รายการอ้างอิง

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ประวัติผู้เขียน

นางสาวปราณี มาตรตุรังคกุล เกิดวันที่ 26 มกราคม พ.ศ. 2501 ที่กรุงเทพมหานคร สำเร็จการศึกษาปริญญาตรี แพทยศาสตร์บัณฑิต จุฬาลงกรณ์มหาวิทยาลัย ในปีการศึกษา 2524 เป็นแพทย์ฝึกหัดที่โรงพยาบาลจุฬาลงกรณ์ ระหว่างปี พ.ศ. 2525 ถึง 2526 หลังจากนั้นได้ปฏิบัติงานในฝ่ายอายุรกรรม โรงพยาบาลประจำจังหวัดน่าน ระหว่าง พ.ศ. 2526 ถึง 2528

เข้าฝึกอบรมเป็นแพทย์ประจำบ้าน สาขาอายุรศาสตร์ ระหว่างปี พ.ศ. 2528 ถึง 2531 เป็นหัวหน้าแพทย์ประจำบ้านของภาควิชาอายุรศาสตร์ ระหว่าง พ.ศ. 2530 ถึง พ.ศ. 2531 ได้เข้าศึกษาต่อในหลักสูตรมหาบัณฑิต สาขาวิชาต่อมไร้ท่อ และเมตาบอลิสม ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ ในปีการศึกษา 2529 และได้วุฒิบัตรจากการฝึกอบรมเป็นแพทย์ประจำบ้านสาขาโลหิตวิทยา ภาควิชาอายุรศาสตร์ ในปี พ.ศ. 2533

ปัจจุบันรับราชการในตำแหน่ง อาจารย์ประจำหน่วยโลหิตวิทยา ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ภาคผนวก

C-Peptide ^{125}I RIA Kit

Human C-Peptide of Insulin

Catalog #45065 or #45130

FOR IN VITRO DIAGNOSTIC USE ONLY.

NOT FOR INTERNAL OR EXTERNAL USE IN HUMANS OR ANIMALS.

INCSTAR Corporation
Stillwater, Minnesota 55082
Customer Service: 1-800-328-1482
In Minnesota: 1-612-439-9710
In Canada: 416-889-0396 or (Collect) 1-612-439-9711
The Netherlands: (31) 20-242888
United Kingdom: 44-734-772693

Revised: 5/89
Part #10404

1. INTRODUCTION

Intended Use
This kit is intended for **in vitro** diagnostic use only.

It contains instructions and materials for the quantitative analysis of human connecting peptide (C-peptide) of insulin by radioimmunoassay (RIA) in serum and urine. Endogenous insulin antibodies and other competitive substances are removed from the serum or urine specimen prior to measurement of C-peptide by precipitation with polyethylene glycol (PEG).

Summary and Explanation
Human insulin and C-peptide originate as a single polypeptide chain known as proinsulin, which is formed on the surface of the rough endoplasmic reticulum in the beta cells of the islet of Langerhans. Proinsulin (M.W. 9000) is then transported to the Golgi complex of the beta cells, where it is packed into granules. It is in these granules that proinsulin is cleaved proteolytically into insulin and C-peptide. Insulin and C-peptide are stored in these beta cell granules until their secretion is stimulated, at which time approximately equimolar amounts of each are released into the portal vein. Because of differences in uptake in the liver and in clearance times of these peptides, peripheral levels of C-peptide are higher than insulin.

While insulin has a pervasive influence on the body, affecting virtually every organ and biochemical component, C-peptide has no known physiologic function, other than possibly facilitating correct insulin conformation. Insulin and C-peptide do not share basic antigenic components and, therefore, antisera directed against insulin will not cross-react with C-peptide. It is this lack of immunoreactivity that makes C-peptide measurement quite valuable (6).

Exogenous insulin administration in diabetics frequently causes the formation of antibodies against insulin, which also bind proinsulin. This renders insulin assay ineffective in the determination of endogenous insulin secretion or general beta cell function. However, because C-peptide and insulin levels correlate strongly in most sera, assessment of C-peptide immunoreactivity will provide sound evaluation of beta cell function and, often, endogenous insulin secretion in these patients when appropriate steps are taken to eliminate the effect of endogenous insulin antibodies (5, 2). This can be accomplished effectively by preliminary precipitation with polyethylene glycol (10, 9).

In transient diabetes mellitus, the return of C-peptide levels to the normal range could provide evidence of remission. C-peptide measurement can be used to separate insulin-dependent diabetics into groups of those with and without some residual islet function, which might be useful in assessment of "brittleness" (4, 8, 12).

C-peptide measurements are useful in detecting suppression of insulin secretion following a challenge of exogenous insulin administration (7). It has been suggested that this test is useful in the diagnosis of insulin-secreting islet tumors or cancer (1). C-peptide measurement can also replace insulin assay in almost all provocative tests of islet function, including glucose tolerance and tolbutamide tolerance (8, 5).

C-peptide assessment after pancreatectomy is utilized to evaluate the existence of residual tissue. In pancreatic transplant, C-peptide measurement will be useful in testing for transplant function even in the presence of administered insulin and antibodies.

A final, less common use of the C-peptide assay is in the diagnosis of hypoglycemia brought on by surreptitious insulin administration. These cases can be identified by high insulin levels with coexisting low C-peptide levels (in contrast to elevated levels of both in endogenous hyperinsulinism) (11).

Method Description
The INCSTAR RIA kit for C-peptide of insulin utilizes a highly specific antibody to human C-peptide of insulin (residues 33-63 of the human proinsulin molecule). The assay can be performed by one of two method options.

Option A is an equilibrium assay employing simultaneous addition of sample, antibody and tracer followed by an overnight incubation at 2-8°C. A pre-precipitated second antibody complex is then added as a single step. The assay can be centrifuged after 15-25 minutes incubation at 20-25°C.

Option B is a disequilibrium assay in which sample and antibody are added and incubated for 16-24 hours at 2-8°C. Tracer is then added, followed by a second incubation for 16-24 hours at 2-8°C. A pre-precipitated second antibody is added in a single step to separate bound from free antigen. Option B is the most sensitive of the two procedures.

Quality Control will be performed at INCSTAR using the procedure outlined in Option A with PEG-treated samples.

Normal specimens need not be pretreated with PEG if one has determined previously that they do not contain endogenous insulin antibodies and other interfering substances. If one is not certain if the specimen contains antibodies and other interfering substances, we recommend performing the PEG-pretreatment.

2. REAGENTS

Catalog number 45065 sufficient for 65 determinations.
Catalog number 45130 sufficient for 130 determinations.
Upon receipt, reagents must be stored at 2-8°C.

1. 0 Standard
BSA-borate buffer contains Merthiolate as a preservative (lyophilized). Reconstitute the vial with 20 ml of distilled or deionized water, mix and allow it to stand for 15-20 minutes until the contents are completely dissolved; mix thoroughly before using. Store the reconstituted reagent at -15°C or lower.

2. Human C-Peptide of Insulin Standards
Human C-peptide of insulin (Tyr 17-31) standards at nominal concentrations ranging from 0.6 - 20 ng/ml are prediluted in BSA-borate buffer containing stabilizers (lyophilized). Exact values are assigned according to each lot. If Option B is performed, an optional low standard with a nominal concentration of 0.15 ng/ml is available upon request (Cat. No. 20258). Reconstitute each vial with 1.0 ml of distilled or deionized water, mix and allow them to stand for 15-20 minutes until the contents are completely dissolved; mix thoroughly before using. Store the reconstituted reagent at -15°C or lower.

3. Rabbit Anti-C-Peptide of Insulin Serum (Green)
Rabbit anti-C-peptide of insulin serum is diluted in BSA-borate buffer containing Merthiolate (lyophilized). Green dye is added. Reconstitute the vial with 14 ml of distilled or deionized water, mix and allow it to stand for 15-20 minutes until the contents are completely dissolved; mix thoroughly before using. Store the reconstituted reagent at -15°C or lower.

4. ^{125}I C-Peptide of Insulin (Red)
Human C-peptide of insulin (Tyr 17-31) is labeled with iodine-125 and is diluted in BSA-borate-EDTA buffer containing Merthiolate (lyophilized). Red dye is added. Reconstitute the vial with 14 ml of distilled or deionized water, mix and allow it to stand for 15-20 minutes until the contents are completely dissolved; mix thoroughly before using. Store the reconstituted reagent at -15°C or lower.

