Laboratory Procedure Manual

Analyte: **Insulin**

Matrix: **Serum**

Method: **Berthold Models LB 2111 and LB2104 Multi-Crystal Gamma Counter with Sodium Iodide 1-1/8" x 1-1/4" Crystals**

Method No.:

Revised:

as performed by: *Department of Child Health*
University of Missouri-Columbia

Contact: *Ms. Hsio-Mei Wiedmeyer*
1-573-882-2705

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 2001–2002 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
l10am_b	LBXIN	Insulin (Uμ/mL)
	LBXINSI	Insulin (pmol/L)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Human insulin is a polypeptide hormone that originates in the ß-cells of the pancreas and serves 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.

Insulin radioimmunoassay (RIA) is a double-antibody batch method. Insulin in the specimen competes with a fixed amount of ^{125}I-labelled insulin for the binding sites of the specific insulin antibodies. Bound and free insulin are separated by adding a second antibody, centrifuging, and decanting. The radioactivity in the pellet is then measured. The radioactivity is inversely proportional to the quantity of insulin in the specimen (1–3).

This test is used to measure insulin levels in the bloodstream and is also useful in determining pancreatic ß-cell activity.

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

2. SAFETY PRECAUTIONS

Wear gloves, a laboratory coat, and safety glasses when handling all human blood specimens. Discard all plastic tips, sample cups, gloves and contaminated assay materials into a biohazard waste container. Discard all disposable glassware into a waste container for sharps. These containers are collected and processed twice a week by the University of Missouri-Columbia (UMC) hazardous waste management personnel.

^{125}I-labelled insulin has approximately 2 µCi ^{125}I radioactivity per kit. A laboratory coat, safety glasses, and gloves are required while handling all radioactive materials. Wear a film badge that monitors radioisotope dosage on the lapel during the RIA procedures. The work area is surveyed for contamination monthly. Discard liquid and solid radioactive waste into their properly labeled containers. The containers are collected and disposed of by the UMC health physics/environmental health personnel.

Protect all work surfaces with disposable absorbent bench top paper, which is discarded into biohazard waste containers weekly, or whenever blood contamination occurs. Wipe all work surfaces with Envirocide solution weekly.

> Dispose of all biological samples and diluted specimens in a biohazard waste container at the end of the analysis. Smoking, eating or drinking is not permitted in areas where radioactive materials are being handled. Dispose of all radioactive waste in properly labeled radioactive waste containers.

Material safety data sheets (MSDSs) for Envirocide are available at Diabetes Diagnostic Laboratory at the University of Missouri, Columbia (UMC).

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

The integrity of specimen and analytical data generated by this method is maintained by the following protocol:

A. Each NHANES IV shipment is labeled with a unique container number. An electronic shipment file is sent to the laboratory at the time when samples are shipped. This file corresponds with the Shipping

Manifest Report (SMR) included in each shipment of specimens. The electronic file contains sample ID, slot ID, collection date, time, and comment code associated with each specimen. The file is formatted as a comma delimited file with an .shp extension.

B. The electronic file is saved to a network drive with a .txt extension. A backup copy is created for each file.

C. A Microsoft Access database (Hanes4.mdb) has been established on the network drive. The shipment file is first imported into a temporary import table in the database. After the data is verified with the SMR, the file is then imported into the RIA (Insulin/C-peptide) analyte table.

D. A batch number is assigned to each shipment. A unique and sequential laboratory accession number is assigned to each specimen. A blank "Data Check Sheet" (work list) is generated by batch number and by analyte for the laboratory technologists.

E. All test result and quality control (QC) files are stored on the network server. Files are backed up daily on tape and monthly on CDs.

F. Records of specimen tracking are kept on Sample Flow Tables located in the same database.

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

A. Fasting blood specimens are collected for insulin analysis in accordance with NHANES IV sample collection protocol.

B. Specimen type: serum without anticoagulants or preservatives.

C. The optimal volume for this test is 1.5 mL; the minimum volume is 0.5 mL.

D. Whole blood, 3 to 5 mL, is collected in a vacuum tube (red-top or serum-separator tube). Specimens are allowed to clot at room temperature for 15–30 min, then centrifuged at 1500 × g for 10 min. Serum is transferred to a 2-mL cryogenic screw-top vial and frozen at –20°C. Frozen serum samples are sent weekly in batches in Styrofoam-insulated shipping containers with dry ice to the University of Missouri Diabetes Diagnostic Laboratory via overnight courier.

