Laboratory Procedure Manual

Analyte: **C-Peptide**

Matrix: **Serum**

Method: **Radioimmunoassay (RIA)**

Method No.:

Revised:

as performed by: *Department of Child Health*
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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 1999–2000 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
lab10am	LBXCPSI	C-peptide (nmol/L)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Human C-Peptide is a peptide chain 31 amino acids in length. Metabolically inert, it originates in the pancreatic beta cells as a by-product of the enzymatic cleavage of proinsulin to insulin. (1, 2)

The modified Diagnostic Products Corporation (Los Angeles, CA) C-Peptide radioimmunoassay (RIA) is a double-antibody batch method. C-Peptide in the specimen competes with I-125-labeled C-Peptide for antibody sites. After incubation for a fixed time, separation of bound from free is achieved by the addition of a PEG-secondary antibody complex.

Serum C-peptide is useful for determination of pancreatic beta cell activity and, in the presence of anti-insulin antibodies, indirectly monitoring insulin secretion. (3)

2. SPECIAL 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), Biohazard Waste (non-sharp biohazard items), Broken Glass (clean, non-contaminated broken glass), and Radioactive Waste (any item which is radioactive. This includes biohazardous radioactive waste which is disposed of by Environmental Health and Safety as Mixed Waste).

I-125 labeled C-peptide has less than 3 microcuries (µCi) of radioactivity per assay. Laboratory coat and gloves are required while handling all radioactive materials. A film badge, which monitors radiation dose, is worn on the lapel during the RIA procedures. The work area is surveyed for contamination monthly. Discard liquid and solid radioactive waste into properly labeled containers. The containers are collected and disposed of monthly (or sooner if needed) by the University of Missouri health physics/environmental health personnel. Declared pregnant technicians also wear fetal radiation dose badges.

All work surfaces are protected by disposable absorbent benchtop 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.

DPC lyophilized reagents (standards, Antiserum and I-125) contain less than 0.2 g/dL of sodium azide. Dispose of excess reagent in the biohazard waste, or if also radioactive, dispose as mixed waste in the radioisotope waste container. Do not flush down the drains.

Smoking, eating or drinking is not permitted in radioactive work areas.

Dispose of all radioactive waste into properly labeled radioactive waste containers. Discard all biohazardous waste into properly labeled containers (sharp, non-sharp)

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 ▹ 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 code (DD 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

Enter the PS code, specimen accession number, and press ENTER.

Choose form 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

(1) At the results entry screen, type in the numerical test value for that specimen, and any comments needed.

(2) Press F5 to release the test value, and press F5 again to confirm.

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

A. Laboratory services are requested through the Pathology Computer terminal Specimens are sent to IR Processing, which routes the sample to the appropriate lab. Each requisition must include:

(1) Patient name
(2) Patient's birthday and sex
(3) Patient ID number
(4) Source of specimen
(5) Specimen collection date and time
(6) Name of test or profile requested (do not abbreviate)
(7) Name of requesting physician
(8) Test status (Stat, Expedite, Routine)
(9) Phone number
(10) Comments (if any)

B. Specimens must be labeled with a minimum of the following information:

(1) Patient name
(2) Patient ID number
(3) Collection date and time

C. Specimen collection and processing

(1) 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 (no more than 30 minutes) and centrifuge in a refrigerated centrifuge at 4oC at 2000 g for 10 minutes. Draw off the serum and store in a plastic cryovial at -20oC until the specimen can be transported to the laboratory. Frozen serum specimens should be delivered within 24 hours of collection.
(2) 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.
(3) The specimen may 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.
(4) Upon receipt by the Diabetes lab, the specimen will be logged in and stored at -70oC until analysis. Analyzed specimens will be stored at -70oC for one month, after which they may be discarded.

D. Criteria for acceptance of specimens.

(1) All specimens must be clearly labeled and legible.
(2) Specimens with any of the following conditions will be rejected:

- Blood is collected in the wrong type of tube
- The specimen quantity is insufficient for analysis
- The specimen has not been stored or transported correctly or in a timely manner
- The specimen label and the requisition have conflicting identifying information
- The specimen is spilled on the outside of the container or on the requisition

- The test requested is not one performed by the Diabetes Diagnostic Laboratory (will then be re-routed through Processing to the appropriate lab)
- The specimen is not labeled correctly (e.g., no patient name, or in the case of multiple specimens from the same patient, each sample is not labeled with draw dates and times)
- If any specimens are rejected, it will be noted in the Pathology Log book and the requesting physician or nursing station will be notified. If the sample rejection is due to IR Processing error, then that laboratory's supervisor will also be notified.

5. PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

A. Reagent Preparation

(1) All reagents required for this assay method are provided by Diagnostic Products Corporation (DPC, Los Angeles, CA). Each DPC C-Peptide kit (Catalog # KPED2) contains reagents for assaying 200 tubes. For assays of more than 200 tubes, pool and mix packages bearing identical lot numbers before use.

(2) All reagents, except for the precipitation solution, must be at room temperature before using. The precipitation solution must be kept cold (2–8°C) until use. Reconstitution of kit components is as follows:

C-Peptide Antiserum (color coded red)

Lyophilized, rabbit anti-human C-Peptide

Reconstitute with 11.0 mL of Type II reagent grade water. Allow to dissolve for 10 minutes, and invert gently to mix. Before reconstitution, it is stable at 4°C until the expiration date printed on the bottle. Upon reconstitution, it may be stored at 2–8°C and used for 30 days.

I-125 C-Peptide (color coded yellow)

Lyophilized, iodinated C-Peptide

Reconstitute with 11.0 mL of Type II reagent grade water. Allow to dissolve for 10 minutes, and invert gently to mix. Before reconstitution, it is stable at 4°C until the expiration date printed on the bottle. Upon reconstitution, it may be stored 2–8°C and used for 30 days.

Precipitating Reagent/Secondary Antibody (color coded blue-green)

Goat anti-rabbit anti-human C-Peptide, in dilute polyethylene glycol and saline solution

No reconstitution is required. Store at 4°C and mix well prior to use. Unopened, it is stable at 4°C until the expiration date printed on the bottle. After opening, it is stable for 30 days at 4°C.

B. Standards Preparation

(1) Standard A/Assay Diluent/Zero Calibrator

Reconstitute with 5.0 mL Type II reagent grade water. Allow to dissolve for 30 minutes. Lyophilized, it is stable at 4°C until the expiration date printed on the bottle. Once reconstituted, it may be aliquoted and frozen at –20°C for 30 days. Thaw and use each aliquot only one time.

(2) Standards B–G (Calibrator values are lot-specific: refer to the vial label).

Reconstitute each standard with 2.0 mL Type II reagent grade water. Allow to dissolve for 30 minutes. Lyophilized, it is stable at 4°C until the expiration date printed on the bottle. Once reconstituted, it may be aliquoted and frozen at –20°C for 30 days. Thaw and use each aliquot only one time.

C. Other Materials

(1) Waterproof markers for labeling tubes (any vendor)

(2) Graduated Cylinders, Single Metric Scales (100, 250, 1000 mL in volume, Fisher Scientific, St. Louis, MO)

(3) Class A Volumetric Pipettes, volumes 2.0 mL, 5.0 mL, 10.0 mL "To Deliver" (Fisher Scientific, St. Louis, MO)

(4) Latex examination gloves (any vendor)

(5) 12-well plastic rack for Gamma Counter (Berthold, Nashua, NH)

(6) Berthold multi-calibrator matched I-125 sources (Berthold, Nashua, NH)

(7) Aluminum foil (any vendor)

(8) Absorbent benchtop paper (University of Missouri)

(9) Bleach (sodium hypochlorite) (any vendor) or Unicide detergent (University)

(10) Computer printout paper (any vendor)

(11) Calculator

D. Instrumentation

(1) Berthold (Nashua, NH 03063) Multi-Crystal Gamma Counter Model LB 2111 or Model LB2104 with Sodium Iodide 1-1/8" x 1-1/4" crystals. The efficiency is approximately 75% for I-125 using glass tubes. The measuring range is up to 250,000 CPM per detector.