5. Goat Anti-Rabbit Precipitating Complex (GAR-PPT)
Normal rabbit serum, pre-precipitated with goat anti-rabbit serum and polyethylene glycol (PEG), is diluted in BSA-borate buffer with preservatives added (lyophilized). Reconstitute each vial with 35 ml of distilled or deionized water; mix THOROUGHLY until the suspension appears homogeneous, and then allow it to stand for a minimum of 30 minutes at room temperature with occasional mixing. Store the reconstituted reagent at -15°C or lower.

6. Quality Control Serum (Level 1)
Human serum is spiked, if necessary, with the appropriate amount of C-peptide of insulin to obtain a concentration within a specified range. Sodium azide and other stabilizers are added (lyophilized). Reconstitute the vial with 1.0 ml of distilled or deionized water, mix and allow it to stand for 15-20 minutes until the contents are completely dissolved; mix thoroughly and treat the quality control samples as unknown samples. Values for each lot can be found on the quality control graph included with each kit. Store the reconstituted reagents at -15°C or lower.

7. Quality Control Serum (Level 2)
Human serum is spiked, if necessary, with the appropriate amount of C-peptide of insulin to obtain a concentration within a specified range. Sodium azide and other stabilizers are added (lyophilized). Reconstitute the vial with 1.0 ml of distilled or deionized water, mix and allow it to stand for 15-20 minutes until the contents are completely dissolved; mix thoroughly and treat the quality control sera as unknown samples. Values for each lot can be found on the quality control graph included with each kit. Store the reconstituted reagents at -15°C or lower.

8. PEG (25%)
No reconstitution is necessary. Mix well before using. Store this reagent at 2-8°C or lower.

3. WARNINGS AND PRECAUTIONS

This kit is intended for **in vitro** diagnostic use only.
It is not for internal or external use in humans or animals.

Reagents Containing Human Serum or Plasma
Human serum or plasma is used in the preparation of some of the reagents contained in this kit. This material was tested by an FDA approved method for the presence of the antibody to HTLV-III/LAV as well as for hepatitis B surface antigen and found to be negative.

Because no test method can offer complete assurance that HTLV-III/LAV virus, hepatitis B virus or other infectious agents are absent, the reagents should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 1984.

Reagents Containing Sodium Azide
CAUTION: Some reagents in this kit contain sodium azide. Sodium azide may react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up. For further information, refer to the Manual-Guide issued by the Centers for Disease Control (3).

Reagents Containing Iodine-125
This kit contains radioactive material which does not exceed 2 microcuries (74 kilobecquerels) of iodine-125. Appropriate precautions and good laboratory practices should be used in the storage, handling, and disposal of this material.

For practitioners or institutions receiving radioisotopes under a general license:
This radioactive material may be received, acquired, possessed, and used only by physicians, clinical laboratories, or hospitals, and only for **in vitro** clinical or laboratory tests not involving internal or external administration of the material, or the radiation therefrom, to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations and the general license of the U.S. Nuclear Regulatory Commission or of the state with which the Commission has entered into an agreement for the exercise of regulatory authority.

1. Storage of radioactive material should be limited to a specifically designated area.
2. Access to radioactive materials must be limited to authorized personnel only.
3. Do not pipette radioactive material by mouth.
4. Do not eat or drink within designated radioactive work area.
5. Areas where spills may occur should be wiped up and then washed with an alkali detergent or radiological decontamination solution. Any glassware used must be rinsed completely with water before washing with other laboratory glassware.

For practitioners or institutions receiving radioisotopes under a specific license:
The receipt, use, transfer and disposal are subject to the provisions of 10 CRF Part 20 and your specific license.

Indications of Possible Deterioration of Kit Reagents
1. The presence of abnormal particulate matter in any of the reagents.
2. A shift in the slope or position of the standard curve from what is normally obtained.
3. A decrease in maximum binding.
4. A high nonspecific binding.

4. SPECIMEN REQUIREMENTS

One hundred microliters in duplicate of serum or urine are required for the assay.

Collection and Storage of Serum
Collect blood by venipuncture in a 5 or 10 ml evacuated glass tube. Allow the blood to clot at room temperature. Centrifuge for 15 minutes using 760 x g* to obtain hemolysis-free serum. Separate the serum from the cells and place in storage tubes. No additives or preservatives are required to maintain integrity of the sample. If the specimen is not used immediately, store at -15°C or lower. All plastics, glassware or other material coming into contact with the specimen should be entirely free of any contamination. Specimens should not be repeatedly frozen and thawed.

Collection and Storage of Urine
C-peptide concentrations in urine samples are frequently calculated from 24 hour urine collections. A 100 ml aliquot of the 24 hour urine collection should be neutralized to pH 7.0 - 7.5 with 1 N NaOH and centrifuged for 15 minutes using 760 x g* to remove particulate matter. Supernatants may then be stored at -15°C or lower until assayed. We recommend assaying the urine C-peptide of insulin at whole, 1:5, 1:10 and 1:50 dilutions using 0.85% saline as the diluent.

* $g = (1118 \times 10^{-8})$ (radius in cm) $(rpm)^2$

5. PROCEDURE

Materials and Reagents Supplied with Each Kit

	Cat No. 45065 65 tubes	Cat. No. 45130 130 tubes
1. 0 STANDARD	1 vial 20 ml	1 vial 20 ml
2. hC-PEPTIDE OF INSULIN STANDARDS* (A - E)	5 vials 1.0 ml	5 vials 1.0 ml
3. RABBIT ANTI-C-PEPTIDE OF INSULIN SERUM (GREEN)	1 vial 14 ml	2 vials 14 ml
4. ^{125}I C-PEPTIDE OF INSULIN (RED)	1 vial 14 ml	2 vials 14 ml
5. GOAT ANTI-RABBIT PRECIPITATING COMPLEX (GAR-PPT)	1 vial 35 ml	2 vials 35 ml
6. C-PEPTIDE OF INSULIN CONTROL SERUM (LEVEL 1)	1 vial 1.0 ml	1 vial 1.0 ml
7. C-PEPTIDE ON INSULIN CONTROL SERUM (LEVEL 2)	1 vial 1.0 ml	1 vial 1.0 ml
8. POLYETHYLENE GLYCOL (PEG)	1 vial 10 ml	2 vials 10 ml

* The low standard (Cat. No. 20258) must be requested when ordering. See Insert, Option B, for details of the 48 hour procedure.

Materials and Reagents Required but not Provided
In addition to the reagents supplied with the INCSTAR C-peptide of Insulin RIA Kit, the following are suggested or required:

1. Disposable borosilicate glass tubes, 12 x 75 mm
2. Temperature controlled centrifuge to accommodate 12 x 75 mm tubes
3. Gamma scintillation counter capable of counting iodine-125
4. Vortex
5. Pipetting devices
   a. Micropipettors calibrated to deliver 100 µl, 200 µl and 250 µl
   b. Repeating dispensers, to deliver 200 µl and 500 µl
6. A low C-peptide of insulin standard is available from INCSTAR for use in the 48 hour assay procedure. Customers wishing to use the 48 hour option should request Cat. No. 20258 when ordering.

Preliminary PEG Treatment
PEG-treatment is performed to remove endogenous insulin antibody or other interfering substances. Normal specimens need not be pretreated with PEG if one has determined previously that they do not contain endogenous insulin antibodies or other interfering substances. If one is not certain if the specimen contains antibodies and/or other interfering substances, we recommend performing the PEG-treatment.

NOTE: If one has decided to subject patient samples to PEG-treatment, the standards and controls must also be PEG-treated.

1. Pipette 250 µl of each patient sample, standard, and control into appropriately labeled 12 x 75 mm glass tubes. If a urine specimen is assayed, it must be centrifuged prior to use.
2. Add 250 µl of 25% PEG to each unknown sample, standard, and control.
3. Vortex all tubes vigorously.
4. Centrifuge for 20 minutes using 760 x g* at 20-25°C.
5. Pipette 100 µl samples in duplicate from the clear supernatant into appropriately labeled 12 x 75 mm glass assay tubes. Due to the viscosity of the PEG-treated samples, pipette slowly to ensure precision.

CAUTION: Do not disturb the pellet. If pellet is accidentally disturbed, repeat step 4.
NOTE: Because PEG is soluble with the standards, no precipitate will form after centrifugation. Pipette 100 µl in duplicate from the solution.

6. Assay samples according to the assay procedure.

* See Section 4, Specimen Requirements for the formula for the calculation of g's.

REFER TO LAST 2 PAGES FOR ASSAY PROCEDURES AND FLOW TABLES.

