

Laboratory Procedure Manual

Analyte: **Insulin**

Matrix: **Serum**

Method: **Two-site immunoenzymometric
assay**

Method No.:

Revised:

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Important Information for Users

The University of Missouri-Columbia periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for NHANES 2003–2004 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
I10am_c	LBXIN	Insulin (U μ /mL)
	LBXINSI	Insulin (pmol/L)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Human insulin is a polypeptide hormone originating in the β -cells of the pancreas and serving as a principal regulator for the storage and production of carbohydrates. Its secretion is normally stimulated by increases in the amount of glucose in the circulation.

The AIA-PACK IRI is a two-site immunoenzymometric assay which is performed entirely in the AIA-PACK. Insulin present in the test sample is bound with monoclonal antibody immobilized on a magnetic solid phase and enzyme-labeled monoclonal antibody in the AIA-PACK. The magnetic beads are washed to remove unbound enzyme-labeled monoclonal antibody and are then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labeled monoclonal antibody that binds to the beads is directly proportional to the IRI concentration in the test sample. A standard curve is constructed, and unknown sample concentrations are calculated using this curve. Amount of sample required for Tosoh insulin analysis is 25uL.

2. SAFETY PRECAUTIONS

Gloves and laboratory coat are required for handling all human blood specimens. Dispose of all waste properly. Waste is segregated according to risk: Regular Trash (non-biohazardous, non-radioactive, non-sharp waste), Sharps Waste (sharp objects such as needles and contaminated glass), Broken Glass (clean, non-contaminated broken glass), biohazard waste (all plastic tips, sample cups and gloves that contact blood) and washing waste (include diluents and wash solution).

All work surfaces are protected by disposable absorbent bench top paper which is discarded into biohazard waste containers at least weekly or whenever blood contamination occurs. All work surfaces and instruments are cleaned with a disinfecting detergent (Unicide) or bleach (10% sodium hypochlorite solution) daily.

Note: Smoking, eating or drinking is not permitted in work areas. Discard all biohazardous waste into properly labeled containers (sharp, non-sharp). Dispose of washing waste in a sink flushing with large volumes of the water to prevent azide build-up.

Body Substance Precautions: All body substances (blood, serum, plasma, urine, etc.) should be treated as potentially infectious. Gloves and lab coat should be worn at all times when handling specimens. Discard contaminated gloves after use: do not touch doors, use phone or computer, nor touch any non-contaminated surface with latex gloves! Wash hands thoroughly after each procedure.

Hepatitis B vaccines are offered at no charge to the employee. Should a technician become exposed to a potential pathogen, such as an accidental needle stick, contact of blood on an open wound, etc., the Diabetes Lab will arrange for appropriate infectious disease testing (HIV, Hepatitis, etc.).

3. COMPUTERIZATION: DATA SYSTEM MANAGEMENT

Pathology tests are requested and resulted through the Hewlett Packard 700/90S laboratory information system. Each authorized person is assigned a unique, confidential password for log-in purposes. To log onto the system, type the password at the highlighted prompt on the main screen. Each function (Receipt Verify, Results, etc) is on a separate page, and may be accessed by typing the page number at the prompt. The main menu is Page 00. To access one page from another, press the Home Key (symbol \triangleright on the first line of the keyboard), a period "." and the two digit page number you wish to reference, followed by <Enter>. To completely log off of the system, type two periods ".." at the main menu screen and then press <Enter>.

A. Receipt verify

Each specimen arriving at the Diabetes Diagnostic Laboratory is accompanied by a transport list, and must be verified upon receipt. Verification may be performed by either the patient ID number (for single specimens) or by transport list number (for multiple specimens).

- (1) Press A to verify by Accession Number, or L to verify by Transport List Number.
- (2) At the next screen displayed, enter the PS (processing site) code (DD, IR or other processing site) and the specimen accession number (or, alternatively, the transport list number).
- (3) Under the title "Change Status To", type in the letter A (for received/accepted).
- (4) Press F4 to verify the receipt.

B. Hospital Order Entry

If a specimen is received which has not been assigned an accession number by IR Processing, it is necessary to enter the specimen information into the Pathology ALS system.

- (1) At A/P, enter P for patient ID number.
- (2) Under Patient #, enter the patient's hospital identification number.
- (3) Tab to the Test field: enter **C101** for C-Peptide or **C218** for Insulin.
- (4) Enter in the collection date, collection time, receipt date and time, and priority level (PL = S for Stat, R for Routine, E for Expedite). The Diabetes Lab does not perform Stat tests, so the PL should be Routine for all specimens.
- (5) Press F5 to save all information and exit the order entry screen. Write down the ALS-assigned accession number on the test request card. All future procedures for this specimen (for results, etc) should reference this number.