E. Insulin is stable for at least 1 month at –20°C and 1 year at –-70°C (4). Specimens should be processed within 1 hour of collection. Upon receipt, the processing laboratory will store the specimens in a –-70°C freezer until analysis. Repeated freeze/thaw cycles, except as needed for analysis, should be avoided.

F. Samples with insufficient volume or samples that arrive thawed are rejected. No analyses are performed on specimens that do not meet acceptance criteria. These conditions are noted in the assay comment codes.

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. Instrumentation

(1) Berthold models LB 2111 and LB2104 multi-crystal gamma counter with sodium iodide 1-1/8" x 1-1/4" crystals (Wallac Inc. Gaithersburg, MD). Counting efficiency is approximately 75% for ^{125}I ; the measuring range is up to 250,000 CPM per detector.

(2) Jouan refrigerated centrifuge models GR4-22 and K110 (Jouan, Winchester, VA). Temperature range: –8°C to 60°C, temperature accuracy: ≤2°C, maximum RPM: 8,000 maximum, timer range: 0–99 min in 1-min increments.

(3) Eppendorf tip ejector fixed-volume pipettetors, 50 and 100-µL volume (Fisher Scientific, St. Louis. MO).

(4) Eppendorf repeater pipette, range from 10 µL to 5 mL, precision to 0.1% (Fisher Scientific, St. Louis, MO).

(5) Combitips for Eppendorf repeater with adapter, 2.5-mL tip graduated in 50-µL increments (Fisher Scientific, St. Louis, MO).

(6) Cornwall repeating dispenser, 2-mL volume (Fisher Scientific, St. Louis, MO).

(7) Pipetteman adjustable pipette, 200–1000 µL (Rainin Instruments, Woburn, MA).

(8) Milli-Q Plus ultrapure water system (Millipore, Bedford, MA).

B. Materials

(1) Pharmacia Insulin RIA kit (Pharmacia Diagnostics AB, Uppsala, Sweden). Reagents are stable until the expiration date, which is printed on each bottle. The recommended storage temperature for all reagents is 2–8°C5. Each kit contains reagents for assaying 100 tubes. Each kit contains the following:

Standards: 6 levels [0, 3, 10, 30, 100, and 240 micro-International Units (µIU)] of human insulin standards

Diluent: 50 mL of phosphate buffer containing bovine serum albumin, EDTA, and detergent, pH 7.0.

Insulin antiserum (guinea pig): Guinea pig antiserum in assay buffer, color-coded yellow.

^{125}I-Insulin : approximately 2.6 ng (1.0 µCi at date of manufacture), color-coded blue.

Decanting suspension: Sepharose anti-guinea pig IgG raised in sheep.

Store standards at 4–8°C and mix well before use.

(2) Veronal buffer (World Health Organization (WHO), Geneva).

(3) 12- × 75-mm Borosilicate glass culture tubes (any vendor).

(4) 1.8 mL Nalgene cryogenic Vials (Nalgene Company, Rochester, NY).

(5) Racks for radioimmunoassay tubes (Fisher Scientific, St. Louis, MO).

(6) Waterproof markers for labeling tubes (any vendor).

(7) Pipette stand (Fisher Scientific, St. Louis, MO).

(8) Class A 5.0-mL volumetric pipette, calibrated "to deliver" (Fisher Scientific, St. Louis, MO).

(9) Reusable glass beakers, various volumes, accurate to 5% (Fisher Scientific, St. Louis, MO).

(10) Disposable gloves (any vendor).

(11) RIA decanting rack (Pharmacia, Uppsala, Sweden).

(12) 12-well plastic counting rack for gamma counter (Berthold, Gaithersburg, MD).

(13) Berthold multi-calibrator matched ^{125}I sources (Berthold, Gaithersburg, MD).

(14) Prescored general purpose ampules, 1-mL (Fisher Scientific, St. Louis, MO).

(15) Aluminum foil (any vendor).

(16) Absorbent bench top paper (Whatman Lab Sales, Hillsboro, OR).

(17) Bleach (10% sodium hypochlorite) (any vendor).

(18) Viro Research Envirocide Disinfectant Decontaminant Cleaner (Fisher Scientific, St. Louis, MO).

(19) Computer printout paper (any vendor).

C. Reagent Preparation

For assays of more than 100 tubes, pool kit reagents bearing identical lot numbers and mix well before use. Mix gently to avoid foaming.

D. Standards Preparation

Human insulin standard materials, 0, 3, 10, 30, 100, and 240 µIU/mL, purchased from Pharmacia Diagnostics. Standards are calibrated against 1st International Reference Preparation 66/304. Ready for use.