Procedural Comments

1. Assay all samples in duplicate to ensure confidence in values obtained.
2. Reagents used must be within the proper dating.
3. Add each aliquot of reagent to the lower third of the assay tube to ensure complete mixture of reagents.
4. Some manufacturers' disposable borosilicate glass tubes yield elevated nonspecific bindings.
5. If you choose to aspirate the supernatant from the precipitate, be careful not to disturb the precipitate pellet.

Standardization
The assay was standardized with synthetic C-peptide of insulin from INCSTAR.

6. QUALITY CONTROL

Each laboratory should include control sera in every assay to ensure the validity of each assay's results. A mean and standard deviation should then be determined for each control using a minimum of ten (10) assays. An acceptable range of values may then be obtained for these controls using ± two standard deviations of the values previously determined. The INCSTAR Quality Control Laboratory has determined a range for the control included in this kit. The values may be found on the quality control graph included with the kit.

In order for a laboratory to completely monitor the consistent performance of an RIA assay there are additional factors which must be checked. INCSTAR suggests a regular check of the following parameters to ensure consistent kit performance.

1. Total Counts
Counts obtained should approximate the expected dpms given in the quality control graph when adjusted for counter efficiency and radioactive decay.

2. Maximum Binding
CPM of 0 Standard Tube / Average CPM of Total Count Tubes.

3. Nonspecific Binding
CPM of NSB Tube / Average CPM of Total Count Tubes.

The quality control graph included with each kit gives the performance characteristics obtained by our quality control laboratory using the reagent lots included in that particular kit. The ranges obtained for each particular lot of INCSTAR quality control sample are also printed on the enclosed kit graph. Significant deviation of these values from obtained results can indicate unnoticed variations in experimental conditions or degradation of kit reagents. In order to evaluate the cause of any variations, the laboratory should use fresh reagents.

7. RESULTS

Procedure for Calculating Values of Unknowns
There are many methods in existence for calculating results of RIAs. Each is based on obtaining a calibration curve by plotting the extent of binding against stated concentrations of the calibration standards. This graph may be either linear or logarithmic scale. Each of these methods gives essentially the same values for controls and samples, although certain assays may "fit" better into one particular method versus another. The calculating method for INCSTAR laboratories is % B/B_0 versus log concentration.

1. Calculate the average CPM for each standard, control and unknown sample.
2. Subtract the average CPM of the NSB tubes from all counts.
3. Divide the corrected CPM of each standard, control or unknown sample by the corrected CPM of the 0 standard.

$$B/B_0\ (\%) = \frac{\text{CPM of Standard or Unknown Sample - CPM of NSB}}{\text{CPM of 0 Standard - CPM of NSB}} \times 100$$

4. Using 3 cycle semi-log or log-logit graph paper, plot percent B/B_0 for the C-peptide of insulin standards (vertical axis) versus the concentration (horizontal axis).
5. Draw a best-fit line through the points.
6. Interpolate the levels of C-peptide of insulin in the unknown samples from the plot.
7. If any unknown serum or plasma sample reads greater than the highest standard it should be diluted appropriately with 0 standard and assayed again. If an unknown urine sample reads greater than the highest standard, it should be diluted appropriately in 0.85% saline. If PEG treatment is being used, treat samples following sample dilution.
8. If a sample has been diluted, correct for the appropriate concentration factor.
9. Calculate maximum binding be dividing CPM of 0 standard by the average total counts obtained in the total count tubes.

TABLE III
INCSTAR C-peptide of Insulin RIA Sample Data
Option A

Tube	Duplicate CPM	Average CPM	Corrected CPM	Percent Bound (B/T)	Percent (B/B_0)	Conc. (ng/ml)
Total Count	27,526 26,953	27,239				
NSB	439 536	488		2.0		
0 Standard	13,743 13,348	13,561	13,073	49.8	100	
Standards (ng/mL)						
A (0.7)	11,229 11,357	11,293	10,805		82.7	
B (1.7)	8,978 8,616	8,797	8,309		63.6	
C (3.0)	6,779 6,727	6,753	6,265		47.9	
D (7.0)	4,736 4,552	4,644	4,156		31.8	
E (17.4)	2,611 2,578	2,595	2,107		16.1	
Unknown Samples						
1	9,367 8,723	9,045	8,557		65.4	1.5
2	5,864 5,804	5,834	5,346		40.9	4.2

Typical sample data and a standard curve for Option A are shown in Table III and Figure 1; this information is for reference only and should not be used for the calculation of any value.

TABLE IV
INCSTAR C-peptide of Insulin RIA Sample Data
Option B

Tube	Duplicate CPM	Average CPM	Corrected CPM	Percent Bound (B/T)	Percent (B/B_0)	Conc. (ng/ml)
Total Count	30,694 30,468	30,581				
NSB	889 799	844		2.8		
0 Standard	18,238 17,060	17,649	16,805	57.7	100	
Standards (ng/mL)						
A (0.15)	16,050 16,373	16,212	15,367		91.4	
B (0.6)	10,340 11,181	10,760	9,916		59.0	
C (1.5)	5,170 4,995	5,082	4,238		25.2	
D (3.0)	3,314 3,672	3,493	2,649		15.8	
E (7.0)	2,456 2,522	2,489	1,645		9.8	
Unknown Samples						
1	2,877 3,162	3,019	2,175		12.9	4.0

Typical sample data and a standard curve for Option B are shown in Table IV and Figure 2; this information is for reference only and should not be used for the calculation of any value.

C-PEPTIDE SAMPLE STANDARD CURVE
Option A

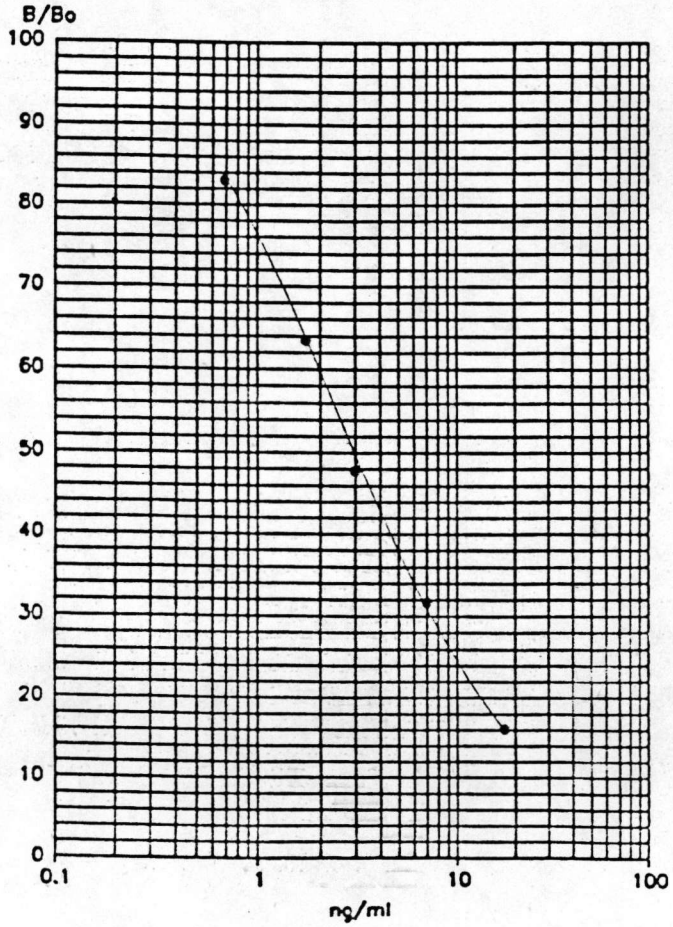

FIGURE 1

C-PEPTIDE SAMPLE STANDARD CURVE
Option B

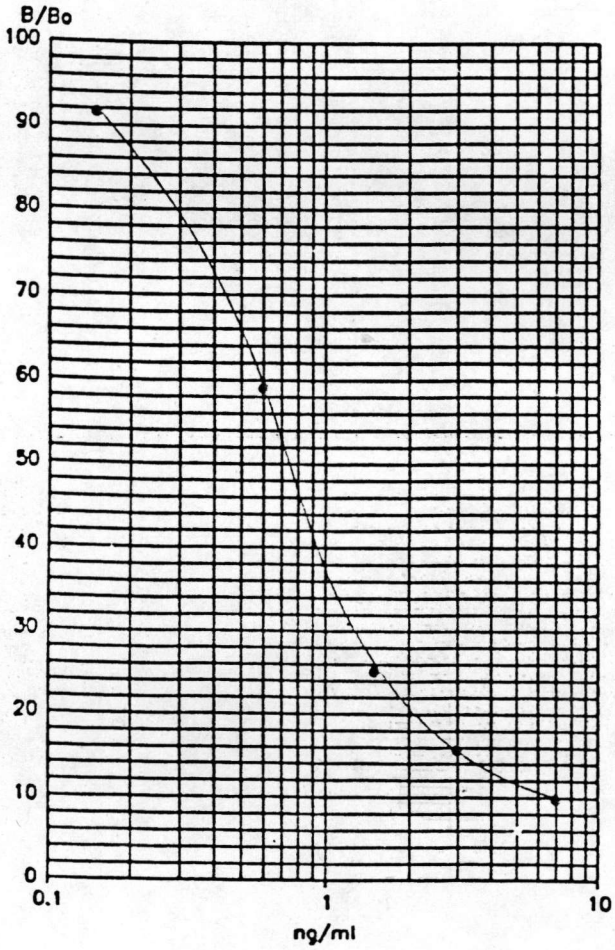

FIGURE 2

Limitations of the Procedure

1. Counting times should be sufficient to prevent the introduction of statistical error in counting (for example, accumulation of 2,000 CPM will yield 5% error; 10,000 CPM will yield 1% error).

