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Anti-Insulin

ORG 520

96 Tests

**Immunometric Enzyme
Immunoassay for the
quantitative determination
of autoantibodies to bovine,
porcine and recombinant
human insulin**

Instruction for use

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WARNINGS AND PRECAUTIONS

All reagents of this test kit are strictly intended for in vitro use only.

Please adhere strictly to the sequence of pipetting steps provided in this protocol. Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.

All reagents should be stored refrigerated at 2 - 8 °C in their original container.

Do not interchange kit components from different lots. The expiration dates stated on the labels of the shipping container and all vials have to be observed. Do not use kit components beyond their expiration dates.

Allow all kit components and specimen to reach room temperature prior to use and mix well.

During handling of all kit reagents, controls and serum samples observe the existing legal regulations. The following precautions should be taken handling potentially infectious materials:

- do not eat, drink or smoke in areas where specimens or kit reagents are handled
- do not pipette by mouth
- wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.

The test kit contains components of human origin which, when tested by FDA-licensed methods, were found negative for hepatitis B surface antigen and for HIV antibody. No known test can guarantee, however, that products derived from human blood will not be infectious. Handle, therefore, all reagents and human blood derivatives, like plasma or serum samples, as if capable of transmitting infection.

Avoid contact with the TMB (3,3',5,5'-Tetramethyl-benzidine). If TMB comes into contact with skin wash thoroughly with water and soap.

The stop solution contains hydrochloric acid. If it comes into contact with skin, wash thoroughly with water and seek medical attention.

Avoid contact between the buffered Peroxide Solution and easily oxidized materials; extreme temperatures may initiate spontaneous combustion.

MATERIALS SUPPLIED

Package size	96 determ.
divisible microplate consisting of 12 modules of 8 wells each,.....	1
coated with a mixture of highly purified preparations of bovine, porcine and recombinant human insulin	
anti-Insulin calibrators in a PBS/BSA matrix.....	6 vials, 1.5 ml each
containing: 0; 6.3; 12.5; 25; 50 and 100 U/ml (A - F)	
anti-Insulin controls in a PBS/BSA matrix (positive and negative),	2 vials, 1.5 ml each
for the respective concentrations see the enclosed package insert.	
sample buffer, yellow, Concentrate	1 vial, 20 ml
enzyme conjugate solution, (light red) containing polyclonal	1 vial, 15 ml
rabbit anti-h-IgG-IgG; labelled with horseradish peroxidase	
TMB substrate solution	1 vial, 15 ml
stop solution (1 M hydrochloric acid)	1 vial, 15 ml
buffered wash solution, Concentrate	1 vial, 20 ml

CONTROLS

A set of two controls is provided with the kit.

TECHNICAL DATA

Sample material:	serum or plasma
Required sample volume:	10 µl of sample to be diluted 1:100 with sample buffer 100 µl prediluted sample per single determination
Total incubation time:	60 minutes at room temperature (20 - 28 °C)
Calibration range:	6.3 - 100 U/ml
Sensitivity:	0.5 U/ml
Storage:	refrigerated at 2 - 8 °C
Shelf life:	12 months after manufacturing or until the expiration date printed on the labels
Package size:	96 tests

PRINCIPLE OF THE PROCEDURE

Anti-Insulin is an indirect solid phase enzyme immunometric assay (ELISA). It is designed for the quantitative measurement of IgG class autoantibodies directed against insulin. The assay is based on microplates coated with a mixture of highly purified preparations of bovine, porcine and recombinant human insulin. The assay is useful in the primary diagnosis of Type I Diabetes, as well as for screening purposes to detect developing insulin autoantibodies in patients under insulin therapy. The microplate can be divided into 12 modules of 8 wells each or can be used completely for 96 determinations.

During this procedure the binding of present autoantibodies, as well as the formation of the sandwich complexes and enzymatic colour reaction take place during three different reaction phases:

Phase 1:

Calibrators, controls and prediluted patient samples are pipetted into the wells of the microplate. Any present antibodies bind to the inner surface of the wells. After a 30 minutes incubation the microplate is washed with wash buffer for removing non-reactive serum components.

Phase 2:

An anti-human-IgG horseradish peroxidase conjugate solution is pipetted into the wells of the microplate to recognize the autoantibodies bound to the immobilized antigens. After a 15 minutes incubation any excessive enzyme conjugate, which is not specifically bound is washed away with wash buffer.

Phase 3:

A chromogenic substrate solution containing TMB (3,3',5,5'-Tetramethyl-benzidine) is dispensed into the wells. During 15 minutes of incubation the color of the solutions change into blue. Color development is stopped by adding 1 M hydrochloric acid as stop solution. The solutions color change into yellow. The amount of colour is directly proportional to the concentration of IgG present in the original sample.

To read the optical density a microplate reader with a 450 nm filter is required. Bi-chromatic measurement with a 600-690 nm reference is recommended. The optical density for each calibrator may be graphically plotted against the concentration of IgG and unknowns extrapolated from the curve.

CLINICAL RELEVANCE

Type I Diabetes is mainly characterised by limited or fully missing secretion of the hormone insulin. Morphological studies demonstrated a destruction of the beta cells of the so-called Langerhans'sche Cells (Islet Cells) in Type I diabetics. Numerous researchers described the appearance of antibodies directed against the islet cells and insulin as the causal reason for the onset of the disease.

Anti-Insulin antibodies are found in 37 percent of patients with newly detected Type I Diabetes, in 4 percent of their relatives of the first degree and in up to 1,5 percent of healthy controls. A positive correlation between the appearance of anti-Insulin and anti-Islet Cell antibodies has been reported.

