in split samples would be considered acceptable if the DT60 analyses are within +0.9% to +7.9% of the Laboratory values for two-thirds of the samples; within -2.6% to + 11.4% for an additional 28% of the samples, etc, in a group of split samples analyzed over a 6 month period.

12.7 Equipment Maintenance Checklist

1. Daily Maintenance: Complete maintenance log (Appendix 1).
   - A. Daily at start up:
     - a. turn machine on 20 minutes prior to desired time of operation
     - b. empty slide disposal box
     - c. wipe DT pipette with a clean absorbent cloth
     - d. check paper supply
     - e. check DT pipette operation
     - f. check printer operation
   - B. Daily at end of day:
     - a. empty slide disposal box
     - b. wipe DT pipette with clean absorbent cloth
2. Weekly maintenance:
   - A. Use only warm distilled H_2O for cleaning
   - B. Clean the pipette locator and all visible slide track areas
   - C. Clean bar code reader and drop detector surfaces
3. Every three months or as required.

   Calibrate DT60 and record calibration on Calibration Log (Appendix 1).

4. As required.

   A. When prompted by analyzer, clean fiber optics reflectance system (FORS) head.

   B. Follow detailed instructions in manufacturers manual for each item of maintenance and calibration.

5. Instrument Service.

   Whenever the instrument is serviced, record the problem and corrective action taken on the Service Log (see Appendix #1).

12.8 Certification & Recertification of DT60 Operators for the DISC Study

The following table shows the minimum acceptable level of training for DT60 analyzer operators and blood sampling personnel. Training should be supervised and certified by the Master Trainer for DT60 operations at each Clinical Center.

A record of this certification should be maintained with the DT60 quality control records.

In addition, the Master Trainer should complete the DT60 Procedures Checklist Form (Exhibit 12.1) for each trainee to be certified. This form is to be mailed to the Coordinating Center when completed.

Each certified operator must be recertified annually. This recertification would include the following:

1. Set up and run 10 actual samples with observation by the Master Trainer.
2. Completion of the DT60 Procedures Checklist Form. (Exhibit 12.1).

Table 12.1 Minimum Level of Training for DISC Study DT60 Operators and Blood Sampling Personnel

Instruction	Location	Activity
1 Day	Class (2-3 hours)	1. Principles of Operation 2. Operating Instructions 3. Calibration 4. Quality Control Testing 5. Instrument Care and cleaning 6. Status Messages 7. Maintenance 8. Record Keeping/Log Sheets
	Laboratory (2 hours)	1. Practice a. Operating/sampling technique b. Setting up, maintaining and shutting down the instrument
Field Training	**Location**	**Activity**
3-5 Days	Screening Site	Day 1 - Run 10 to 20 split Quality Controls and subject samples in parallel with an experienced operator. (Trainees results are not to be used for the study.) Days 2-5 - Run actual samples under the supervision of an experienced operator.

Training: New Operators are to be trained by the Master Trainer.

Certification: Successful completion of above as certified by the Master Trainer.

12.8.1 Certification of DT60 Analyzers for On-site cholesterol analysis in the DISC Study

The following items are required before certification of the Kodak Ektachem DT60.

1. Calibration, test/reagent, service and maintenance logs are on hand and current (Appendix 1).
2. Manufacturers test reference for cholesterol is on hand.
3. Operator's Manual is on hand and near the DT60.
4. Quality control (QC) records (charts, logs) are on hand and current.
5. Quality control limits have been established. These limits are set following the analysis of two levels of QC material in duplicate on each of 5-10 days. Quality control limits are calculated and QC charts are constructed as described as in the DISC Manual of Operations.
6. Instrument must meet the criteria for acceptable accuracy and precision specified by the Laboratory Standardization Panel of the National Cholesterol Education Program. Current criteria: average accuracy, within 5% of reference values, CV $\leq$ 5%. Ultimate criteria: average accuracy with 3% of reference values; CV $\leq$ 3% [1].

12.8.2 References

1. Current Status of Blood Cholesterol Measurements in Clinical Laboratories in the United States. A Report from the Laboratory Standardization Panel of the National Cholesterol Education Program. NIH Publ No. 88-2928, Bethesda, Jan 1988.

APPENDIX 1

KODAK EKTACHEM DT60 ANALYZER

MAINTENANCE LOG

Serial Numbers:
DT60 Analyzer _____ Pipette _____
DTE Module _____ Pipette _____
DTSC Module _____ Pipette _____

Daily Cleaning		Weekly Cleaning							As Needed	
DT60 & DTE		DT60, DTE, & DTSC		DT60 Only		DTE Only	DTSC Only		DT60 Only	
Empty & Clean Slide Disposal Box	Pipette	Pipette Locator	Visible Slide Track	Bar Code Surface Reader	Drop Detector Sensor	Rubber Boot	Sapphire Read Window	White Reference Cap	FORS Head	Initial & Date

Kodak, Ektachem, DTE, and DTSC are trademarks.