2. If any serum sample reads greater than the highest standard, it should be diluted appropriately in 0 standard and assayed again. If any urine sample reads greater than the highest standard, it should be diluted appropriately in 0.85% saline and assayed again.

3. Control values have been established using PEG-treated samples, Option A, the overnight assay procedure. Values obtained using the procedure outlined in Option B may give slightly different results.

8. EXPECTED VALUES

Normal Range for Serum

Each laboratory should establish its own normal range. Normal values observed in the INCSTAR laboratory are as follows:

Untreated serum: 1.0 - 3.5 ng/ml
PEG-treated serum: 0.5 - 3.0 ng/ml

Normal Range for Urine

Values obtained on urine specimens should be assessed considering factors such as diet, medication, etc. (See Section 10, References). Each laboratory should establish their own normal range.

Polyethylene Glycol (PEG) Pre-treatment of Samples

All C-peptide of insulin antisera cross-react with varying amounts of proinsulin. When there is a possibility of the presence of endogenous insulin antibodies and other interfering substances, measures must be taken to eliminate proinsulin bound to these antibodies from the serum or urine sample. Pre-treating serum or urine samples with 25% PEG will precipitate such antibodies and other interfering substances along with their associated proinsulin. C-peptide of insulin does not precipitate with PEG-treatment, and we have demonstrated that standards of C-peptide are completely recovered after such treatment. When pre-treated with PEG, sera from fasting individuals returned an average value of 84% of the C-peptide immunoreactivity observed in the original sera. During glucose tolerance tests, when C-peptide levels rise, the percentage of observed C-peptide immunoreactivity in PEG-treated sera rises and then declines as the C-peptide levels return to normal.

9. PERFORMANCE DATA

Precision

Within Assay Variation (values = ng/ml)

	Mean Value	S.D.	% C.V.
Low	1.4	0.08	5.7
Medium	5.1	0.35	6.7
High	10.5	0.64	6.1

Between Assay Variation (values = ng/ml)

	Mean Value	S.D.	% C.V.
Low	1.3	0.19	15.3
High	4.9	0.70	14.3

Parallelism

Serial Dilution Study of 5 Patient Samples (values = ng/ml)

Sample Number	Undiluted	1/2	1/4	1/8
Serum				
1	4.7	4.4	5.6	5.6
2	9.9	9.2	10.0	11.2
Urine				
3	2.7	2.4	2.0	4.0
4	16.4	13.4	14.4	14.4
5	6.7	6.4	7.2	5.6

Accuracy

Serum Recovery Study (values = ng/ml)

Background	Standard Added	Expected Value	Measured Value	Percent Recovery
Set No. 1				
1.5	2.5	4.0	3.9	98
1.5	5.0	6.5	5.7	88
1.5	10.0	11.5	11.0	96
Set No. 2				
1.9	2.5	4.4	4.2	95
1.9	5.0	6.9	6.5	94
1.9	10.0	11.9	10.0	84
Set No. 3				
1.0	2.5	3.5	3.3	94
1.0	5.0	6.0	5.3	88
1.0	10.0	11.0	9.0	82

Urine Recovery Study (values = ng/ml)

Background	Standard Added	Expected Value	Measured Value	Percent Recovery
Set No. 1				
1.7	2.5	4.2	4.2	100
1.7	5.0	6.7	6.5	97
1.7	10.0	11.9	12.0	103
Set No. 2				
3.6	2.5	6.1	6.1	100
3.6	5.0	8.6	8.5	99
3.6	10.0	13.6	13.0	96
Set No. 3				
3.8	2.5	6.3	6.0	95
3.8	5.0	8.8	8.6	98
3.8	10.0	13.8	13.0	94

Sensitivity

When defined as the apparent concentration at three standard deviations from the counts at maximum binding, the minimum detectable amount is 0.02 ng/tube or 0.1 ng/ml for Option A, and 0.01 ng/tube or 0.05 ng/ml for Option B.

Specificity

Comparison to the cross-reactivity of the C-peptide antibody was made with the following peptides:

Peptide	% Cross-reactivity
Human C-peptide of Insulin	100
Human Insulin	<0.01
C-peptide of Somatomedin C	<0.01
Human Proinsulin	4.0

10. REFERENCES

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INCSTAR C-peptide of Insulin Assay Procedure and Flow Table

TABLE I Option A

	Total Count	NSB	STANDARDS nanograms/ml						CONTROL AND UNKNOWN SAMPLES			
			0	A	B	C	D	E	1	2	3	4
Tube number	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
0 Standard		100	100									
Standards				100	100	100	100	100				
Control and unknown samples									100	100	100	100
Rabbit anti-C-peptide of insulin serum			← 200 µl →									
^{125}I C-peptide of insulin	← 200 µl →											
	Vortex gently. Incubate for 16-24 hours at 2-8°C.											
Goat anti-rabbit precipitating complex (GAR-PPT)		← 500 µl →										
		Vortex gently. Incubate for 15-25 minutes at 20-25°C.										
All volumes are in microliters		Centrifuge using 760 x g for 20 minutes. Decant or aspirate supernatant.										
	Count each tube for 60 seconds or longer.											

ASSAY PROCEDURE - OPTION A

1. Reconstitute the lyophilized reagents and allow any frozen reagents to thaw completely. Do not allow reagents to reach temperatures above 25°C. Mix gently before using.

2. Set up labeled 12 x 75 mm disposable tubes in duplicate according to the protocol in Table I.

3. Add reagents as follows:

a. Total count tubes
Set aside until step 4.

b. Nonspecific binding tubes (NSB)
100 µl of 0 standard (PEG-treated or untreated)

c. 0 standard
100 µl of 0 standard (PEG-treated or untreated)
200 µl of rabbit anti-C-peptide of insulin serum (green)

d. C-peptide of insulin standards (A - E)
100 µl of C-peptide of insulin standard (PEG-treated or untreated)
200 µl of rabbit anti-C-peptide of insulin serum (green)

e. Control and unknown samples
100 µl of either serum or urine (PEG-treated or untreated)
200 µl of rabbit anti-C-peptide of insulin serum (green)

4. Add 200 µl of ^{125}I C-peptide of insulin (red) to all the tubes.

5. Mix the tubes gently without foaming and incubate for 16-24 hours at 2-8°C.

6. Vigorously mix the GAR-PPT; add 500 µl to all the tubes except the total count tubes.

7. Vortex the tubes without foaming and incubate them for 15-25 minutes at 20-25°C.

8. Centrifuge the tubes for 20 minutes at 760 x g* at 20-25°C.

9. Immediately decant the supernatant from all the tubes except the total count tubes by inverting them for a minimum time of 2 minutes. Blot the tubes on absorbent paper to remove any drops of supernatant that may be remaining on the rims before turning the tubes upright.

10. Using a gamma scintillation counter, count the precipitate of each tube, and the total count tubes, for a sufficient time to achieve statistical accuracy.
(See Section 7, Limitations of the Procedure).

* See Section 4, Specimen Requirements, for the formula for the calculation of g's.