E. Preparation of QC Materials

Three levels (low, medium, high) of lyophilized human serum controls are purchased from Bio-Rad Laboratories, Irvine, CA. Using a Class A volumetric pipette, reconstitute each vial of control serum at room temperature by adding 5.0 mL distilled water. Allow the vials to stand for 10 minutes; invert the vials several times to mix contents.

Do not shake the vials. If more than one vial of each control is reconstituted, pool and mix vials with identical lot numbers together. Transfer 750-µL aliquots of each pool into cryogenic storage tubes. Cap the tubes tightly and freeze the aliquots at –70°C. Thaw each aliquot one time only. Stable until expiration date.

The In-House control Lot # 10 (IH10) was prepared by collecting one unit of whole blood from three non-diabetic volunteers. All blood was screened for HIV and hepatitis B. Serum was separated from red blood cells, then pooled and transferred 750 µL aliquot into cryogenic storage tubes. The tubes were tightly capped and frozen at –70°C. Reconstitution of IH10 is not required. Thaw each aliquot one time only.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Calibration Curve

(1) A calibration curve is constructed by using the linear %B/B0 (B = %bound, B0 = maximum binding) of standards at 0, 3, 10, 30, 100, and 240 µIU/mL plotted against the logit of insulin concentrations.

(2) A cubic spline curve option is chosen in the Berthold "Create Protocol" option in the gamma-counter software.

(3) The calibration curve is displayed immediately following the standard curve summarization. To verify the mathematical fit, the smoothing factor must be less than 16 for assay acceptance.

(4) Percent B0/total counts (TC) is monitored to verify the binding activity of the antibody and labeled ^{125}I insulin solution. It should be between 45% to 55%. If the B0/TC value is outside of those limits, notify the supervisor prior to accepting a run.

B. Verification

The World Health Organization's (WHO's) international standard for insulin (first International Reference Preparation, 66/304) is used monthly to verify the insulin calibration curve. Prepare the stock solution by dissolving the ampoule supplied by WHO in 0.75 mL of 0.07 M veronal buffer (pH 8.6). Class A volumetric pipettes are used to prepare subsequent working standards of 3, 9, 30, 100, 120, 150 and 240 µIU/mL with Pharmacia diluent (WHO Lot#2, prepared at the University of Missouri in November 1997) . Store both stock and working standards in sealed 1-mL ampule vials at –20°C.

The stock and working solutions are stable at –20°C for years. Thaw one set of working standard monthly and, in a regular insulin assay, analyzes it simultaneously with Pharmacia standard. The validation is monitored in two areas:

(1) The Pharmacia kit standard curve is compared directly with WHO Lot #2 insulin standard curve. The relationship between the two standard curves over time should be consistent with the initial relationship established in November 1997.

(2) A group of samples is analyzed by using both sources of standards. The means and linear regression relationships between the paired insulin values are calculated and charted. For low levels of insulin (<20 µIU/mL), the mean insulin value obtained from Pharmacia should be within 25% of the mean value obtained from WHO. For medium (20 to 60 µIU/mL) and high (>60 µIU/mL) levels of insulin, the mean insulin values from Pharmacia should be within 15% of the mean values from WHO.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

(1) Allow frozen samples and quality control materials (three levels of Bio-Rad controls and one level of in-house control) to reach ambient temperature. Invert each gently to mix.

(2) Allow the standards, antibody, ^{125}I tracer, and assay buffer to reach ambient temperature. (Note: keep the precipitating reagent cold until ready to be used).

(3) Label a set of 12- × 75-mm borosilicate glass tubes in a RIA rack in the following sequence: Total Count (TC), nonspecific binding (NSB), and 6 levels of standards (all in triplicate); followed by one set of controls, specimens, and another set of controls (all in duplicate).

B. Sample preparation

(1) All samples are analyzed initially without dilution.

(2) For information regarding the range of linearity and how to handle results outside this range, refer to the calculations section of this document.

C. Procedure

All single-volume dispensing (standards, controls and unknowns) is performed with Eppendorf fixed-volume pipettes. Use a new tip for each new specimen. All multiple-volume dispensing (^{125}I insulin, insulin antibody, decanting suspension) is done with an Eppendorf 2.5-mL Repipete with Combitips graduated in 50-µL increments (e.g., a setting of "1" will dispense a volume of 50 µL; a setting of "3" will dispense a volume of 150 µL).

(1) Pipette 150 µL of diluent in triplicate into the NSB tubes.

(2) Transfer 100 µL of standards, controls, and unknown specimens into their respective borosilicate test tubes.

(3) Add 50 µL of ^{125}I-insulin to all tubes.