(2) Jouan (Winchester, VA) Refrigerated Centrifuge Model GR4-22 or Model K-110

Temperature Range: –8 to 60°C

Temperature Accuracy: <± 2°C

Maximum RPM: 8,000

Timer Range: 0–99 minutes in 1 min. increments

(3) Mettler (Hightstown, NJ) electronic balance, Model AT200.

(4) Thermolyne (Dubuque IA) Maxi Mix vortexer Model I.

(5) Fisher Accumet Basic pH meter; pH Range: 0.0 to 14.00; pH Accuracy: ±0.05 pH units; temperature Range: –5 to 100.0°C

(6) Eppendorf Tip Ejector Fixed Volume Pipetters (50, 100, 200, 300 µL volume Fisher Scientific, St. Louis, MO)

(7) Eppendorf Repeater Pipette (range from 50 µL to 12.5 mL, precision to 0.1%, Fisher Scientific, St. Louis, MO)

(8) Combitips for Eppendorf Repeater with Adapter (2.5 mL tip graduated in 50 µL increment, Fisher Scientific, St. Louis, MO)

(9) Combitips for Eppendorf Repeater with Adapter (5 mL tip graduated in 100 µL increment, Fisher Scientific, St. Louis, MO)

(10) Combitips for Eppendorf Repeater with Adapter (12.5 mL tip graduated in 250 µL increment, Fisher Scientific, St. Louis, MO)

(11) Cornwall Repeating Dispenser (2 mL and 5 mL volumes, Fisher Scientific, St. Louis, MO)

(12) Pipetman Adjustable Pipette (200–1000 µL, Rainin Instrument, Woburn, MA)

(13) Milli-Q Plus Ultra Pure Water System (Millipore, Bedford, MA), Type II Reagent Grade

6. CALIBRATION

A. A calibration curve is constructed using the linear B/Bo (B = % bound, Bo = maximum binding) of standards plotted against the log of C-peptide concentrations (pmol/mL) . Individual C- peptide standard values will vary with the lot number (as no universal reference material is available), but the approximate values are as follows:

Table 1.

Standard	Approximate ng/mL	Approximate pmol/mL
A	0.0	0.000
B	0.1	0.033
C	0.5	0.166
D	1.0	0.331
E	3.0	0.993
F	5.0	1.655
G	10.0	3.310

B. A cubic spline curve option is chosen in the Berthold "Create Protocol" option in the gamma counter software.

C. The calibration curve is displayed immediately following the standard curve summarization. To verify the mathematical fit, the smoothing factor must be less than 32 for assay acceptance.

D. Percent B0/TC are used to verify the binding activity of the antibody and labeled I-125 insulin solution. It usually is in the range of 45% and 75%. If the % B0/TC is outside of these limits, notify the supervisor prior to accepting a run.

7. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

(1) Allow frozen samples and controls (three levels of commercial lyophilized serum controls, purchased from Bio-Rad Inc. Hercules, CA, lot number 40030 expiration 02/98, and one level of In-House pooled non-diabetic serum control) to reach ambient temperature. Invert gently to mix.

(2) The C-peptide RIA is a batch method. All standards, controls and specimens are treated in the exact same manner throughout the entire assay procedure.

(3) Label 12×75 mm Borosilicate glass tubes in the following sequence:

(a) Total Counts (triplicate)

(b) Non-Specific Binding (triplicate)

(c) Standards (triplicate)

(d) First set of controls (duplicate)

(e) Samples (duplicate)

(f) Second set of controls (duplicate)

(4) Reconstitute the assay standards, Antibody and I-125 with Type II reagent grade water as described under Reagent Preparation (Sections 5.A. and 5.B.). Mix well prior to use.

For assays of more than 200 tubes, pool and mix packages bearing identical lot numbers before use. Mix gently to avoid foaming.

B. Sample preparation

(1) All samples are analyzed initially without dilution (DF1)

(2) Samples with a previous assay value greater than the upper assay limit (3.000 pmol/mL) must be re-analyzed with an appropriate dilution factor: DF2 (1 part sample + 1 part diluent), DF4 (1 part sample + 3 parts diluent), etc. Refer to the Calculations (Section 7.H.(4)) section of this document for information regarding correction of values.