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INCSTAR C-peptide of Insulin
Assay Procedure and Flow Table

TABLE II
Option B

	Total Count	NSB	STANDARDS nanograms/ml						CONTROL AND UNKNOWN SAMPLES			
			0	A	B	C	D	E	1	2	3	4
Tube number	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
0 Standard		100	100									
Standards				100	100	100	100	100				
Control and unknown samples									100	100	100	100
Rabbit anti-C-peptide of insulin serum			← 200 µl →									
	Vortex gently. Incubate for 16-24 hours at 2-8°C.											
^{125}I C-peptide of insulin	← 200 µl →											
	Vortex gently. Incubate for 16-24 hours at 2-8°C.											
Goat anti-rabbit precipitating complex (GAR-PPT)		← 500 µl →										
		Vortex gently. Incubate for 15-25 minutes at 20-25°C.										
		Centrifuge using 760 x g for 20 minutes. Decant or aspirate supernatant.										
	Count each tube for 60 seconds or longer.											

All volumes are in microliters

ASSAY PROCEDURE - OPTION B

1. Reconstitute the lyophilized reagents and allow any frozen reagents to thaw completely. Do not allow reagents to reach temperatures above 25°C. Mix gently before using.

2. Set up labeled 12 x 75 mm disposable tubes in duplicate according to the protocol in Table II.

3. Add reagents as follows:

a. Total count tubes
Set aside until step 5

b. Nonspecific binding tubes (NSB)
100 µl of 0 standard (PEG-treated or untreated)

c. 0 standard
100 µl of 0 standard (PEG-treated or untreated)
200 µl of rabbit anti-C-peptide of insulin serum (green)

d. C-peptide of insulin standards (A - E)
100 µl of C-peptide of insulin standard (PEG-treated or untreated)
200 µl of rabbit anti-C-peptide of insulin serum (green)

e. Control and unknown samples
100 µl of either serum or urine (PEG-treated or untreated)
200 µl of rabbit anti-C-peptide of insulin serum (green)

4. Incubate for 16-24 hours at 2-8°C.

5. Add 200 µl of ^{125}I C-peptide of insulin (red) to all tubes.

6. Mix the tubes gently without foaming and incubate them for 16-24 hours at 2-8°C.

7. Vigorously mix the GAR-PPT; add 500 µl to all the tubes except the total count tubes.

8. Vortex the tubes without foaming and incubate them for 15-25 minutes at 20-25°C.

9. Centrifuge the tubes for 20 minutes at 760 x g* at 20-25°C.

10. Immediately decant the supernatant from all the tubes except the total count tubes by inverting them for a minimum time of 2 minutes. Blot the tubes on absorbent paper to remove any drops of supernatant that may be remaining on the rims before turning the tubes upright.

11. Using a gamma scintillation counter, count the precipitate of each tube, and the total count tubes, for a sufficient time to achieve statistical accuracy.
(See Section 7, Limitations of the Procedure).

* See Section 4, Specimen Requirements, for the formula for the calculation of g's.

Coat-A-Count®
INSULIN

is a solid-phase ^{125}I radioimmunoassay designed for the quantitative measurement of insulin in serum. It is intended strictly for *in vitro* diagnostic use as an aid in clinical diagnosis.

Catalog Numbers: **TKIN1** (100 tubes) **TKIN2** (200 tubes) **TKIN5** (500 tubes) **TKINX** (1000 tubes).

The 100-tube kit contains not more than 3 microcuries (111 kilobecquerels) of radioactive ^{125}I-insulin, and the 200-tube kit contains not more than 6 microcuries (222 kilobecquerels); the 500-tube kit contains not more than 15 microcuries (555 kilobecquerels); and the 1000-tube kit contains not more than 30 microcuries (1110 kilobecquerels).

Introduction

In the Coat-A-Count Insulin procedure, ^{125}I-labeled insulin competes with insulin in the patient sample for sites on insulin-specific antibody immobilized to the wall of a polypropylene tube. After incubation, isolation of the antibody-bound fraction is achieved simply by decanting the supernatant. The tube is then counted in a gamma counter, the counts being inversely related to the amount of insulin present in the patient sample. The quantity of insulin in the sample is then determined by comparing the counts to a standard curve.

Procedure There is only one reagent to dispense, and a single overnight (or 3-hour) incubation. No centrifuge is required. The simplicity of the Coat-A-Count procedure makes it ideal for high-volume testing.

Separation The coated-tube methodology offers significant advantages in reliability, since the tubes can be vigorously decanted without loss of antibody-bound material. This results in a clean separation, with negligible misclassification of bound and free.

Data Reduction Conventional RIA techniques of calculation and quality control are applicable. The assay has been optimized for linearity in a logit-log representation throughout the range of its calibrators. The computation can be simplified by omitting the correction for nonspecific binding, without compromising results or quality control.

Calibration The calibrators have been prepared in processed *human serum,* to insure full compatibility with patient serum samples. This approach offers significant advantages, particularly with respect to interlaboratory precision.[10] The calibrators, which are supplied lyophilized for maximum stability, have insulin values ranging from 5 to 400 μIU/ml. The assay is standardized in terms of the World Health Organization's First International Reference Preparation of Insulin for Immunoassay, number 66/304.

Counts The tracer has a high specific activity with total counts of approximately 45,000 cpm at iodination. Maximum binding is approximately 50%.

Accuracy Extensive experiments have shown that the assay is accurate over a broad spectrum of insulin values. Its accuracy has been further verified in a patient comparison study against another insulin radioimmunoassay.

Specificity The antiserum is highly specific for insulin, with very low crossreactivity to other compounds that might be present in patient samples. Crossreactivity with pro-insulin at mid-curve is approximately 40%. The kit's freedom from "matrix effects" is demonstrated by patient sample dilution experiments and by studies on the effect of bilirubin and lipemia.

Sensitivity The procedure can detect as little as 1 μIU/ml using the overnight procedure, or 3 μIU/ml using the same-day procedure.

Materials Supplied—Initial Preparation

■ *Precautions:* Before opening the kit, review the paragraphs on safety printed on the inside front cover as they relate to the safe handling and disposal of reagents containing radioactivity, human serum and sodium azide.

1 INSULIN ANTIBODY-COATED TUBES **TIN1**

100 (200,* 500†, 1000‡) polypropylene tubes coated with antibodies to insulin and packaged in zip-lock bags. Store refrigerated and protected from moisture, carefully resealing the bags after opening: stable at 2–8°C for at least one year from the date of manufacture. *Color:* lime green.

2 BUFFERED [^{125}I] INSULIN **TIN2**

One vial (two vials,* five vials†, ten vials‡) of a *concentrate,* consisting of iodinated insulin. To each vial add a measured **100 ml** of distilled water, and mix by gentle inversion. Store refrigerated: stable at 2–8°C for at least 30 days after reconstitution, or until the expiration date marked on the vial.

3 INSULIN CALIBRATORS **INC3–9**

One set (two sets†, three sets‡) of seven vials, labeled A through G, of lyophilized processed human serum. *At least 30 minutes before use,* reconstitute the zero calibrator **A with 6.0 ml** of distilled water, and each of the remaining calibrators **B through G with 3.0 ml** of distilled water. Use volumetric pipets and mix by gentle swirling. *Store frozen:* stable at −10°C for at least 30 days after reconstitution. Aliquot if necessary to avoid repeated freezing and thawing.

The reconstituted calibrators contain respectively 0, 5, 15, 50, 100, 200 and 400 micro-International Units of insulin per milliliter (μIU/ml, 1st IRP 66/304) in processed human serum. Intermediate calibration points can be obtained by mixing the calibrators in suitable proportions.

*Pertains to the 200-tube TKIN2 kit.
†Pertains to the 500-tube TKIN5 kit.
‡Pertains to the 1000-tube TKINX kit.

Materials Required But Not Provided

- Gamma counter—compatible with standard 12×75mm tubes
- Vortex mixer

Reagent Preparation:

- Distilled or deionized water
- Graduated cylinder: 100 ml
- Volumetric pipets: 3.0 and 6.0 ml

Radioimmunoassay:

- Plain 12×75mm polypropylene tubes—for use as NSB tubes, available from DPC
- Micropipets: 200 μl and 1000 μl

  For the 1.0 ml reagent addition, a reliable repeating dispenser (Nichiryo, Eppendorf or equivalent) is also suitable.

- Waterbath, capable of maintaining 37°C—required only for the alternate 3-hour procedure

  Neither an oven nor a heat block is suitable

- Foam decanting rack—available from DPC

A tri-level, human serum-based immunoassay control, containing insulin as one of over 25 assayed constituents, is available from DPC (catalog number: CON6).

Specimen Collection

No special preparations are necessary. Collect blood by venipuncture into plain tubes, noting the time of collection, and separate the serum from the cells. The procedure calls for 200 µl of serum per tube. (See the Performance Data section on anticoagulants.)