C. Result Entry (Page 20)

- (1) Enter the PS code, specimen accession number, and press ENTER.
- (2) Choose from the displayed list which specimen you want to enter results for, and type in the function to be performed under "FNC":

A = Add
E = Edit
B = Browse

- (3) At the results entry screen, type in the numerical test value for that specimen, and any comments needed.
- (4) Press F5 to release the test value, and press F5 again to confirm.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

Serum is required for the assay. EDTA and citrated plasmas SHOULD NOT BE USED.

No special patient preparation is necessary. A venous blood sample is collected aseptically without additives. Store at room temperature until a clot has formed (usually 15–45 minutes), then centrifuge to obtain the serum specimen for assay.

Samples may be stored at 2–4 °C for up to 24 hours prior to analysis. If the analysis cannot be done within 24 hours, the sample should be stored frozen at -70 °C.

Repeated freeze-thaw cycles should be avoided. Turbid serum samples or samples containing particulate matter should be centrifuged prior to testing. Prior to assay, slowly bring frozen samples to room temperature (18-25°C) and mix gently.

Serum, obtained from venous blood, is required for this procedure. Draw 7 mL venous blood in a serum clot tube (red top), allow to clot at room temperature for 20 minutes and centrifuge in a refrigerated centrifuge at 4°C at 2000 g for 10 minutes. Draw off the serum and store in a plastic cryovial at -20°C until the specimen can be transported to the laboratory. Frozen serum specimens should be delivered within 24 hours of collection.

Specimens should be transported to the laboratory in a double-containment system to minimize the chance of leaking or spills: the cryovial containing the frozen serum should itself be placed in a cup or other suitable container.

The specimen may also be drawn in a serum separator tube and given directly to the Diabetes Laboratory without freezing, if the sample is brought to the lab immediately after collection.

Upon receipt by the Diabetes lab, the specimen will be logged in and stored at -70°C until analysis. Analyzed specimens will be stored at -70°C for one month, after which they may be discarded. The stability of serum insulin is one year at -70°C.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

A. Materials (Provided by Tosoh)	Cat. No.
(1) AIA-PACK (Stored 2-8°C)	
(a) Substrate Set II (Substrate/Reconstituent)	020968
(b) IRI Test Cup	020260
(c) IRI Calibrator Set	020360
(d) IRI Sample Diluting Solution	020560
(e) Wash Concentrate Set	020955
(f) Diluents Concentrate	020956
(g) Detector Standardization test Cups	020970
(h) Sample Treatment Cup	020971

All above materials if they are unopened are stable until the expiration date on the label when stored at the specified temperature.

(2) AIA-600 II Sample Cups	018581
(3) AIA-600 II Pipette Tips	019215
(4) AIA-600 II Tip Rack	019216
(5) AIA-600 II printer paper	

B. Other Materials (not provided by tosoh)

- (1) Waterproof markers (for labeling tubes) (any vendor)
- (2) Pipette Stand (any vendor)
- (3) Class A 20 mL Volumetric Pipette, calibrated "To Deliver" (Fisher Scientific, St. Louis, MO)

- (4) Contrad (any vendor)
- (5) Latex examination gloves (any vendor)
- (6) Absorbent benchtop paper (Whatman Lab Sales, Hillsboro, OR)
- (7) Unicide disinfecting detergent (hospital distribution)
- (8) Ethyl Alcohol (any vendor)

C. Instrumentation

- (1) AIA-600 II
- (2) Eppendorf Tip Ejector Fixed Volume Pipettes (50, 100 mL in volume, Fisher Scientific, St. Louis, MO)
- (3) Eppendorf Repeater Pipet (range from 10 mL to 5 mL, precision to 0.1%, Fisher Scientific, St. Louis, MO)
- (4) Combitips for Eppendorf Repeater with Adapter (2.5 mL tip graduated in 50 mL increment, Fisher Scientific, St. Louis, MO)
- (5) Pipet Aid XP (Drummond scientific company)
- (6) Pipetman Adjustable Pipet (200-1000 mL, Rainin Instrument, Woburn, MA)
- (7) Milli-Q Plus Ultra Pure Water System (Millipore, Bedford, MA)

Warning

The AIA-PACK IRI is intended for in vitro diagnostic use only.

- (1) Does not use beyond the expiration date.
- (2) The AIA-PACK IRI has been designed so that the high dose “hook effect” is not a problem for the vast majority of samples. Samples with insulin concentrations between 320 and 30000 uU/mL will read > 320 uU/mL. The “hook effect” phenomenon may occur at insulin concentrations >30000 uU/mL.
- (3) The materials provided by Tosoh contain solution azide, which may react with lead or copper plumbing to form potentially explosive metal azides. When disposing of such reagents, always flush with large volumes of water to prevent azide build-up.

D. Reagent Preparation

All reagents are supplied by Tosoh Medics INC. The unopened reagents are stable until the expiration date which is printed on each bottle. The recommended storage temperature for all reagents is 2-8°C.