(4) Add 50 µL of insulin antiserum to all tubes except the TC and NSB tubes. Checkpoint: Contents of all tubes should now be green.

(5) Shake the rack vigorously to ensure that the samples are well mixed. Cover the rack with aluminum foil and incubate for 2 hours at room temperature.

(6) At the end of the incubation period, remove the bottles containing the decanting solution from the cold room; however, do not allow the solution to warm up to room temperature. Shake the bottles vigorously to make the suspension homogenous before using the solution.

(7) Using a Cornwell repeating syringe, add 2 mL of the COLD (4–8°C) decanting reagent to each

tube except the TC tube.

(8) Vortex all tubes. Cover the rack with aluminum foil and incubate the tubes for an additional 30 min at room temperature.

(9) Centrifuge the tubes for 10 min at 2000 rpm at 4°C. Do not allow the tubes to stand after centrifugation.

(10) Transfer all tubes, except the TC tube, to a specially designed decanting rack. Press the tubes down so they are seated securely inside the rack. Invert the rack in one smooth motion and decant the supernatant into a waste container. Invert the tubes for 30 sec on plastic-backed absorbent paper to blot off excess liquid, then turn the tubes upright. Dispose of contaminated waste liquid properly.

(11) Check the background activity of the counting racks by counting the empty racks for 2 min prior to placing any RIA tubes in the racks. Background activity should be less than 100 CPM. If the background exceeds 100 CPM, do not use the rack for the assay. The rack must be set aside or cleaned with decontaminant wash until its radioactivity has decreased below 100 CPM. See Section 8.G.2 for instrument calibration and efficiency measurement procedures.

(12) Count the pellets for 2 min. See section 8.D and 8.E for how to set up and operate the Berthold gamma counter.

D. Instrument Set-Up

(1) Berthold Multi-Crystal Gamma Counter (6)

(a) An evaluation method has to be assigned to each protocol number prior to operation. At the main menu, choose CHANGE DIRECTORY.

(b) Using the space bar, select RIA for the protocol number that is used for RIA INSULIN. Enter it into the directory by pressing RETURN.

(c) Create a protocol for insulin RIA by choosing CREATE PROTOCOL from the main menu.

(d) Enter the parameters as indicated in Table 1.

Table 1. Parameters for the Berthold Gamma Counter

Parameter	Setting	Parameter	Setting
Name of test	Insulin	Number of standards	5
Measurement time	2.00 min	Units of Concentration	µU/mL
Isotope	^{125}I	Standard 1	3
Curve fit	Spl-auto	Standard 2	10
Type of assay	Bound	Standard 3	30
		Standard 4	100
TC	3 Replicates	Standard 5	240
NSB	3 Replicates	Lower threshold	1.00
B_0	3 Replicates	Upper threshold	100.00
Standards	3 Replicates	No. of QC Controls*	4
Pat-NSB	0 Replicates	NSB/T	Calculated
Unknowns	2 Replicates	B_0/T	Calculated
Dilution factor	1	Slope at 50%	Calculated
Ether interference	Off	ED20	Calculated

Quality control	Full QC	ED50	Calculated
		ED80	Calculated

* Maximum allowed by the program is 3; if additional controls are used, specify the number used in assay set-up mode under OPERATION.

(2) Jouan Refrigerated Centrifuge model GR4-22

(a) All controls and indicators are located on the front panel.

(b) Press PROG to program the parameters for various centrifuge conditions. The initial set up is needed only once for each program.

(c) Press ENTER to move from one parameter to next.

(d) Make changes when it is necessary. Press ENTER.

(e) Press the program number for SAVE PROG NUMBER.

(f) After the initial set up has been completed, choose the required centrifuge parameters for RIA procedures by pressing RCL and PROG NUMBER.

Table 2. Parameters for Jouan Centrifuge GR4-22

Parameter	Setting	Parameter	Setting
Program number	(1–9)	Acceleration rate	9
Radius	172 mm	Brake	9
Time	10 min 00 sec	RPM/RCF	RPM
Temperature	4°C	Speed	3000 RPM
Temp deviation	0	Saved program #	(1–9)

(3) Jouan Refrigerated Centrifuge Model K-110

For this instrument, the desired time, temperature and speed settings are controlled with a knob on the front of the instrument panel. Adjust the knob to the desired value, and press the yellow button to start the centrifuge.

Parameter	Setting
Temperature	4°C
Time	30 mins
Speed	200 × 10/min

E. Operation of Gamma Counter

(1) At the main menu, select MEASUREMENT UNINTERRUPTED.

(2) Choose the protocol number identified as "RIA Insulin".