C. Instrument Setup

(1) Berthold Multi-Crystal Gamma Counter (Model LB 2104 or Model LB 2111)

(a) An evaluation method has to be defined for each protocol number prior to operation. At the "MAIN MENU", choose "CHANGE DIRECTORY". At the next screen, choose an unused number for a new protocol.

(b) Select "RIA" with the space bar. Enter it into the DIRECTORY with RETURN.

(c) Create a protocol for DPC C-peptide RIA by choosing "CREATE PROTOCOL" under the "MAIN MENU".

(d) Enter the parameters as follows:

INSTRUMENT PARAMETER VALUE	
Name of test	DPC CPEP
Measurement time	2.00 minutes
Isotope	I-125
Curve fit	Spl-auto
Type of assay	Bound
Replicates:	
TC	2
NSB	2
Standards	2
Pat-NSB	0
Unknowns	2
Dilution factor	1
Ether interference	Off
Quality Control	Full QC
Number of Standards	6
Units of Concentration	pmol/mL
Standard 1	
Standard 2	
Standard 3	
Standard 4	
Standard 5	
Standard 6	
Lower Threshold	

Upper Threshold	
Number of QC controls*	3
NSB/T	Calculated
B0/T	Calculated
Bn/REF	Calculated
Slope at 50%	Calculated
ED20	Calculated
ED50	Calculated
ED80	Calculated

* Maximum allowed by program is 3; for additional controls, specify in assay setup mode explained under Operation (Section 7.E.).

(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. This initial set up is needed only once for each program.

INSTRUMENT PARAMETER VALUE	
Program Number	(1–9)
Radius	172 mm
Time sec	30 min 00
Temperature	4°C
Temp Deviation	0
Acceleration Rate	9
Brake	9
RPM/RCF	RCF
Speed	2000 × *g*
Saved Program #	(1–9)

(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 then the desired program number.

(3) Jouan Refrigerated Centrifuge Model K-110

For this instrument, the desired time, temperature and speed settings are controlled with a knob on the instrument panel. Adjust the knob to the desired value, and press the yellow button to start the centrifuge. To stop it before the time is up, simply press the red button.

D. Procedure

All single volume dispensing is done with Eppendorf fixed volume pipettes. A new tip is used for each new specimen. All multiple volume dispensing is done with an Eppendorf 5.0 mL Repipet with Combitip Tips graduated in 100-µL increments (e.g. a setting of "1" will dispense a volume of 100 µL; a setting of "3" will dispense a volume of 300 µL).

Each assay is accompanied by an Assay Log Sheet, on which all assay-related information is recorded, such as date, sample ID numbers, technician name, reagent/standard/control lot numbers and expiration dates, Standard values (printed on bottle labels). After the assay is counted on the gamma counter, the following information is recorded: total counts, % B0/TC, smoothing factor, detection limit, goodness of fit, and control results. The mean and range of each control are used to determine assay acceptance or rejection.

Day 1

(1) Pipette 100 µL of the zero calibrator A into the NSB and A tubes, and 100 µL of each of the calibrators B through G into correspondingly labeled tubes. Pipette 100 µL of each sample and control into the appropriate tubes.

(2) Add 100 µL of I-125-C-Peptide (color coded yellow) to all tubes. Shake rack.

(3) Add 100 µL of C-Peptide Antiserum (color coded red) to all tubes except NSB and TC. Vortex all tubes well. Checkpoint: all tubes (except TC) should be orange.

(4) Cover the rack with foil, label with radioactive labeling tape, and mark with the date and time. Incubate for 20–24 hours at 4°C.

Day 2

(5) Using a Cornwall Repeating Syringe, add 1.0 mL of cold Precipitating Solution (color coded blue-green) to all tubes except TC. Vortex.

(6) Centrifuge for 30 minutes at 2000 × *g* and 4°C.

(7) Place all tubes except TC into a decanting rack, and invert onto a radioactive waste receptacle. Allow the rack to remain inverted on a stack of paper towels for 10 minutes, and then blot gently.

(8) Check the background activity of the gamma counter counting racks by counting the empty racks for two minutes 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 for the assay. The rack is set aside until its radioactivity has deteriorated below the limit.

(9) Count the radioactivity in each tube for 2 minutes. Consult Sections 7.C and 7.E for how to set up and operate the Berthold Gamma Counter.