All reagents must be at room temperature before using. Mix all reagents thoroughly before use.

When new reagents are received, they need to be initialed and dated by technician who received them. When they are opened, they should be initialed and dated by technician.

(1) Substrate Solution

- (a) Bring substrate and substrate reconstituent to room temperature.
- (b) Add 1 bottle of reconstituent to 1 bottle of lyophilized powder. Mix thoroughly.
- (c) Let stand for 20 minutes to dissolve contents completely.
- (d) Label the bottle with the preparation date.

The stability after reconstitution is 3 days On-board (room temperature) and 7 days in the refrigerator. Protect the substrate solution from light.

(2) Wash Solution

- (a) Add 40 mL of the concentrate to 800 mL of Reagent Grade, Type 1 water.

- (b) Fill to 1 Liter with Reagent Grade, Type 1 water.
- (c) Mix thoroughly.
- (d) Label the container with the lot number and expiration date.
- (e) Stable for 30 days on the instrument at room temperature.

(3) Diluent

- (a) Add 20 mL of the concentrate to 800 mL of Reagent Grade, Type 1 water.
- (b) Fill to 1 Liter with Reagent Grade, Type 1 water.
- (c) Mix thoroughly.
- (d) Label the container with the lot number and expiration date.
- (e) Stable for 30 days on the instrument at room temperature.

(4) Test Cups (10 tray * 20 test cups)

Plastic test cups containing lyophilized magnetic beads coated with anti-insulin mouse monoclonal antibody and mouse monoclonal antibody (to human insulin) conjugated to bovine alkaline phosphates with 0.1% sodium azide as a preservative. Stable up to 72 hours at room temperature and until expiration date at 2-8 °C.

E. Standards (Calibrator) Preparation

The Calibrator set contains human serum with six assigned levels of Insulin.(0 and approx. 20, 40, 80, 160 and 320 uU/mL)

Using the Pipetman Adjustable Pipet, reconstitute each level calibrator at room temperature with 1mL of CAP Class I or NCCLS Type I Reagent Grade water except 0 level calibrator. Allow stand for 10 minutes, inverting the vial several times to mix contents. Wait for calibrator completely dissolved. After opening, the calibrators should be used within 24 hours.

F. Quality Control Materials and Preparation

The three levels of lyophilized controls are purchased from Bio-Rad Laboratories Allow one box to reach room temperature. Using a Class A volumetric pipet, reconstitute each control at room temperature with 5.0 mL distilled water. Allow to stand for 10 minutes and invert the vial several times to mix contents. Waiting for completely dissolved. Avoid shaking. Combine the four bottles of same level (identical Lot numbers) into a beaker and gently mix. Transfer 500 µL aliquots into polypropylene storage tubes. Cap tightly, label (technician and date) and freeze at -70°C. Enter information into the Controls Log Book. Thaw each aliquot one time only.

The In-House control is prepared by collecting one unit each of whole blood from three non-diabetic volunteers. All blood is screened for HIV and Hepatitis. Serum is separated from red blood cells and serum from all donors is pooled. Transfer 500 µL aliquots to polypropylene storage tubes. Cap tightly and frozen at -70°C. Reconstitution of the In-House control is not required. Thaw each aliquot one time only. Enter information into the In-House Control Log Book (room M767).

7. CALIBRATION AND CALIBRATION PROCEDURES

A. Calibration curve

- (1) The calibrators for use with the AIA-PACK IRI have been standardized against WHO 1st IRP 66/304 (1974)
- (2) The calibration curve for the AIA-PACK IRI is stable for up to 90 days. Calibration stability is monitored by quality control performance and is dependent on proper reagent handling and AIA System maintenance according to the manufacturer's instruction.
- (3) Recalibration may be necessary more frequently if controls are out of the established range for this assay or if certain service procedures are performed (e.g. temperature adjustment, sampling mechanism changes, or detector lamp adjustment or change) and the Test cup lot number is changed.
- (4) The calibration curve type from the AIA-600 II is linear-lin.