(3) The parameter inputs for operational set up are shown in Table 3.

Table 3. Operational Set Up for Gamma Counter

Parameter	Setting
Comment	
Outlier Rejection	On
Standard Curve Used	Create new curve
Curve Plot	Linear-log
Patient ID File	not used
Auto Incr. Numbers Start with	(1st Sample ID)
Blank lines	1
Control Position Setup Total #	4
Position of Controls QC1 QC2 QC3 QC4 QC5 QC6 QC7 QC8	 1 2 3 4

Operational set up for gamma counter.

Press HOME to exit.

(4) After the protocol information is entered, the gamma counter screen displays the tube sequence. Place the first 12 RIA tubes in plastic counting rack. Match the tube sequence with the screen display. Insert the rack into the gamma counter.

(5) Press the red button to initiate counting. The counting time is indicated on the gamma counter.

(6) At the end of 2 min, a beep will sound to alert the operator to place the next rack of 12 RIA tubes in the gamma counter.

(7) Proceed until all tubes have been counted.

(8) All data reduction is done automatically.

F. Recording of Data

(1) Quality Control Data

All replicate values of QC data plus all pertinent assay information (date of analysis, reagent lot number, technician ID, samples ID etc.) are recorded in the Microsoft Access Insulin Daily Diary Log database located on the network drive.

Enter the data under the form “Diary Sheet Entry Form”. The Microsoft Access program will automatically calculate the daily mean and range for each control and determine if a run is accepted or rejected. The current above or below the mean trend is also calculated. The program will print out a diary sheet for each run and the information is checked and signed by a supervisor. The daily mean and range for each control are calculated in the Diary Log Sheet database.

(2) Analytical Results

Record the fasting serum insulin results in µIU/mL in the "Data Check List", matching the sequential UMC numbers on the gamma counter print out with corresponding numbers on the data check list.

(3) Insulin results are entered in HANES4.mdb database. During the data entry process, check the lab accession number.

NHANES IV has established a list of assay comment codes for reporting results. If a result is below the assay detection limit (2.5 µIU/mL), or a sample is missing, or there is not enough sample to run the test, leave the result blank and record the appropriate comment code in the assay comment field. Lipemic or hemolyzed samples are also coded in the comment field.

(4) A second Data Check List with test results is printed. Test results are verified against the gamma counter printer out. A copy of the data check list is kept in the NHANES IV RIA Data Book at the Diabetes Diagnostic Laboratory at the University of Missouri.

(5) A comma delimited test file (container id.txt) is generated in Hanes.mdb with an export query. The file follows the format specified by NHANES IV. A copy of the text file is printed and the information is validated against data check list.

(6) The data files are exported by batch within three weeks of receipt of the specimens. The text file is sent to Westat via electronic mail.

The quality control information and the assay information are entered into the Microsoft Access RIA Diary Log Sheet database located on the network drive. A QC file (INSmmyy.txt) is generated from the RIA Diary Lot Sheet database following the format specified by NHANES IV. The file is sent monthly to Westat via electronic mail.

G. Replacement and Periodic Maintenance of Key Components

(1) The centrifuge RPM and timer are checked and the centrifuge buckets are greased yearly by a commercial company (DiRuscio Associates, Manchester, MO). The brushes are checked semiannually and changed as needed. Centrifuge maintenance is recorded in the equipment maintenance log book.

(2) A monthly calibration program is performed on the Berthold gamma counter by using a matching set of multicalibrator reference sources. Choose CALIBRATION OPTIONS under the gamma counter main menu. Place the 12 matching ^{125}I reference sources in the counting rack. Perform INSTRUMENT QC, STANDARDIZATION, and HV ADJUST following the instructions displayed on the screens. Press START, and the calibration program will be performed automatically. A report will be generated listing the efficiency and the background measurements of all 12 wells. Standardization of all wells is performed via high-voltage standardization. The report is kept in the gamma counter log book. If the calibration program fails, the supervisor is notified immediately. The air filter for the gamma counter is cleaned monthly. All calibrations and maintenance records are recorded in the equipment maintenance log book.

(3) All pipettes are calibrated quarterly by using the gravimetric method. The method measures the performance of a pipette by using an analytical balance with distilled water. The accuracy and precision of the pipette at a specified volume are calculated by using a Quattro spreadsheet program. Pipettes that do not meet the accuracy and precision criteria are returned to the manufacturer for repair. All calibration results are recorded in the pipette calibration log book.