It is important to avoid hemolysis in collecting samples for insulin determinations, since it has been shown that visible hemolysis characteristically interferes with insulin radioimmunoassays incorporating any of the standard separation methods.

The samples may be stored under refrigeration for seven days, or for up to three months frozen at −20°C. Before assay, allow the samples to come to room temperature and mix by *gentle* swirling or inversion. Aliquot, if necessary, to avoid repeated thawing and freezing. Do not attempt to thaw frozen specimens by heating them in a water bath. Samples contaminated from the administration of radioactivity to the patient are unsuitable for use.

Radioimmunoassay Procedure

All components must be at normal room temperature before use. An automatic pipetter-diluter is not recommended for this assay.

1 **Plain Tubes:** Label four plain (uncoated) 12×75mm polypropylene tubes T (total counts) and NSB (nonspecific binding) in duplicate.

Because nonspecific binding in the Coat-A-Count procedure is characteristically low, the NSB tubes may be safely omitted without compromising accuracy or quality control.

Coated Tubes: Label fourteen Insulin Antibody-Coated Tubes A (maximum binding) and B through G in duplicate. Label additional antibody-coated tubes, also in duplicate, for controls and patient samples.

Calibrators	µIU/ml 1st IRP [66/304]
A(MB)	0
B*	5
C	15
D	50
E	100
F	200
G	400

* Omit calibrator B if the alternate, 3-hour, 37°C incubation will be used at step 4.

2 Pipet **200 µl** of the zero calibrator A into the NSB and A tubes, and **200 µl** of each remaining calibrator, control and patient sample into the tubes prepared. **Pipet directly to the bottom.**

3 Add **1.0 ml** of Buffered [^{125}I] Insulin to every tube. Vortex.

Samples should not be left in the tubes for extended periods of time. Following step 2 (sample addition), step 3 (tracer addition) should be completed with minimal delay, with no more than 40 minutes elapsing between the addition of the first sample and the completion of tracer addition. Set the T tubes aside for counting (at step 6); they require no further processing.

4 Incubate for **18 – 24 hours at room temperature.**

Alternatively, incubate for 3 hours at 37°C, using a waterbath—neither an oven nor a heat block is suitable—and omitting the 5 µIU/ml calibrator (Calibrator B). This approach allows for same-day results at some sacrifice in low-end sensitivity and precision. (See the sensitivity table and precision profiles in the Performance Data section.)

5 Decant thoroughly.

Removing all visible moisture will greatly enhance precision. Using a foam decanting rack, decant the contents of all tubes (except the T tubes) and allow them to drain for 2 or 3 minutes. Then strike the tubes sharply on absorbent paper to shake off all residual droplets.

6 Count for 1 minute in a gamma counter.

Calculation of Results

To calculate insulin concentrations from a logit-log representation of the calibration curve, first calculate for each pair of tubes the average NSB-corrected counts per minute:

Net Counts = Average CPM *minus* Average NSB CPM

Then determine the binding of each pair of tubes as a percent of maximum binding (MB), with the NSB-corrected counts of the A tubes taken as 100%:

$$\text{Percent Bound} = \frac{\text{Net Counts}}{\text{Net MB Counts}} \times 100$$

The calculation can be simplified by omitting the correction for nonspecific binding; samples within range of the calibrators yield virtually the same results when Percent Bound is calculated directly from Average CPM.

Using the logit-log graph paper provided with the kit, plot Percent Bound on the vertical axis against Concentration on the horizontal axis for each of the calibrators B through G, and draw a straight line approximating the path of these six points. (The standard curve for the alternate 3-hour, 37°C procedure should be based on calibrators C through G only.) Insulin concentrations for the unknowns may then be estimated from the line by interpolation.

Although other approaches are acceptable, data reduction by the logit-log method just described has certain advantages in this context—for example, in allowing easier recognition of deviant calibration points—since the Coat-A-Count Insulin procedure has been optimized for linearity in that representation.

Example: The figures tabulated below were generated with the overnight procedure. They are for illustration only and should not be used to calculate results from another assay.

Tube	Duplicate CPM	Average CPM	Net CPM	Percent Bound	Insulin µIU/ml
T	30,546 30,840	30,693			
NSB	341 327	334	0		
A(MB)	15,285 15,591	15,438	15,104	100.0%	0
B	13,561 13,091	13,326	12,992	86.0%	5
C	11,499 11,183	11,341	11,007	72.9%	15
D	7,757 7,841	7,799	7,465	49.4%	50
E	5,481 5,499	5,490	5,156	34.1%	100
F	3,899 3,905	3,902	3,568	23.6%	200
G	2,620 2,318	2,469	2,135	14.1%	400
Unknowns:					
X1	12,663 12,305	12,484	12,150	80.4%	9.5
X2	9,575 9,779	9,677	9,343	61.9%	28
X3	3,466 3,798	3,632	3,298	21.8%	220

Quality Control Parameters: T = 30,693 cpm. %NSB = 1.1%. %MB = 49%.
20% Intercept = 251 µIU/ml. 50% Intercept = 51 µIU/ml. 80% Intercept = 10 µIU/ml.

Quality Control

Record Keeping: It is good laboratory practice to record for each assay the lot numbers and reconstitution dates of the components used.

Sample Handling: The instructions for handling and storing patient samples and components should be carefully observed. Dilute high patient serum samples with the kit's zero calibrator before assay. All samples, including the calibrators and controls, should be assayed in duplicate. Pairs of control tubes may be spaced throughout the assay to help verify the absence of significant drift. Inspect the results for agreement within tube pairs, and take care to avoid carry-over from sample to sample.

Controls: Controls or serum pools with low, intermediate and high insulin concentrations should routinely be assayed as unknowns, and the results charted from day to day as described in J.O. Westgard et al, "A multi-rule chart for quality control" *Clinical Chemistry 27* (1981) 493–501. See also *Scandinavian Journal of Clinical and Laboratory Investigation 44* (1984) Suppl 171 and 172. Repeat samples are a valuable additional tool for monitoring interassay precision.

Data Reduction: It is good practice to construct a graph of the calibration curve as a visual check on the appropriateness of the transformation used, even where the calculation of results is handled by computer. See further S.E. Davis et al, "Radioimmunoassay data processing with a small programmable calculator" *Journal of Immunoassay 1* (1980) 15–25; and R.A. Dudley et al, "Guidelines for immunoassay data reduction" *Clinical Chemistry 31* (1985) 1264–71.

Q. C. Parameters: We recommend keeping track of these performance measures:

- T = Total Counts (as counts per minute)
- %NSB = 100 × (Average NSB Counts ÷ Total Counts)
- %MB = 100 × ((Average MB Counts *minus* Average NSB Counts) ÷ Total Counts)

And the 20, 50 and 80 percent "intercepts," where

- 20% = Insulin Concentration at 20 Percent Bound, etc.

Performance Characteristics

In the sections below, insulin results are expressed as micro-International Units of insulin per milliliter in terms of the First International Reference Preparation of Insulin for Immunoassay, number 66/304 (μIU/ml 1st IRP 66/304). Except as noted, all results were obtained using an overnight, room-temperature incubation.

Sensitivity

Forty zero calibrator (maximum binding) tubes were processed in a single assay, along with a set of non-zero calibrators and controls. Mean and standard deviation were calculated for the counts-per-minute of the forty zero tubes. Then, from a standard curve prepared by the logit-log technique and using this mean as the zero point, the apparent insulin concentration was determined at increasing standard deviations from the mean.

Incubation	Mean ± SD of 40 MB tubes	Mean *minus*	% B/B_0	Apparent Concentration	Approximate Sensitivity
Overnight	24,731 ± 271	2SD	97.8%	0.3	1 μIU/ml
		3SD	96.7%	0.5	
		4SD	95.6%	0.7	
3 hr 37°C	15,506 ± 566	2SD	92.4%	2.4	3 μIU/ml
		3SD	88.7%	4.1	
		4SD	84.9%	6.2	

The detection limit (or "least detectable dose") of an assay is commonly defined as the apparent concentration two standard deviations below the counts at maximum binding or as the concentration at 95% B/B_0. By the more conservative definition, the overnight and 3-hour procedures have detection limits of approximately 1 and 3 μIU/ml, respectively.