B. Calibration Procedure

- (1) Select SPECIAL MENU from the keypad by pressing the SPECIAL MENU key. Then press '2' to select ASSAY SPECIFICATIONS.
- (2) Press the down arrow key until the cursor is at IRI.
- (3) Press the right arrow once to move the cursor to the calibrator lot number. Verify the calibrator lot number. It is the last two digits of the lot number located on the calibrator box or vial. If the lot number is same skip to step 8.
- (4) 4.* To enter a new calibrator lot number, press F4 (COCCECT). Enter the calibrator lot number using the keypad. When finished, press ENTER.
- (5) 5.* Press the right arrow to move the cursor to the calibrator which need change the concentration value. Press F4 to select CORRECT.
- (6) 6.* Type in the calibrator concentration located on the bottle or the calibrator box. Then press ENTER. Verify that the concentration is correct.
- (7) 7.* Repeat step 5 and 6 until all the calibrators' value are verified.
- (8) Select ASSAY MONITOR from the keypad by pressing the ASSAY MONITOR key.
- (9) Touch the CALIB key on the keyboard. Select the analyte to be calibrated by using the numeric keyboard to enter the analyte code 55 or by positioning the cursor under the name of the analyte in the Analyte Table window. Press ENTER. The triplicate of each calibrator will be show in the screen.
- (10) Print a work list by pressing F-1, <WORK LIST>. Verify that the calibrator values programmed in the system are the same as the concentrations printed on the vials. If not, update the calibrator file under SPECIAL MEUN, ASSAY SPECIFICATIONS.
- (11) Follow the sequence of the work list and pipette enough calibrator into each of the specified cups to satisfy the total sample volume requirement printed on the work list (pipette more 20uL than the work list show will be better). Avoid bubble formation in the cups. Load calibrators and test cups according work list in the proper positions on the sample chain.
- (12) Press the ASSAY START key.

* For these steps, also can use Barcode scanner to read the barcode from the barcode sheet instead typing.

C. Calibration Acceptability Criteria

- (1) The mean rate for the zero calibrator should be <3.0 nM/sec and the rate of the highest should be >30nM/sec.
- (2) Since there is a direct relationship between concentration and rate, the rates should increase as the concentration increases.
- (3) The replicate values should be within a 10% range.

D. Calibration Review and Acceptance

Calibration data is available for review after one of two events occurs:

- (1) A result is output after the calibration data is printed.
- (2) The run is completed and the chain returns to the home position.

Following below step to accept the calibration:

- (1) Press CALIB. REVIEW key. The calibration data will be displayed one assay at a time, in the order performed. Each data point, the assigned value, and the rate will be displayed.
- (2) If the current calibration is not on the screen, press F1 <NEXT CALIB> until find.
- (3) Press F4 <CALIBRATE> to display the graph and the curve equation.
Review the curve and rate according to the Calibration Acceptance Criteria. If a data point is out of the Criteria, move the cursor to the point to be rejected by touching the right arrow key. Reject will then flash on the screen and that point will be excluded from the calibrations used to create the standard curve. If a data point is changed from accept to reject (or visa versa), the F4 <CALIBRATE> key must be pressed to recalculate the curve.
- (4) When the all data is acceptable, press F3 <ACCEPT>. The LOT SELECT window will appear.
- (5) Place the cursor on the left or right side in the location where the new calibration is to be stored. Press ENTER to store the calibration. Press ENTER when the confirmation box is displayed.

Calibration Facts

- The AIA-600 II will store two calibration curves simultaneously for different pack lots of the same analyte.
- The first time an analyte is calibrated, it should be stored on the left side.
- Always place the cursor on top of the same test cup lot number or the test cup lot that is no longer in use.
- If both stored curves are the same lot number, the curve designated as the “left lot” will always be the active curve even if the lot is the expired lot. The AIA-600II will not use the “right lot” unless the “left lot” is inactive.
- The calibration curve and rate results will not automatically print if the instrument is performing assays. Calibration curve can be printed on demand when the instrument is idle.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

- (1) Allow frozen samples and controls (three levels of commercial lyophilized serum controls and one level of pooled non-diabetic serum In-House control) to reach ambient temperature. Invert gently to mix.
- (2) Bring the substrate reagent, calibrators if necessary and test cups to room temperature.
- (3) Label sample cups (provided by Tosoh) using a fine-point permanent marker with sample ID and control ID.
- (4) Check the Diluent solution and wash solution bottle level. Fill the bottles as needed.
- (5) Empty the waste bottle and solid waste container.

B. Instrument set-up

- (1) Power on. Wait for Log On screen.
- (2) Logon by pressing F2 <SKIP> to retain current operator ID. For new operator, press F1 <CORRECT> and enter an operator name. Use the arrow key to select letters; press the enter key to accept the letter selection. Continue this process until the operator name is correct. Press F5 <QUIT> once operator name registration is complete. Use the up/down arrow key to select the current operator and press Enter key.
- (3) Follow the instructions on the Daily Maintenance 1 screen. When complete, press F1 <OK>.
- (4) Place Substrate in substrate compartment and observe the volume on the Inventory screen. Press ENTER. Press the INVENTORY key to remove the Inventory screen from the display.
- (5) When the Daily Maintenance 2 screen appears, place 1 test cup adapter with a Standardization Cup (STD) on the chain in position 2. Press F1 <START>. Automated maintenance will begin.
- (6) Record results of the Substrate Background Measurement on the Substrate Background form and keep the printed result for troubleshooting.
- (7) Press the Reag/Tip Pause key. Refill tip rack and update tip inventory using the keypad (only Tosoh brand tips can be used). Register any required reagent(s) and place in correct reagent rack position(s). When finished, move the rack assembly to the left, past the sensor. Press the Reag/Tip Pause key to remove the screen from the display.
- (8) To choose a calibration curve by pressing the SPECIAL MENU key. Then press '2' to select ASSAY SPECIFICATIONS. On the IRI line using the arrow key moves the cursor right. Under TEST CUP, there is LOT L and LOT R. Move the cursor on the LOT L and press ENTER to erase the '*' if choosing LOT R as a current curve otherwise LOT L always is active curve.