H. Calculations

(1) The Berthold gamma counter has full data reduction capabilities. The calibration curve is obtained with a smoothed cubic spline function. The curve plots linear $\%B/B_0$ versus logit of the concentrations, where

$$\% B/B_0 = (B - NSB)/(B_0 - NSB) \times 100.$$

B_0 = Mean bound counts at maximum binding

B = Mean bound counts of specimen

NSB = Mean bound counts of non-specific binding

The Berthold data-reduction program generates a 6-point sigmoidal splined standard curve (B_0

included). Control and unknown sample values are obtained from the curve.

The program also provides a smoothing factor, which indicates the deviation between measuring points and curve points, or the deviation required for a good curve fit. Generally, the smoothing factor should be between 0.125 and 4. Smoothing factors exceeding 16 require the supervisor's approval before the assay is accepted.

(2) Freshly iodinated ^{125}I-insulin should provide at least 10,000 CPM for total counts (TC). Do not use any labeled materials in which the TC activity has deteriorated below 3,000 CPM.

(3) Percent B_0/TC can be used as a check on the binding activity between the antiserum and the labeled materials. For Pharmacia insulin antiserum, %B_0/TC usually is in the range of 45% to 55%. If it falls outside those limits, notify the supervisor.

(4) Test results are expressed as micro International Units of Insulin per milliliter of serum (µIU/mL). The insulin RIA has a low detection limit of 2.5 µIL/mL and is linear up to 100 µIU/mL.

(5) Dilute and reanalyze any specimen with a concentration greater than 100 µIU/mL. Depending on the concentration, prepare a new specimen using either 1 part serum with 1 part assay buffer with a dilution factor of 2 (DF2) or 1 part of serum with 3 parts of assay buffer (DF4). Multiply the measured value by the appropriate dilution factor before reporting.

(6) The low detection limit, based on 10 repeat measurements of zero standard and serial dilution of a sample containing a low insulin concentration, is determined to be 2.5 µIU/mL. All specimens with insulin value less than 2.5 µIU/mL are reanalyzed for confirmation. If the result is confirmed, leave the result field blank and enter the appropriate comment code.

(7) Duplicate specimen tubes for which the coefficient of variation is greater than 10%, indicating unacceptable imprecision, are flagged by the gamma counter software. These specimens are reanalyzed.

9. REPORTABLE RANGE OF RESULTS

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

10. QUALITY CONTROL (QC) PROCEDURES

Two types of QC systems are used in this analytical method: 1) "batch QC" specimens that are placed in each run, and 2) "sample QC" specimens (2% of the total specimens) that are randomly selected from each run and analyzed either within-assay or between-assay for quality assurance purposes. If the deviation between duplicates is greater than 10%, the specimen is reanalyzed.

The batch QC pools consist of four levels of control pools that cover the spectrum of C-peptide ranges for both normal and diabetic populations. Three are commercial lyophilized serum controls, L19, 20 and 21 (Lyphchek, Lot 40081, 40082 and 40083, expiration 12/2001) are purchased from Bio--Rad Laboratories (Irvine, CA). The other control, IH10 was prepared in-house and stored at -70 °C. One vial of each is thawed and used in each assay. Reconstitution is not required for in-house control. All four levels of controls are assayed at the beginning and end of each analytical run.

If the stock of these controls becomes low, another batch is ordered or prepared in time to analyze it concurrently with the current QC materials. The new controls are used only after their means and the ranges have been established by performing 20 characterization runs. All updates of control means and ranges are performed after approval from NCHS.

Daily means and ranges of the controls are calculated from 20 interassay determinations. The bias ranges of the daily means are set at ±1 SD or the 67% confidence interval (CI); the warning limits (WL) are the ±2 SD or the 95% CI and the control limits (CL) are the ±3 SD or the 99% CI. For the daily ranges, the bias limit is the mean + 1 SD with warning and control limits set at the mean +2 SD and the mean + 3 SD, respectively.

Examples of precision and accuracy estimates established for controls during NHANES IV are shown in Table 4.

Table 4. Daily Means and Ranges

Pool	Mean	95% Limits	99% Limits	95% Limits (Range)	99% Limits (Range)	Runs	CV,%
L19	12.45	11.21–13.70	10.58–14.32	2.30	2.90	20	4.98
L20	42.09	37.88–46.30	35.78–48.40	7.88	9.88	20	5.00
L21	106.22	95.60–116.84	90.29–122.15	21.12	26.17	20	5.00
IH10	27.06	24.96–29.16	23.91–30.21	6.08	7.51	20	3.88

(L19, L20, and L21 were established on 10/1/99, IH10 was established on 09/24/98.)