E. Operation of the Berthold Gamma Counter (4)

(1) At the MAIN MENU, select "MEASUREMENT UNINTERRUPTED"

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

(3) On the Operational Setup, the parameter inputs are:

Table 2.

Comment	<Tech comments>
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 #	8
Position of Controls	
QC1	1
QC2	2
QC3	3
QC4	4
QC5	# of samples + 5
QC6	# of samples + 6
QC7	# of samples + 7
QC8	# of samples + 8

Press "Home" to exit without counting the assay.

Press "Enter" to confirm.

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

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

(6) At the end of two minutes, a beep will sound to alert you to place the next twelve RIA tubes in the rack.

(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 quality control data plus all pertinent assay information are recorded on the C-peptide Assay Log Sheet. The calculated daily mean and range for each control are plotted on Levey-Jennings graphs.

(2) Analytical Results.

Test results are recorded in the Pathology Log book. Results are then recorded onto the test request form.

Results on the test request form are checked against the original gamma counter printout prior to data transmission.

Results are entered from the test request forms onto the Department of Pathology computer system using an HP 700/96 terminal with the AdVantage program (version 4.2):

(a) Log onto the system by typing the password at the password prompt. Each technician is assigned a unique, confidential password.

(b) Select Option .20 (Results) from the main menu

(c) Enter in the Processing Site Code (DD) and the accession number for the specimen. Then tab over to the FNC field and enter 'A' for adding new data. To edit previously-transmitted data, enter 'E' at the FNC field.

(d) Enter in the test value, making sure that all patient information is correct.

(e) Press F5 to transmit the data, and then press F5 again to confirm.

G. Replacement and periodic maintenance of key components

(1) Centrifuge. The centrifuge RPM and timer are checked and the centrifuge buckets are greased quarterly. The brushes are checked semi-annually and they are changed as needed. Centrifuge maintenance checks are recorded in the Equipment Maintenance Log book.

(2) Gamma Counter. A monthly calibration program is performed on Berthold Gamma Counter using a matching set of multi-calibrator reference sources. Choose "CALIBRATION OPTIONS" under the Gamma Counter MAIN MENU. Place the 12 matching I-125 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 are performed via high voltage standardization. The report is kept in the Gamma Counter Log Book. If the calibration program fails, notify the supervisor immediately. The air filter for the Gamma Counter is cleaned monthly. All calibrations and maintenance checks are recorded in the Equipment Maintenance Log book.

(3) Pipettes. All pipettes are gravimetrically calibrated quarterly. This method measures the performance of a pipette using an analytical balance with distilled water. The accuracy and precision of the pipette at a specified volume are calculated using a Quattro Pro for Windows spreadsheet program. Pipettes which do not meet the accuracy and precision criteria are returned to the manufacturer for replacement or repair. All calibration results are recorded in the Calibration Log Book.

H. Calculations

(1) The Berthold Gamma Counter has full data-reduction capabilities. The calibration curve is obtained with smoothed cubic spline function. The curve plots linear % B/B0 versus logit of the concentrations where:

% B/B0	=	[(B – NSB)/(B0 – NSB)]*100
B0	=	Mean Bound Counts at Maximum Binding
B	=	Mean Bound Counts of Specimen
NSB	=	Mean Bound Counts of Non-Specific Binding

Berthold data reduction programs generates a 6-point sigmoidal splined standard curve (B0 included). Control and unknown sample values are obtained from the curve.

The program also provides a smoothing factor which indicates the deviation permitted 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 16. Smoothing factors exceeding 32 require the supervisor's approval prior to assay acceptance.

(2) Freshly iodinated I-125 C-peptide provides approximately 30,000 CPM for total counts (TC). Do not use any labeled materials where the TC activity has deteriorated below 5,000 CPM.

(3) Percent B0/TC can be used to verify the binding activity between the antiserum and the labeled materials. For the DPC method, % B0/TC usually is in the range of 45–75%. If it falls outside those limits, notify the supervisor.

(4) Specimens with a previous assay value above the assay limit (3.000 pmol/mL) need to be repeated with an appropriate dilution factor: DF2 (1 part sample + 1 part diluent), DF4 (1 part sample + 3 parts diluent), etc. The raw gamma counter result must then be multiplied by the dilution factor (2, 4, etc) to correct for dilution.