C. Processing Samples:

- (1) Press ASSAY MONITOR. Enter sample ID and the control ID (for control by press the CONTROL key and type the ID number). Press ENTER. Type in analyte test code 55. (For each run need two sets control, one set is placed in front and other set are place in end).
- (2) Press ENTER to store tests and sample ID.
- (3) Repeat above steps for remaining samples.
- (4) Press F1< WORKLIST> to print a list defining the required volume (including dead volume).
- (5) Using an Eppendorf pipette, transfer the required volume of controls and samples into the corresponding sample cups. Remove any bubbles. (For better result, adding more 20uL then required volume)
- (6) Place appropriate sample cups on the chain with the required test cups. (Verify the sample ID on the sample cup against the woe list.)
- (7) Press ASSAY START.

D. Procedural Notes

- (1) The AIA-6000 II has a sample/test cup chain there are 56 positions. Do not use last 4 which are labeled "STAT" in daily. Those 4 positions for a sample result needed immediately.
- (2) If need run more than one load samples, wait for the chain return to the home position then repeat the PROCEDURE steps.
- (3) If a serum specimen Insulin concentration is found to be greater than the 300 uU/mL, the specimen should be diluted with the IRI Sample Diluting solution and reassayed. The AIA-6000 II will automatically perform dilutions and calculate results if the dilution factors are entered into the software. The dilution steps are:

- After enter the sample ID and test code, using the left Arrow key to move the cursor back over the test code (55).
- Press F5 <DILUTION>. To display the dilution menu.
- Select option 3, <OTHER>, using the keypad. Type in the desired dilution factor and press ENTER.
- Place sample, which the dilution ordered, and Sample Treatment Cup (STC) on the chain prior to the test cup. If the dilution factor higher than 51 require two STC cups. (The AIA-600 II is capable of performing dilutions up to 625)

E. Printing After Calibration

The AIA System performs all sample and reagent handling operations automatically. The AIA Systems read the rate of fluorescence produced by the reaction and automatically convert the rate to insulin concentration on uU/mL then print out both rate and concentration.

If the calibration curve is undetermined before the analysis, using below steps to recalculate results after a calibration curve is determined.

- (1) Press RESULTS REVIEW.
- (2) Place the cursor line under the first sequence number desired.
- (3) Press F2 <ARAE SELECT>
- (4) Use the down arrow key to move the cursor to the last sequence number desired.
- (5) Press F2 <ARAE SELECT> again. This will highlight the range so result selected.
- (6) Press F4 <EXECUTE>. A small pop-up window will appear with 4 options. Select option 3 (RECALCULATE), and press ENTER.
- (7) A small pop-up window will appear to confirm your selection. Press ENTER to select YES for the highlighted samples to be recalculate.
- (8) Press F4 <EXECUTE>. Select option 1 (PRINT OUT), and press ENTER. The result will be printed on the printer tape.

For samples requiring dilution, the AIA-600 II will automatically perform dilutions and calculate result if the dilution factor are entered into the software.

F. Recording of Data

(1) Quality Control Data.

All replicate values of quality control data plus all pertinent assay information are recorded on the Tosoh Insulin Assay Log Database located on the network drive. Print out the Insulin Diary Sheet.

(2) Analytical Results.

When the assay is accepted, record the results.

For clinical and Nhanes samples:

- (a). Results on the corresponding test request form then enter the result in the corresponding Database located on network drive.
- (b). During the data entry process, check the lab accession number and enter any comments associated with the specimen in the comment field.
- (c). A data check sheet with test result is printed. Test result is checked against the instrument print out by the supervisor. A copy of the data check sheet is kept in the appropriate book at Diabetes Diagnostic Laboratory.

G. Replacement and periodic maintenance of key components

- (1) Perform the Daily maintenance on the day of assay by following the DAILY MAINTENANCE¹. Discard the used sample cups when the analysis was complete. Turn off the instrument once per 24 hours.
- (2) Clean the B/F Wash Probe Filter weekly with deproteinizing solution (5% Contrad 70) then rinse with a small amount of DI water.
- (3) Clean sample area with Ethanol and diluents and wash reservoirs with 1:5 dilution of Clorox (bleach) then rinse reservoirs with DI water monthly.
- (4) AIA-600 II performs a substrate background measurement each time daily maintenance is run and the results are automatically printed out. If the substrate background measurement is within specifications, an OK will be displayed next to 4MU Background. If the substrate background is too high a "BH" (blank high) error flag will be printed and Substrate Replacement will be incomplete. Prime or replace the substrate and repeat daily maintenance. If the lamp intensity level is within specifications an OK will be displayed next to Lamp Intensity Level. If the lamp intensity level is too low an "LL" (lamp low) error flag will be printed. The "LL" is warning that the lamp will need to replace soon.
- (5) The pipets, which are used for calibrator reconstitution is gravimetrically calibrated semiannually. This method measures the performance of a pipet using an analytical balance with distilled water. The accuracy and precision of the pipet at a specified volume are calculated using a Microsoft Excel program. Pipets which do not meet the accuracy and precision criteria are returned to the manufacture for replacement or repair. All calibration results are recorded in Pipet Log Book.