After each assay run, all control data are recorded on the daily worksheet. The analysis is accepted or rejected according to the guidelines established by NHANES, with a slight modification on the determination of a trend.

After each assay run, all control data are recorded on the daily worksheet. The analysis is accepted or rejected according to the guidelines established by NHANES, with a slight modification on the determination of a trend.

The quality of an assay is assessed by two types of Levey-Jennings quality chart plots. 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 20 characterization runs.

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

- The mean for one control from a single run falls outside the 99% confidence limits.
- The means for two controls from a single run fall outside the 95% confidence limits.
- The daily means for one control from eight successive runs (excluding the runs in which the mean is within1 SD or bias range) falls either all above or all below the center line.

The second type of QC 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 20 characteristic runs.

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

- The daily range for one control from a single run exceeds the 99% confidence limit.
- The daily ranges for two controls from a single run exceed the 95% confidence limits.
- The daily ranges for one control from eight successive runs (excluding the runs in which the mean is within 1 SD or bias range) are all above the center line.

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 further analysis of specimens.

The Diabetes Diagnostic Laboratory participates in an external QC program conducted by the College of American Pathologists (CAP). Two levels of survey materials are analyzed 3 times a year for insulin in a routine RIA run, and results are submitted to CAP for inter-laboratory comparison.

The Laboratory also participates in a second external QC program (Unity) offered by Bio-Rad Laboratories. The individual control values obtained in all insulin assays performed each month are submitted to Bio-Rad. These values are then compared with our own cumulative mean as well as the group cumulative mean (grouped by method). Up to 12 months of statistical data is available in each monthly report. The external QC reports are reviewed and approved by the supervisor monthly.

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

When the system is declared "out of control", take the following steps:

A. Recount the run.

B. Troubleshoot the system to locate probable cause of the problem (i.e., spilled tubes, partial loss of a pellet, pipetting error, or problem with one level of standard, etc.). If a cause can be identified and the problem 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, reject the run and reanalyze all of the specimens.

D. Document the problem and any actions taken on the daily diary log sheet.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

No significant interference from specimens containing anticoagulants, or from those that are lipemic or show hemolysis has been observed. However, patients taking insulin injections typically develop anti-insulin antibodies that will interfere with the assay. The cross-reactivity of Pharmacia insulin antibody with proinsulin is approximately 40%.

Obesity, consumption of a high carbohydrate diet, and inactivity tend to increase expected normal values. Values are increased after food intake and are higher among patients with acromegaly, Cushing's syndrome, or thyrotoxicosis. An extensive review and additional factors that may interfere with the determination of insulin are reported by Friedman (6) and Tryding (7).

13. REFERENCE RANGES (NORMAL VALUES)

A. The manufacturer indicates that fasting levels for healthy individuals lie below 20 µIU/mL (5).

B. The Diabetes Diagnostic Laboratory performed an internal reference range in August 1999 on non-obese, non diabetic adults (n = 23, mean age = 28 year). Subjects with a BMI greater than 30 kg/m^2 and fasting glucose greater than 110 mg/dL were excluded from the calculations. The mean and observed reference range are shown in Table 5.

Table 5. Mean and Observed Ranges for Insulin (µIU/mL)

	Fasting
Mean	8
Range	3-15

14. CRITICAL CALL RESULTS (PANIC VALUES)

Not applicable for this procedure.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens should reach and maintain ambient temperature during analysis. Return specimens to –70°C storage as soon as analysis is completed. Avoid repeated freeze/thawing except when reanalysis is required.

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

Because the radioimmunoassay is complex and the characteristics of antibodies are very different from one another, there is no acceptable alternative method of analysis. If the analytical system fails, return all specimens to storage at ≤ –70°C. Reanalyze the specimens when the system is back in control.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable.