Kinetics

To determine the effect of employing incubation times other than those specified on page 3, assays were set up in parallel, using incubations of 2; 3 and 6 hours at 37°C; and 6, 18 and 24 hours at room temperature. Various quality control performance measures were monitored, including: nonspecific binding and maximum binding (percent of total counts); the slope, intercept and correlation coefficient (rho) of the logit-log line; the assay CV, based on the binding of replicates; the sensitivity of the assay (concentration at two standard deviations from the counts per minute at zero binding); the 20, 50 and 80 percent intercepts (μIU/ml); and the binding of the calibrators (% B/B_0). In addition, several samples were assayed as unknowns in each of the assays.

Parameter	2 hr 37°C	3 hr 37°C	6 hr 37°C	6 hr RT	18 hr RT	24 hr RT
Total Counts	44,208 cpm	44,344 cpm	45,560 cpm	43,787 cpm	46,354 cpm	45,291 cpm
% NSB	1.4%	1.1%	1.2%	2.0%	2.1%	1.6%
% MB	30%	33%	39%	38%	53%	57%
Slope	−0.853	−0.855	−0.851	−0.904	−0.853	−0.895
Intercept	1.61	1.56	1.48	1.71	1.45	1.52
rho	−0.997	−0.997	−0.997	−0.997	−0.998	−0.998
Assay CV	2.1%	1.5%	1.6%	1.9%	1.9%	1.2%
Sensitivity	1.9 μIU/ml	1.9 μIU/ml	0.5 μIU/ml	2.0 μIU/ml	0.7 μIU/ml	1.4 μIU/ml
Intercepts:						
20% B/B_0	388 μIU/ml	339 μIU/ml	279 μIU/ml	358 μIU/ml	257 μIU/ml	233 μIU/ml
50% B/B_0	76	67	55	77	51	49
80% B/B_0	15	13	11	17	10	11
Calibrators:						
B—5 μIU/ml	(91%)	(91%)	(89%)	94%	88%	89%
C—15	81%	76%	76%	82%	74%	75%
D—50	58%	55%	51%	59%	50%	48%
E—100	43%	41%	36%	43%	36%	34%
F—200	30%	28%	26%	31%	23%	23%
G—400	21%	20%	17%	19%	16%	15%
Controls:						
1	21 μIU/ml	21 μIU/ml	19 μIU/ml	20 μIU/ml	13 μIU/ml	14 μIU/ml
2	19	20	24	22	24	24
3	68	71	68	70	67	68
Samples:						
1	5 μIU/ml	7 μIU/ml	6 μIU/ml	7 μIU/ml	3 μIU/ml	3 μIU/ml
2	6	9	10	9	4	6
3	9	14	13	10	7	8
4	16	15	17	12	10	11
5	15	17	15	10	10	10
6	20	19	21	14	12	13
7	17	19	19	14	13	14
8	11	17	20	16	13	15
9	19	20	25	20	17	20
10	32	30	37	28	28	30

Drift

To determine whether there is any position (or "end of run") effect due to delays in the addition of reagents, pairs of tubes were placed at various locations throughout a long assay, for each of four samples. The table below indicates the time (in minutes) and the number of tubes intervening between each set of tubes.

Sample	1st pair of tubes	Intervening time	Intervening tubes	2nd pair of tubes	Intervening time	Intervening tubes	3rd pair of tubes
1	10	13	136	11	10	144	8
2	20	13	136	17	10	144	16
3	55	13	136	53	10	144	51
4	136	13	136	129	10	144	125

It took 34 minutes to pipet the entire assay, and 15 minutes to add the tracer to all tubes following sample addition. In the Coat-A-Count Insulin procedure the *time* intervening appears to be more important than the number of tubes. The actual effect is dependent both on the time the sample sits in the tube by itself, and on the delay in the addition of tracer. It is recommended that the user not attempt to process more tubes in one batch than can be comfortably pipetted (both sample and tracer additions) in 40 minutes.

Precision

The reliability of DPC's Coat-A-Count Insulin procedure was assessed by examining its reproducibility on samples selected to represent a range of insulin levels.

Intraassay: Precision profiles, based on approximately 20 degrees of freedom and representing the intraassay CVs to be expected for samples assayed in duplicate, are displayed on page 13.

Interassay: Statistics were calculated for each of four samples from the results of pairs of tubes in 20 different assays.

Sample	Mean	SD	CV
1	16	1.6	10.0%
2	35	2.5	7.1%
3	76	5.5	7.2%
4	95	4.7	4.9%

Effect of Bilirubin and Hemolysis

To simulate severe icterus, four serum samples were spiked with 10 and 20 mg/dl of bilirubin. In another experiment, to simulate mild, moderate and severe hemolysis, four serum samples were spiked with 5, 7.5 and 15 µl/ml of packed red blood cells. All samples were assayed both spiked and unspiked by the Coat-A-Count Insulin procedure with the following results.

	Bilirubin mg/dl		
Sample	Unspiked	10	20
1	21	26	23
2	41	39	36
3	90	86	82
4	315	302	301

	Packed Red Blood Cells µl/ml			
Sample	Unspiked	5	7.5	15
1	15	18	17	17
2	34	36	36	35
3	48	47	45	49
4	72	73	74	84

The results show that neither severe icterus (bilirubin up to 20 mg/dl) nor gross hemolysis has any effect on the Coat-A-Count Insulin procedure.

Effect of Anticoagulants

To determine whether anticoagulants interfere with the assay, blood was collected from eleven normal volunteers into plain, heparin and EDTA vacutainer tubes. All samples were assayed by the Coat-A-Count Insulin procedure, with the following results.

(Heparin) = 1.05 (Serum) − 0.6 µIU/ml r = 0.995
(EDTA) = 1.35 (Serum) + 13.2 µIU/ml r = 0.995
n = 11

Means:
Serum: 19.6 µIU/ml
Heparin: 19.9 µIU/ml
EDTA: 39.6 µIU/ml

It is apparent that EDTA plasma yields much higher results than serum or heparinized plasma.

Protein Effect

To simulate various protein concentrations, experiments were performed in which 2.0 ml aliquots of a human serum pool were freeze-dried and then reconstituted with various volumes of water. Each reconstituted aliquot was then assayed by the Coat-A-Count Insulin procedure. Note that aliquots reconstituted with half the original volume represent an extremely high protein concentration, in the order of 14 gm/dl. These results indicate that even wide variations in total protein have virtually no effect on the Coat-A-Count Insulin assay.

Experiment	Reconstitution		Protein Concentration	O Observed	E Expected	% O/E
1	1.0 ml	2.00×	~14.0 gm/dl	257	240	107%
	1.3	1.50×	~10.5	184	180	102%
	2.0	1.00×	~ 7.0	120	—	(100%)
	3.0	0.67×	~ 4.7	79	80	99%
2	1.0 ml	2.00×	~14.0 gm/dl	308	276	112%
	1.3	1.50×	~10.5	199	207	96%
	2.0	1.00×	~ 7.0	138	—	(100%)
	3.0	0.67×	~ 4.7	82	92	89%

Specificity

The Coat-A-Count Insulin antiserum is highly specific for insulin, with a particularly low crossreactivity to compounds (other than insulin and pro-insulin) that might be present in patient samples. Crossreactivity with pro-insulin at mid-curve is approximately 40%.

Compound	Crossreactivity
Human Insulin	100%
C-Peptide	ND
Glucagon	ND

In another study, it was found that bovine insulin was indistinguishable from human insulin in the Coat-A-Count Insulin procedure.

Effect of Lipemia

A 500 µIU/ml spiking solution prepared in the kit's zero calibrator was serially diluted with each of two lipemic serum pools. This causes the degree of lipemia to increase as the insulin concentration (due primarily to the spiking solution) decreases. All dilutions were assayed along with the unspiked lipemic pools. The results show good recoveries even in the presence of severe lipemia.

Lipemic pool	Dilution of 500 µIU/ml	O Observed	E Expected	% O/E
1	*unspiked*	14		
	8 in 16	253	257	98%
	6 in 16	186	196	95%
	4 in 16	128	136	94%
	2 in 16	77	75	103%
	1 in 16	41	45	91%
2	*unspiked*	19		
	8 in 16	236	260	91%
	6 in 16	190	200	95%
	4 in 16	122	139	88%
	2 in 16	70	79	89%
	1 in 16	45	49	92%

Spiking Recovery

Three spiking solutions were prepared using the kit's zero calibrator as diluent. The solutions (A, B and C) were made to represent 438, 1314 and 2628 µIU/ml, respectively. 50 µl of each solution was spiked into 950 µl aliquots of four different patient serum samples, for a spiking ratio of 1 to 19, leaving the serum matrix of the spiked samples relatively intact. All samples were then assayed by the Coat-A-Count Insulin procedure. To calculate expected values, 95% of the unspiked value was added to 5% of the spiking solution concentration (22, 66 and 131 µIU/ml, respectively).