H. Calculations

- (1) Any specimen with a concentration greater than 250 $\mu\text{U}/\text{mL}$ is reanalyzed using a diluted specimen.
- (2) The low detection limit, based on ten repeat measurements of zero standard and serial dilution of a sample containing low insulin concentration, is determined to be 1.0 $\mu\text{U}/\text{mL}$. All specimens with insulin values less than 1.0 $\mu\text{U}/\text{mL}$ are reanalyzed for confirmation and then reported as "<1.0".
- (3) Any sample if the result has any kinds of FLAG sign except "H" and "L" have to be reanalyzed after troubleshooting. (See the table-1)

9. REPORTABLE RANGE OF RESULTS

Serum insulin values are reportable in the range of 2.5 to 100 $\mu\text{IU}/\text{mL}$. Values below the detection limit (2.5 $\mu\text{IU}/\text{mL}$) are repeated for verification and values above 100 $\mu\text{IU}/\text{mL}$ are reanalyzed at an appropriate dilution factor.

10. QUALITY CONTROL (QC) PROCEDURES

Two types of quality control systems are used in this analytical method.

- A. Sample QC: five percent of specimens are randomly selected and analyzed either within-assay or between-assay for quality assurance purposes.

- B. Batch QC: quality control specimens are placed before and after all specimens to be analyzed. The bench quality control consists of four levels of controls, which cover the spectrum of Insulin and C-Peptide ranges for both normal and diabetic populations. Three are commercial lyophilized serum controls purchased from Bio-Rad Laboratories (Irvine, CA). The other control is prepared in-house and stored in -70°C. One vial of each is thawed and used in each assay. Reconstitution is not required for In-House control.

If the stock of these controls becomes low, another batch is ordered or prepared in time to analyze it concurrently with the current quality control materials. The new controls are used only after their means and the ranges are established after twenty characterization runs.

The bias limit is set at 1 SD or the 67% limit; the warning limit (WL) is the 2 SD or the 95% limit and the control limit (CL) is the 3 SD or the 99% limit.

C. QC Guidelines

L22, L23, and L24 control parameters were established on 05/01/03 using 20 inter-assay observations both manual and robot runs. IH11 control parameter was established on 05/01/03 using 20 inter-assay.

Mean	Mean	SD	1 SD	2 SD	3 SD
L22	10.96	0.46	10.51-11.42	10.05-11.88	9.59-12.34
L23	44.02	1.13	42.89-45.15	41.76-46.28	40.63-47.42
L24	113.77	3.29	110.48-117.06	107.18-120.36	103.89-123.65
IH11	51.06	1.43	49.62-52.49	48.19-53.92	46.76-55.35
Range	Mean	SD	+1 SD	+2 SD	+3 SD
L22	0.99	0.49	1.48	1.96	2.45
L23	1.61	1.50	3.11	4.61	6.11
L24	3.81	3.28	7.09	10.37	13.65
IH11	2.06	1.55	3.61	5.17	6.72

After each assay run, all control data are recorded on the Assay Log Sheet. The analysis is judged to be accepted or rejected following the guidelines established by NHANES III (9/21/89 memo from National Health And Nutrition Examination Survey, CDC) with a slight modification on the determination of a trend.

The quality of an assay is assessed by two types of quality chart plots (Levy-Jennings). The first chart plots the *mean* of all the replicate determinations in a run. It is then compared with the target mean which is the overall mean established by the twenty characteristic runs.

The NHANES guideline declares a system "out-of-control" if any of the following events occur for any one of the quality control materials:

- (1) The mean from a single control falls outside the 99% (3SD) confidence limit;
- (2) The means from two controls fall either above or below the 95% (2SD) confidence limit; or

- (3) The daily means of one control from eight successive runs lie either all above +1SD or all below -1SD.

The second type of quality control chart plots the **range** of the replicates (the difference between the highest and lowest value of a single control within a run). It is compared with the target range which is the overall mean of daily ranges established by the twenty characteristic runs.

The NHANES guideline declares a system as "out-of-control" if any of the following events occur for any one of the quality control materials:

- (1) The daily range from a single control falls outside the 99% confidence limit;
- (2) The daily ranges from two controls fall above the 95% confidence limits; or
- (3) The daily ranges of one control from eight successive runs fall all above +1SD.