Make a photocopy of this log sheet prior to use for additional sheets.

KODAK EKTACHEM DT60 ANALYZER

CALIBRATION LOG

Serial Numbers:
DT60 Analyzer _____ Pipette _____
DTE Module _____ Pipette _____
DTSC Module _____ Pipette _____

Date _____ Operator _____ Test _____

CALIBRATOR Kit No. _____ Exp. Date _____

Bottle No. 1 Lot Nos. CAL _____ DIL _____
Bottle No. 2 Lot Nos. CAL _____ DIL _____
Bottle No. 3 Lot Nos. CAL _____ DIL _____
Bottle No. 4 Lot Nos. CAL _____ DIL _____

Electrolyte Reference Fluid: Lot No. _____ Gen. No. _____ Exp. Date _____

CDM Lot No. _____ CLM Lot No. _____

SLIDES				*CALIBRATION COEFFICIENTS				
Date	Lot Number	Gen No.	Exp. Date	CP #1	CP #2	CP #3	CP #4	Remarks

*Affix dated Calibration Printout or manually record values in appropriate spaces.

Make a photocopy of this log sheet prior to use for additional sheets.

KODAK EKTACHEM DT60 ANALYZER

SERVICE LOG

DT60 Serial Numbers: Analyzer _____, Pipette _____

DTE Serial Numbers: Module _____, Pipette _____
DTSC Serial Numbers: Module _____, Pipette _____

Date	Problem Noted	Corrective Action Taken	Initial

Make a photocopy of this log sheet prior to use for additional sheets.

KODAK EKTACHEM DT60 ANALYZER

QUALITY CONTROL LOG

Test _____ Target _____ Range _____

NORMAL Lot Number _____ Expiration Date _____

ABNORMAL Lot Number _____ Expiration Date _____

CHEMISTRY CONTROL

Date	Normal Value	Abnormal Value	Comments	Initial

Date	Normal Value	Abnormal Value	Comments	Initial

Make a photocopy of this log sheet prior to use for additional sheets.

KODAK EKTACHEM DT60 ANALYZER

TEST/REAGENT LOG

SLIDES						ELECTROLYTE REFERENCE FLUID				
Date Put Into Use	Test	Lot Number	Gen No.	Expiration Date	Initials	Date Opened	Lot Number	Gen No.	Exp. Date	Initials

Make a photocopy of this log sheet prior to use for additional sheets.

EXHIBIT 12.1

Dietary Intervention Study in Children

DT-60 Procedures Checklist Form

This form is required for laboratory technician certification and recertification. It is to be completed by the trainer by observing the individual who is to be certified or recertified perform the actual procedures.

The trainer should be located in a position which allows careful observation of these procedures. No comments should be made by the trainer while these procedures are being carried out.

Identifying Information

1. Clinic Location
(circle one)

Johns Hopkins ..... ( )
Northwestern ..... ( )
Iowa ..... ( )
Newark ..... ( )
LSU ..... ( )
Portland ..... ( )

2. Individual to be certified or recertified

Name _____
(please print)

DISC Staff ID ___ - ___ ___ ___

3. Trainer

Name _____
(please print)

DISC Staff ID ___ - ___ ___ ___

4. Person is being:

Certified ..... ( )
Recertified ..... ( )

EXHIBIT 12.1 (Continued)

Dietary Intervention Study in Children

DT60 Analyzer Checklist for the DISC Study

1. Was the DT60 Analyzer allowed to warm-up for 30 minutes?

   Yes _____ No _____

2. Was the slide disposal box emptied?

   Yes _____ No _____

3. Was the analyzer calibrated within the past 3 months?

   Yes _____ No _____

4. Are quality control samples analyzed at least once each day that patient samples are analyzed?

   Yes _____ No _____

5. Were the mean and range for the quality control pool(s) within control limits established for the pool(s)?

   Yes _____ No _____

6. Were the appropriate maintenance procedures performed?

Clean pipette locater?	Yes _____	No _____
Clean barcode reader?	Yes _____	No _____
Clean drop detector surface?	Yes _____	No _____
Clean F.O.R.S. head?	Yes _____	No _____

7. Are the instrument maintenance logs up-to-date?

   Yes _____ No _____

8. Are the quality control records up to date?

   Yes _____ No _____

9. Is the analyst aware of the quality control criteria, and other criteria for accepting the analytical results?

   Yes _____ No _____

Comments:

Date: _____

Name: _____