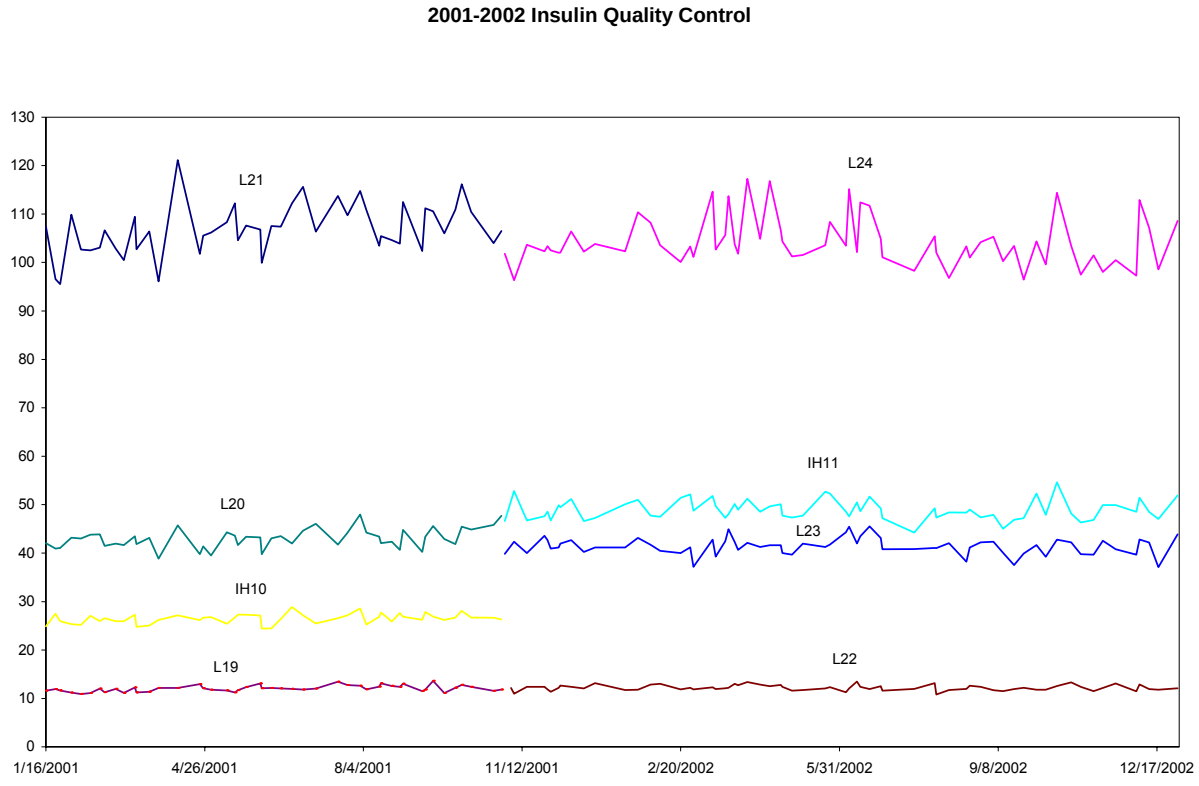
18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

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**
L19	48	1/16/2001	10/30/2001	12.055	0.646	5.362
IH10	48	1/16/2001	10/30/2001	26.481	1.017	3.841
L20	48	1/16/2001	10/30/2001	42.997	2.010	4.674
L21	48	1/16/2001	10/30/2001	106.820	5.239	4.905
L22	64	11/1/2001	12/30/2002	12.204	0.573	4.698
L23	64	11/1/2001	12/30/2002	41.422	1.750	4.226
IH11	64	11/1/2001	12/30/2002	48.957	2.062	4.211
L24	64	11/1/2001	12/30/2002	104.279	5.059	4.852



REFERENCES

1. Hales CN, Randle PJ. Immunoassay of insulin with insulin antibody precipitate. Biochem J. 1963:88;137.

2. Morgan CR, Lazarow A. Immunoassay of insulin: two-antibody system. Diabetes. 1963:12;115.

3. Prout J. Radioisotope measurements of insulin. In: Rothfeld J, ed. Nuclear medicine in vitro. 1974:Philadelphia;J B Lippincott Co, p 267.

4. Livessy JH, Hodgkinson SC, Roud HR, Donald, RA Effect of time, temperature and freezing on the stability of immunoreactive LH, FSH, TSH, growth hormone, prolactin and insulin in plasma. Clin Biochem. 1980:13;151–155.

5. Pharmacia Insulin RIA 100 package insert. Uppasala, Sweden: Pharmacia Diagnostics AB, 1993.

6. Berthold Multi-Crystal Gamma Counter operations manual. Wildbad, West Germany: Laboratorium Prof. Dr. Berthold, 1990.

7. Friedman RB. Effects of Disease on Clinical Laboratory Test. Second ed. AACC Press 1989:3;138–139.

8. Tyding N et al. Editors. Drug effects in Clinical Chemistry. 1992:Stockholm;Apoteksbolaget, 372.

ACKNOWLEDGMENTS

We gratefully acknowledge the contributions of Doughua Huang, B.S., Alethea Tennill, M.S., Xiaoying Wei, Jack England, David E. Goldstein, M.D., and Hsiao-Mei Wiedmeyer, M.S. who assisted in developing the methodology, performing the analysis for insulin in the NHANES IV study, and preparing the manuscript for this chapter.