If the system is declared "out of control", the system (instrument, calibration standards, etc.) is investigated to determine the cause of the problem before any analysis of specimens.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

- A. When the QC results fail to meet the acceptable criteria, check the sample cup containing the QC specimen for bubbles and reanalyze the QC specimen.
- B. If the QC results meet the acceptable criteria, accept the run and report the results. Otherwise, troubleshoot the system to locate probable cause of the problem. If a cause can be identified and corrected, notify the supervisor. The supervisor will evaluate the situation and determine whether to accept or reject the run.
- C. *If no obvious cause of a problem can be identified, the run is rejected and all of the specimens are repeated. Recalibration?*
- D. Document the problem and actions taken, if any, on the daily worksheet.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Hemolysis in samples is known that has a significant effect on the assay.

Samples that are hemolyzed with greater than 100mg Hb/mL may not give reliable insulin concentrations. In this case, the comment : "Exceeds Hemolysis Limit " is written on the Test Request Form.

Lipemia has an insignificant effect on the assay except in the case of gross lipemia where spatial interference may occur.

Specimens from patients who have received preparations of mouse monoclonal antibodies for diagnosis or therapy may contain human anti-mouse antibodies (HAMA). Such specimens may show false elevated values when tested for Insulin.

Conditions such as obesity, high carbohydrate diet and inactivity tend to increase expected normal values. Values are found to be elevated with food intake and in cases of acromegaly, Cushing's Syndrome, and thyrotoxicosis.

For a more complete understanding of the limitation of this procedure, please refer to the Specimen Collection and Handing,

13. REFERENCE RANGES (NON-DIABETIC VALUES)

- A. Tosoh suggested mean fasting levels for healthy individuals lie below 17 uU/mL
- B. 28 Frozen serum collected for the Pharmacia Meal Challenge reference ranges on Tosoh. The reference ranges are comparable to the values obtained from the Pharmacia assay. (Reference ranges for Pharmacia insulin were updated at the Diabetes Diagnostic Laboratory in October 2001 by combining result from two volunteer groups. In August 2001, 28 non-obese, non-diabetic subjects (mean age=34.7, M:F=15:13) fasted overnight (between 10 to 14 hours), donated blood and then drink 360 calorie (1 ½ cans) Sustacal™ dietary supplement. Blood was also drawn at the 30, 60, 90 and 120 minute time points following the Sustacal™ meal. 23 non-obese, non-diabetic subjects (mean age=28, M:F=13:10) were performed in August 1999 for fasting, 30, 60 and 120 minutes. Any subject with BMI greater than 30kg/m² and fasting glucose than 110 mg/dL was excluded from the calculation.) Thus Reference range will remain the same:

The means and observed ranges are:

Insulin Reference Range uU/mL					
	Fasting	30 min	60 min	90 min	120 min
N	51	51	51	28	51
Mean	7	47	30	23	13
Range	3–17	13–113	6–68	5–58	3–28

- C. Proper interpretation of the Insulin and C-Peptide results can be difficult: values are affected by many factors, such as body mass index, age and state of nutrition. Results that are outside of these reference ranges do not necessarily mean the abnormal test result is of clinical significance. This should only be determined by a physician after careful evaluation of the individual person's health record.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

These values aren't reported to the NCHS Medical Officer

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are thawed and maintain ambient temperature during analysis. Specimens are returned to -70°C storage as soon as the analysis is completed. Repeated freeze/thawing, except as necessary for specimen analysis, is avoided.

16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

If the analytical system fails, all specimens are returned to storage at -70°C. The specimens are re-analyzed when the system is back in control.

17. TEST RESULT REPORTING SYSTEM

After the assay has been accepted, the pathology patient sample results are transcribed onto the test request card. The supervisor against the original printout checks this. Results, which have been checked by the supervisor, are entered onto the ALS data management system (as described in **Reporting of Results**, Section 8.f.) Pathology sends a data check sheet, and all patient information and results are checked against the original test request form. Any discrepancies are corrected in the system, and then the final result is sent to the physician

