

**Human Recombinant Insulin Autoantibody
Immuno-Radioassay**

PROTOCOL

Liping Yu and George S. Eisenbarth
Barbara Davis Center for Childhood Diabetes

QA/QC for mIAA Assay

Establish of lab-cut-off

The cutoff index of mIAA was established as the 99th percentile in the control group (106 subjects, an index of 0.010). We constructed a receiver operating characteristic (ROC) curve among the newly diagnosed (within 2 weeks) patients with diabetes (n=104). The cutoff index of 0.010 corresponded to 66% sensitivity among diabetic patients and 99% specificity among normal controls.

Intra-assay CV: 12% (n=10)

Inter-assay CV: 14% (n=283)

- QA:
- 1) All assays are run in duplicate, along with four standard samples (one high positive, one low positive, one ultra-low positive, and a negative control serum samples. Two-well duplicates for each sample will be aliquoted with two separated events (one-well aliquoting per sample for each process). Upon finishing sample aliquoting on the full plate, the plate should be hold against the light and checked from bottom for the wells missing samples. Every sample with index above 0.010 (above 99th percentile of upper limit of normal control) must be repeated in a separated assay. The 3rd will be run if 2nd disagree with 1st. The result will be reported as the 1st value of two agreement (+,+ or +,-,+). The lab does not re-test negative samples since 1) it is rare to see negative results re-test as positive - the frequency is extremely low; 2) most of screening samples are negative so it would be very costly to re-test them.
 - 2) The low positive, and one ultra-low positive must show positive, and negative control must be negative in each assay.
 - 3) Shewart chart is plotted over time.
 - 4) All raw data and their analysis of QC control sets in each assay must be passed through lab director or supervisor.
 - 5) All final reporting results must be double-checked by lab director or supervisor before reported or uploaded.
 - 6) The lab should attend any national or international workshops or efficient evaluations if available.

Principle:

- Incubation of serum with labeled antigen with and without cold insulin overnight
- Precipitation of antibody-bound labeled antigens with protein-A/G Sepharose in a 96-well plate format, with each serum tested in duplicate
- Washing of the 96-well plates to remove unbound labeled antigens
- Counting of each well with a 96-well plate β counter.
- Results expressed as an index that adjusts the delta cpm of the test serum for the delta cpm of positive and negative control sera in a particular assay.

Plan for performing the assay:

<u>Day 1:</u>	morning:	retrieve and thaw sera to be tested
	mid-day:	(1) set up incubation of sera in buffer 1 (Part III) (2) prepare protein-A/G Sepharose in buffer 1 (Part IV) (3) Coating the 96-well plates with buffer 1 (Part IV)
<u>Day 2:</u>	morning:	(1) add incubate to protein-A/G Sepharose in plates (Part V) (2) wash plates with buffer 2 (3) dry plates (4) add scintillation liquid
	afternoon	(4) count (5) analyse data (Part VI)

Part I: Reagents & Supplies

Reagents and Supplies

	<u>Suggested suppliers (cat. no.)</u>
• - Trizma Base	Fisher (BP152-5);
• - NaCl	Fisher (BP358-212);
• - Tween 20	Sigma (P-1379)
• - Bovine Serum Albumin	Sigma (A-7906)
• - Protein A-Sepharose	GE HealthCare (17528003)
• - Protein G-Sepharose	GE HealthCare (17061803)
• - ¹²⁵ I Insulin	Perkin Elmer (NEX420)
• - NAP Column	GE HealthCare (17-0853-02 or 17-0854-02 if separating multiple labeling reactions)
▪ - 5N HCl	
• - Parafilm	Sigma (P7793-1EA)
• - 96-well round bottom plate	Fisher (08408220)
• - 96-well filtration plates	Fisher (07200754)
• - Bottle-Top 500 ml-Filter Units	Fisher (0974064A or B)
• - TopSeal	Perkin-Elmer (6005185)
• - Sealing Foil	USA Scientific (2923-0100)
• - Microscint-20	Perkin-Elmer (6013621)
• - Aluminum foil	