8. REPORTABLE RANGE OF RESULTS

Test results are expressed as picomole of C-peptide per milliliter of serum (pmol/mL). The C-peptide RIA has a low detection limit of 0.030 pmol/mL and it is linear up to 3.000 pmol/mL. Duplicate specimen tubes with a coefficient of variance greater than 10% are flagged by Gamma Counter and the specimen is reanalyzed.

9. QUALITY CONTROL (QC) PROCEDURES

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

(1) Sample QC: five percent of specimens are randomly selected and analyzed either within-assay or between-assay for quality assurance purposes. If the deviation between sample duplicate is greater than 10%, the specimen is repeated.

(2) Batch QC: quality control specimens that 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 C-peptide ranges for both normal and diabetic populations. Three are commercial lyophilized serum controls L10, L11 and L12 purchased from Bio-Rad Laboratories (Hercules, CA).

The other control IH8 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 the 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.

A. Preparation of Quality Control Materials

Three levels of lyophilized controls (L16, L17, L18) are purchased from Bio-Rad Laboratories (Lot# 40061, 40062 and 40063, expiration 10/99). Using a Class A volumetric pipette, reconstitute each vial of control at room temperature with 5.0 mL Type II reagent grade water. Allow to stand for 10 minutes. Invert the vial several times to mix contents. Avoid shaking. If more than one vial of control is used, pool and mix identical lot numbers together prior to aliquoting. Transfer 750 µL aliquots into polypropylene storage tubes. Cap tightly and freeze at –70°C. Thaw each aliquot one time only.

The In-House control is prepared by collecting one unit each of whole blood from six 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. 500 KIU Trasylol/mL of serum is added as a preservative. Transfer 750 µL aliquots to polypropylene storage tubes. Cap tightly and freeze at –70°C. Reconstitution of the In-House control is not required. Thaw each aliquot one time only.

B. QC Guidelines

Permanent L16, L17 and L18 Control Parameters were established 05/01/98 using 66 inter-assay observations. Data includes both manual and robot runs. IH10 control parameter was established on 09/24/99 using 20 inter-assay.

Mean	**Mean**	**SD**	**1 SD**	**2 SD**	**3 SD**
L16	0.549	0.033	0.516-0.582	0.483-0.615	0.450-0.648
L17	2.116	0.134	1.982-2.250	1.848-2.384	1.714-2.518
L18	4.802	0.222	4.580-5.024	4.358-5.246	4.136-5.468
IH10	1.487	0.105	1.382-1.592	1.277-1.697	1.172-1.802
Range	**Mean**	**SD**	**1 SD**	**2 SD**	**3 SD**
L16	0.050	0.025	0.075	0.100	0.125
L17	0.321	0.171	0.492	0.663	0.834

L18	0.792	0.362	1.154	1.516	1.878
IH10	0.203	0.081	0.284	0.365	0.446

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 (Levey-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 system is declared "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% confidence limit;

(2) The means from two controls fall outside of the 95% confidence limits; or

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

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 system is declared "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 outside the 95% confidence limit; 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 subsequent analysis of specimens.

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

When the system is declared "out of control", then the following steps should be taken:

A. Re-count the entire run. Due to the variability of the Gamma Counter, a second count sometimes will solve the problem.

B. Troubleshoot the system to locate probable cause of the problem. If an obvious cause can be determined, such as loss of pellet, problem with one level of standard, etc., 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 and corrected, the run is rejected and all the specimens are repeated.

D. Document the problem and actions taken, if any, in the daily worksheet.

11. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

According to the DPC product insert, the following conditions may affect assay values. Bilirubin, when tested at levels of 100 mg/L and 200 mg/L, was found to interfere with the assay, causing elevation of values. Samples containing high levels of free fatty acid may yield misleadingly high C-peptide results. Accordingly, such sample results should be interpreted with caution. Hemolysis has no clinically significant

effect on this assay. Lipemia may interfere with the assay; hence, lipemic samples should be clarified by ultracentrifugation. Samples from patients receiving radioactive substance therapy may interfere with the test.