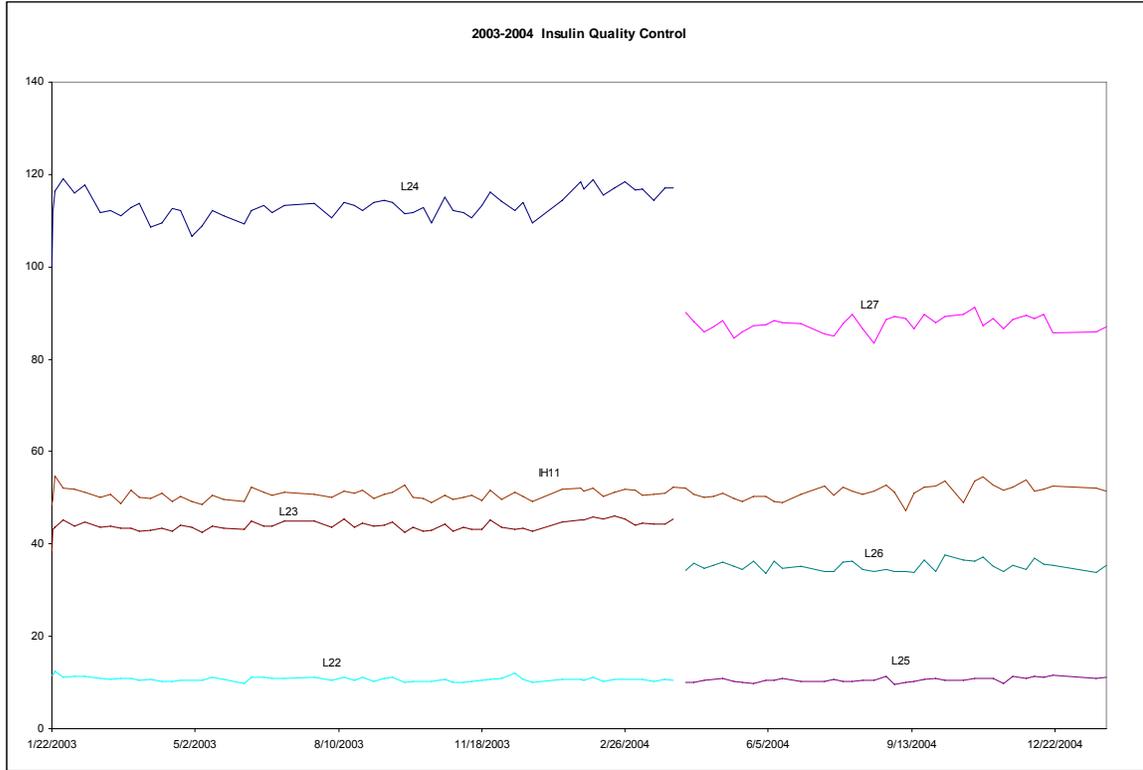
18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

All specimens are tracked on both laboratory log books and electronic data files kept on the Diabetes Diagnostic Laboratory network server and back up CDs. Hard copies of all shipping manifest reports, and data check lists containing the specimen information, test results, and daily assay information is kept in 3-ring binders. The QC diary log sheet data are stored in a separate notebook. Only the NHANES ID numbers are known to the laboratory. Other personal identifiers are not provided to the laboratory in order to protect the confidentiality of study participants.

Residual samples are stored at -70°C for 1 year and periodically shipped to the NCHS serum repository in Rockville, MD.

19. SUMMARY STATISTICS AND GRAPHS

Summary Statistics for Insulin by Lot						
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
L22	58	1/22/2003	3/31/2004	10.719	0.485	4.5
L23	58	1/22/2003	3/31/2004	43.885	1.171	2.7
IH11	95	1/22/2003	1/27/2005	50.926	1.364	2.7
L24	58	1/22/2003	3/31/2004	113.211	3.296	2.9
L25	37	4/8/2004	1/27/2005	10.571	0.487	4.6
L26	37	4/8/2004	1/27/2005	35.180	1.081	3.1
L27	37	4/8/2004	1/27/2005	87.727	1.711	2.0



APPENDIX

Table 1.

Flag and Error Codes

Flag	Description	Rates Output	Result Calculated
SE	System Error	NO	NO
SP	Sampling Pause	NO	NO
IS	STC Shortage or Discrepancy	NO	NO
RO	No Test Cup	NO	NO
ME	Matching Error	NO	NO
AE	Test Code Detection Error	NO	NO
NB	Seal Break Failure	NO	NO
TS	Tip Shortage	NO	NO
DS	Diluents Shortage	NO	NO
RS	Reagent (Cassette) Shortage	NO	NO
WS	Wash Shortage	NO	NO
WU	Washing Incomplete	YES	YES
BS	Substrate Shortage	NO	NO
ES	Pretreatment Shortage	NO	NO
LS	Sample Diluting Solution Shortage	NO	NO
SS	Short Sample	NO	NO

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SC	Sample Clog Detected	NO	NO
LE	Lot Detection Error	NO	NO
DO	Detector Over Range	NO	NO
NC	No Calibration	YES	NO
CE	Calculation Error	YES	NO
<L	Lower Than Assay Range	YES	NO
>H	Higher Than Assay Range	YES	NO
IO	Temperature Error	YES	YES
HB	Substrate Background High	YES	YES
CV	Calibration Void	YES	YES
DL	Lamp Intensity Low	YES	YES
MA	Match Error	YES	YES
L	Lower Than Reference Range	YES	YES
H	Higher Than Reference Range	YES	YES