Equipments

• - TopCount β -counter (or similar)	Perkin-Elmer
• - 96-well Plate Shaker	Wallac - Delfi
• - Fume Hood	
• - Biological & radiation safety cabinets	
• - -20 °C freezers	
• - 4 °C refrigerator	
• - Pipette-Aid	
• - Water purification system	
• - Ice maker	
• - Radioactive contamination monitor	
• - Radiation sink	
• - pH meter	
• - Vortex mixer	
• - Stepper pipette	
• - Pipettes/tips	
• - Ice trays	
• - Vacuum-operated 96-well plate washer	Millipore (MAVM0960R)

Part II: Buffers

(1) buffer 1 (150 mM NaCl, 20 mM Tris-HCl, 1% BSA, 0.15% Tween-20, 0.1% Sodium Azide pH 7.4)

30 ml 5M NaCl
10 ml 2M Tris-HCl pH 7.4
10 gm BSA
1 gm Sodium Azide (essential, to prevent bacterial contamination)
1.5 ml Tween-20
up to 1000 ml

(2) buffer 2 The same as buffer 1 except for 0.1% BSA

Important Points:

- Buffer should be filtered (0.45 micron filter) to prevent any particles blocking the membrane in bottom of the wells of the 96 well plate (which would decrease washing efficiency and increase the assay background)
- Store buffers at 4°C in a sterile bottle for up to 2 weeks

Part III: Incubation of Serum Samples with ¹²⁵I-insulin

Each 96-well plate is sufficient for testing 24 samples in duplicate (24 duplicate with cold insulin and 24 duplicate without cold insulin. Usually, 4-8 plates can easily be run at one time (total of 96 to 192 samples). Six samples: High PC, nc, Low PC1, nc, Low PC2, nc, should be included in each assay.

(1) Spin down sera to remove fibrin clots when necessary (otherwise these may partially block membrane in bottom of wells)

(2) Prepare the stock solution of ¹²⁵I-insulin.

Use 1 ml of 5% BSA in PBS dissolve the powder of each 10 uCi of ¹²⁵I-insulin.

(3) Calculate how much ¹²⁵I-insulin and cold insulin are required.

6.4 ml of buffer 1 for two plates:

48 x 4.2 x 30 = 6 ml (48 samples, with 30 ul/well; in duplicate for both with and without cold insulin wells but multiply by 4.2 rather than 4 to allow for some extra)

20,000 cpm is used for each well.

3040 ul buffer 1
160 ul ¹²⁵I-insulin
3.2 ml

2784 ul buffer 1
 160 ul ¹²⁵I-insulin
256 ul humulin (or Novolin)
3.2 ml

Keep the Buffer-labeled antigen mixture on ice.

(3) Mix each serum sample with Buffer-antigen mixture in a PCR tube (or similar tube).

Serum: 6 ul

Buffer: 30 ul

Each sample for 2 wells.

(5) Vortex and incubate 2 hours at room temperature and overnight at 4°C.

**Part IV: Preparation of MultiScreen Filtration Plates
and Protein A/G-Sepharose**

- (1) Coat the plate with BSA by adding 150 ul of Buffer 1 to each well.

Incubate overnight at room temperature, after placing the plate on aluminium foil.

- (3) Remove buffer 1.
- (4) The plates are now ready to run the assay, but they can be stored at 4°C if necessary.
- (5) Prepare Protein-A/G Sepharose:
- a: prepare Protein A-Sepharose*
 - Use only plastic tubes because Protein-A sticks to glass
 - **For each plate**, take 5 ml Protein-A Sepharose in a 50 ml tube. Spin down and remove the fluid phase. Wash 2x times with buffer 1.
 - Finally add buffer 1 to give **62.5%** concentration of Protein-A Sepharose by volume.
 - b: prepare Protein G-Sepharose*
 - Use only plastic tubes because Protein-G sticks to glass
 - **For each plate**, take 1 ml Protein-G Sepharose in buffer 1 in a 50 ml tube. Spin down and remove the fluid phase. Repeat once with buffer 1.
 - Finally add buffer 1 to give **40%** concentration of Protein-A Sepharose by volume.
 - c: mix Protein A/G Sepharose*
- Mix Protein A/G Sepharose as 4:1 ratio (final concentration: 50% PA/8% PG).