Sample	Spiking Solution	O Observed	E Expected	% O/E
1	—	11		
	A	34	32	106%
	B	81	76	107%
	C	160	141	113%
2	—	12		
	A	36	33	109%
	B	83	77	108%
	C	162	142	114%

Sample	Spiking Solution	O Observed	E Expected	% O/E
3	—	13		
	A	37	34	109%
	B	86	78	110%
	C	165	143	115%
4	—	24		
	A	45	45	100%
	B	95	89	107%
	C	172	154	112%

Parallelism

Two patient serum samples were assayed both undiluted and diluted with the kit's zero calibrator. The observed and expected values are presented below in μIU/ml.

Sample	Dilution	O Observed	E Expected	% O/E
1	8 in 8 (undiluted)	34.7		
	4 in 8	16.4	17.4	94%
	2 in 8	8.9	8.7	102%
	1 in 8	4.2	4.3	98%

Sample	Dilution	O Observed	E Expected	% O/E
2	8 in 8 (undiluted)	71.9		
	4 in 8	36.4	36.0	101%
	2 in 8	18.4	18.0	102%
	1 in 8	9.4	9.0	104%

The results indicate that serum samples maintain good linearity under dilution in the Coat-A-Count Insulin assay. This is due largely to the fact that the calibrators are prepared in (insulin-free) human serum, virtually eliminating matrix effects.

Curve Displacement

Five solutions were prepared using the zero calibrator as diluent and containing increasing amounts of insulin. Four samples were each assayed by the Coat-A-Count Insulin procedure in assays which included one-to-one dilutions of the sample with each of these five solutions.

Sample	O Observed	E Expected	% O/E
1 unspiked	62		
1 + (10)	35	36	97%
1 + (50)	59	56	105%
1 + (100)	84	81	104%
1 + (250)	163	156	105%
1 + (500)	265	281	94%
2 unspiked	120		
2 + (10)	68	65	105%
2 + (50)	88	85	104%
2 + (100)	108	110	98%
2 + (250)	188	185	102%
2 + (500)	289	310	93%

Sample	O Observed	E Expected	% O/E
3 unspiked	131		
3 + (10)	74	71	104%
3 + (50)	94	91	103%
3 + (100)	119	116	103%
3 + (250)	193	191	101%
3 + (500)	275	316	87%
4 unspiked	159		
4 + (10)	87	85	102%
4 + (50)	107	105	102%
4 + (100)	125	130	96%
4 + (250)	189	205	92%
4 + (500)	287	330	87%

Method Comparison

The Coat-A-Count Insulin kit was compared to another commerically available insulin radioimmunoassay on 46 patient samples. Linear regression statistics are displayed below.

(Coat-A-Count) = 0.78 (Kit A) + 0.8 μIU/ml
r = 0.93 SEE = 6.4 n = 46

Means:
Coat-A-Count: 40 μIU/ml
Kit A: 50 μIU/ml

Clinical Applications

Human insulin is a polypeptide hormone originating in the beta 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 circulation. This leads to higher insulin levels and more rapid tissue-assimilation of glucose—followed by a decline in the insulin level as the glucose level subsides.

In a number of conditions, notably insulinoma and diabetes, this relationship is impaired. Insulin tends to circulate at inappropriately high levels in patients with insulin-secreting pancreatic tumors; such tumors can thus be a cause of hypoglycemia. Accordingly, insulin immunoassays—used sometimes in connection with provocative doses of tolbutamide or calcium—play an essential role in the identification (and localization) of insulinomas. The finding of fasting hypoglycemia in association with an *inappropriately high* serum insulin concentration is considered diagnostic.

Insulin levels do not figure in the subclassification of diabetes worked out by the National Diabetes Data Group. Nevertheless, when obtained in the course of a glucose tolerance test, they appear to be of some prognostic value in predicting the benefits of insulin therapy and the likelihood of progression to insulin-dependence and the complications (such as retinopathy) characteristic of diabetes.

The application of insulin radioimmunoassay to patients already undergoing insulin therapy is complicated by the fact that such therapy typically leads to the formation of anti-insulin antibodies capable of interfering with the assay. Some investigators have sought therefore to measure insulin in urine, or in serum samples subjected to column chromatography or PEG precipitation. But the measurement of "free" insulin remains of limited interest as a technique for monitoring insulin therapy in the absence of statistics establishing therapeutic or toxic ranges. So far it appears that glucose control in diabetics cannot in general be achieved by normalizing the insulin profile. Nor is it known at what point abnormally high insulin levels become dangerous.

Expected Values

1 Ninety-three apparently normal individuals were analyzed at a large independent hospital for serum insulin using DPC's Coat-A-Count Insulin procedure. The individuals, none of whom were diabetic, were tested following an overnight fast. The data thus generated yielded an absolute range of 3 - 35 μIU/ml, a mean of 15 μIU/ml and a standard deviation of 7.6 μIU/ml. This indicates that a 2SD range for insulin of 0 - 30 μIU/ml is appropriate for normal individuals.

2 Twenty-two patients submitted to a two-hour glucose tolerance test. Four samples collected after the ingestion of 75 grams of carbohydrate were then assayed for both glucose and insulin. The subjects were classified as normal, using guidelines set by the National Diabetes Data Group, and thus had fasting glucose values of less than 115 mg/dl, two-hour values of less than 140 mg/dl and values which remained below 200 mg/dl throughout the two-hour period. As expected from the literature, the insulin values obtained varied greatly from patient to patient. Using the same protocol, 5 patients with impaired glucose tolerance (fasting glucose less than 140 mg/dl, two-hour between 140 and 200 mg/dl, and 200 mg/dl or above during the two-hour period) and 6 patients with overt diabetes type II (non-insulin dependent) who had never received exogenous insulin were subjected to a glucose tolerance test. The results of these studies are shown graphically on the following page, in Figure 2.

In the graphs showing results for the impaired and diabetic patients, the range of the 22 normals—depicted by shading—has been superimposed for reference. The insulin values of these patients vary to as great an extent as the normal population, and may thus be of minimal diagnostic use. Note that the overt diabetics as a group exhibit a modest insulin response to the ingestion of carbohydrate, and a somewhat elevated basal insulin level.

3 The Coat-A-Count Insulin kit was also used to measure fasting insulin levels in a group of 21 obese nondiabetic adults, weighing approximately 94 to 120 kg. Insulin levels for this group, graphed on this page in Figure 1, ranged from 7 to 74 μIU/ml, with a mean of 35 μIU/ml. Regression of fasting insulin levels on weight demonstrated a modest degree of relatedness between these two parameters. The correlation coefficient was 0.50 (or 0.64, with two points omitted.) Correlation between the insulin level and the ratio of weight to height was somewhat higher, viz. 0.72, with two points omitted: $(y) = 0.82\ (x) - 25$ μIU/ml.

Evidently, for individuals who are significantly overweight, fasting insulin levels are typically somewhat higher than for adults of normal weight.

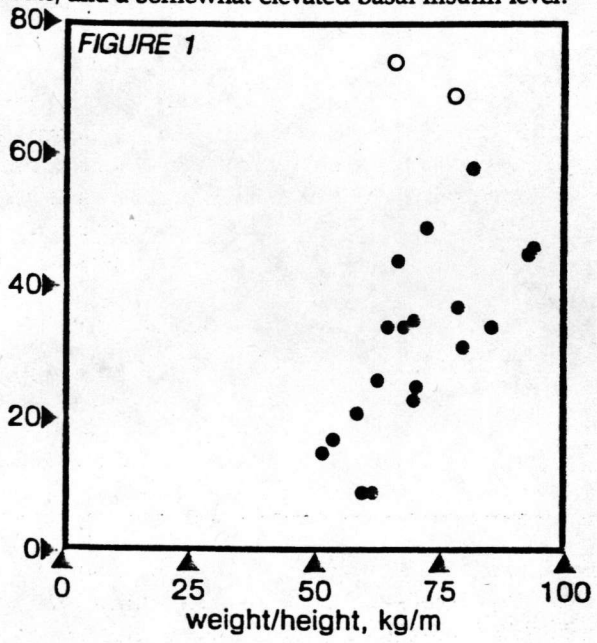

FIGURE 1

Laboratories should consider these results *as guidelines only*. Because of differences which may exist between laboratories and locales with respect to population, diet, laboratory technique and selection of reference groups, it is important for each laboratory to establish by similar means the appropriateness of adopting the normal ranges suggested by this study.