12. REFERENCE RANGES (NON-DIABETIC VALUES)

Reference ranges for C-peptide were established at the Diabetes Diagnostic Laboratory on 23 non-obese, non-diabetic subjects between the ages of 20 and 38 years old (mean age = 28 years). Each person was given a 360 calorie standard mean challenge (Sustacal) after an overnight fast.

Proper interpretation of the Insulin and C-Peptide results can be difficult: values are affected by many factors, such as body mass, 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 be determined only by a physician after careful evaluation of the individual person's health record.

The means and observed ranges are:

Table 4. C-PEPTIDE DPC Double-Antibody Radioimmunoassay (pmol/mL)

	FASTING	3O MIN	1 HOUR	2 HOUR
MEAN	0.56	1.82	1.47	1.01
RANGE	0.22–0.87	1.03–2.83	0.78–2.24	0.37–1.56

13. CRITICAL CALL RESULTS (PANIC VALUES)

Since test values vary depending on the individual's health record, all values are reported to the physician with no further action taken. Specimens may be repeated for verification upon request from the physician.

14. 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.

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

Radioimmunoassay is a complex method, and the characteristic of one antibody is very different from another; therefore there is no acceptable alternative method of analysis. 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.

16. TEST RESULT REPORTING SYSTEM

After the assay has been accepted, the pathology patient sample results are transcribed onto the test request card. This is checked by the supervisor against the original gamma counter printout. Results which have been checked by the supervisor are entered onto the ALS data management system. 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.

17. SUMMARY STATISTICS AND GRAPHS

Summary Statistics for C-Peptide by Lot						
Lot	**N**	**Start Date**	**End Date**	**Mean**	**Standard Deviation**	**Coefficient of Variation**
IH10	109	3/19/1999	12/28/2000	1.438	0.065	4.49
L16	29	3/19/1999	9/29/1999	0.528	0.025	4.80
L17	29	3/19/1999	9/29/1999	2.052	0.100	4.87
L18	29	3/19/1999	9/29/1999	4.700	0.200	4.26
L19	80	10/5/1999	12/28/2000	0.559	0.031	5.52
L20	80	10/5/1999	12/28/2000	2.144	0.118	5.49
L21	80	10/5/1999	12/28/2000	4.549	0.270	5.93

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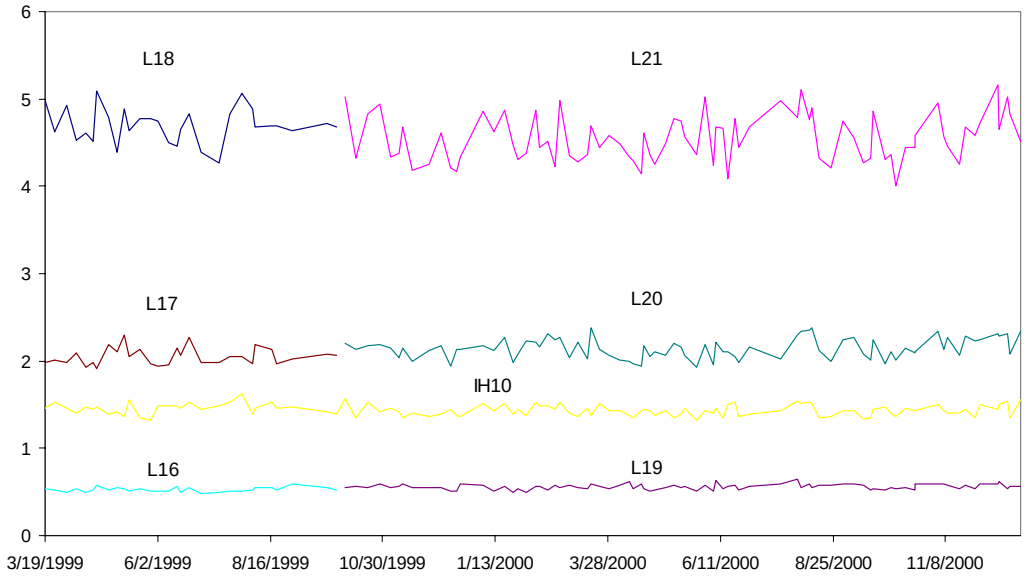

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