Part V: Immunoprecipitation with Protein A-Sepharose

- (1) Add 50 ul of Protein A/G-Sepharose mixture to each well. Use Eppendorf multi-step pipettor and **re-suspend the Protein-A/G Sepharose after each row of the plate is done.** (Will need 5 ml of Protein-A/G Sepharose per plate.)
- (2) Add 35 ul of overnight incubate to each well (i.e., each serum will be tested in duplicate).
- (3) Shake the plate on a Plate Shaker for 45 minutes at 4°C.
- (4) Place the plate on Millipore plate washer device (with vacuum).
- (5) Wash the plate three times in this way with 200 ul of Washing Buffer per well.
- (6) Add 100 ul of Washing Buffer to each well. Shake for at least 5 minutes at 4°C.
- (7) Wash the plate four times with 200 ul of washing buffer per well (change the plate direction after two times of washing at this stage).
- (8) Place the plate in 37 C incubator for 15 minutes. Do not over-dry.
- (9) Add 50 ul of scintillation cocktail (Microscint-20) to each well.
- (10) Count on Top Count 96-well plate β counter.

Part VI: Data Analysis

A. If the signal is completely absorbed by cold insulin (mean cpm of the duplicate with cold insulin is <20 cpm), proceed as below.

(1) Delta cpm:

mean cpm of duplicate without cold insulin - mean cpm of duplicate with cold insulin

(1) CPM Index for each sample:

$$\frac{\text{Sample delta cpm} - \text{NC delta cpm}}{\text{PC delta cpm} - \text{NC delta cpm}}$$

(2) Coefficient of Variation

For Duplicates:

$$\frac{(\text{High CPM} - \text{Low CPM})/1.128}{\text{Mean CPM}} \times 100$$

For Triplicates:

$$\frac{(\text{High CPM} - \text{Low CPM})/1.693}{\text{Mean CPM}} \times 100$$

B. If mean cpm of the duplicate with cold insulin is ≥ 20 cpm, proceed as below.

1) If the count <1,000 cpm and is confirmed by 2nd run, then the sample will be considered Not Reportable.

2) If the count $\geq 1,000$ cpm and is confirmed by 2nd run, then the assay will be re-run with 10 more times of cold insulin.

- If repeat is completely absorbed, proceed with calculation of delta cpm and cpm index as per Part A above.
- If repeat is not completely, absorbed, the sample will be considered Not Reportable.

Amendment for Data Analysis (January 8, 2015)

We and others found some samples, not very often, gave the signal in the mIAA assay not because of the antibodies specifically binding to insulin, but binding to any iodine-proteins, which gave the false positive signals. Our mIAA assay is competitive assay with unlabeled human insulin as competitor. These false positive samples were shown binding to iodine-insulin, but could not be competed with unlabeled insulin, which means the signals from iodine-insulin binding is not from insulin, but from non-specific iodine-peptide. Our final mIAA calculation is based on delta signal between uncompetitive and competitive and theoretically the delta values from these samples with false positive signals should be close to "0", but most of time these false positive samples still gave positive delta values, which were finally reported as positive results in most of cases.

After several months of discussion through TrialNet Laboratory Monitoring Sub-committee (LMS), data analysis, and new ECL-IAA experimental assistance, the laboratory will start the following criteria to assist mIAA result analysis based on the decision of TrialNet LMS at January 8th of 2015.

- 1) If the count $<1,000$ cpm with signal not able to be completely absorbed, the result is confirmed by the 2nd run, and then the sample will be considered not reportable;
- 2) If the count $\geq 1,000$ cpm with signal not able to be completely absorbed, the result is confirmed by the 2nd run. Then the assay will be re-run with 10 more times of cold insulin and we will report the result as it is if completely absorbed or as not reportable if not completely absorbed.