Anti-Insulin autoantibodies may be detected several months and in some cases years before the onset of the fully clinical manifestation of the diseases. Occasionally also autoantibodies to Pro-Insulin may appear.

These "true" anti-Insulin autoantibodies directed against endogenous insulin have to be distinguished from those autoantibodies which are developed in insulin dependent diabetics undergoing therapy with insulin preparations of animal origin. In fact the latter have to be referred to side effects. These side effects may occur as local reactions of the skin by development of insulin-specific autoantibodies. These autoantibodies are causing the formation of an insulin depot and they may simulate a resistance against the hormonal treatment with animal insulin.

Additionally other immunological phenomenon have been reported for Type I diabetics. A lot of other autoantibody specificities have been detected in those patients too, but these antibodies must not cause additional autoimmune phenomenon.

The table below shows the incidence (in percent) of various autoantibodies occurring in Type I diabetics compared to healthy controls:

Autoantibody specificity:	Type I diabetics (%)	healthy controls (%)
Antu-Islet Cell antibodies	32	1
Antibodies against Islet Cell surface antigens	approx. 50	approx. 2
Anti-Insulin antibodies	up to 70	0
Anti-Thyroid peroxidase ab's (Anti-TPO)	18	6
Anti-single stranded DNA ab's (Anti-ssDNA)	85	9

Indications: - anti-Insulin antibodies in Type I diabetics

- development of anti-Insulin antibodies under insulin therapy

NORMAL VALUES

In a normal range study with serum samples from healthy blood donors the following ranges have been established with the anti-Insulin test:

	Anti-Insulin [U/ml]
normal:	< 5
borderline:	5 - 10
elevated:	> 10

Positive results should be verified concerning the entire clinical status of the patient. Also every decision for therapy should be taken individually.

It is recommended that each laboratory establishes its own normal and pathological ranges. The reference ranges below should be regarded as guidelines only.

SPECIFICITY

The microplate is coated with a mixture of highly purified preparations of bovine, porcine and recombinant human insulin. Therefore the anti-Insulin test kit recognises only IgG-class autoantibodies specific for these insulins. No crossreactivities have been observed.

CALIBRATION

Since no international reference preparation for anti-Insulin autoantibodies is available, the assay system is calibrated in relative arbitrary units.

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MATERIALS REQUIRED

Equipment

- Microplate reader capable for endpoint measurements at 450 nm
- Vortex mixer
- Pipets for 10 µl, 100 µl and 1000 µl

Preparation of reagents

- distilled water
- graduated cylinder for 100 and 1000 ml
- plastic container for storage of the wash solution

Optional

- Multi-Chanel Dispenser
- or repeatable pipet for 100 µl
- data reduction software

SPECIMEN COLLECTION AND PREPARATION

For determination of anti-Insulin antibodies serum or plasma are the preferred sample matrices.

All serum and plasma samples are prediluted 1 : 100 with sample buffer. Therefore 10 µl of sample may be diluted with 1,000 µl of sample buffer.

The patients need not to be fasting, and no special preparations are necessary. Collect blood by venipuncture into vacutainers and separate serum or plasma from the cells by centrifugation after clot formation.

Samples may be stored refrigerated at 2 - 8 °C for at least 5 days. For longer storage of up to six months samples should be stored frozen at -20 °C. To avoid repeated thawing and freezing the samples should be aliquoted.

Neither Bilirubin nor Hemolysis have significant effect on the procedure.

PREPARATION AND STORAGE OF REAGENTS

All components of this test kit are supplied in a liquid format and ready to use, except the sample buffer and wash buffer. When stored refrigerated at 2 - 8 °C the components are stable for at least 30 days after opening or until the expiration date printed on the labels.

Remaining modules of the microplate should be stored refrigerated at 2 - 8 °C protected from moisture; store together with desiccant and carefully sealed in the plastic bag.

Preparation of sample buffer

Dilute the contents of each vial of the sample buffer concentrate (5x) with distilled water to a final volume of 100 ml prior to use. Store refrigerated: stable at 2 - 8 °C for at least 30 days after preparation or until the expiration date printed on the label.

Preparation of buffered wash solution

Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled water to a final volume of 1000 ml prior to use. Store refrigerated: stable at 2 - 8 °C for at least 30 days after preparation or until the expiration date printed on the label.

NOTES ON TECHNIQUE

Control sera or pools should routinely be assayed as unknowns to check performance of the reagents and the assay.

For all controls, the respective concentrations are provided on the labels of each vial. Using these concentrations a calibration curve may be calculated to read off the patient results semi-quantitatively.

Pipetting and Sample Handling

Use a disposable-tip micropipette to dispense sera and plasma samples. Pipet directly to the bottom of the wells. To avoid carryover contamination change the tip between samples.

Patient samples expected to contain high concentrations should be additionally diluted with sample buffer before. Additional dilutions must be considered during calculation.

IMMUNOASSAY PROCEDURE

Do not interchange components of different lots.

All components should be at room temperature before use.

Dilute all patient samples 1:100 with sample buffer before assay. Therefore combine 10 µl of sample with 1000 µl of sample buffer in a polystyrene tube. Mix well. Calibrators and controls are ready to use and need not to be diluted.

1. Prepare a sufficient number of microplate modules to accommodate calibrators, controls and prediluted patient samples in duplicates.

	1	2	3	4	5	6
A	SA	SE	P1	P5		
B	SA	SE	P1	P5		
C	SB	SF	P2	P..		
D	SB	SF	P2	P..		
E	SC	C1	P3			
F	SC	C1	P3			
G	SD	C2	P4			
H	SD	C2	P4			

SA - SF: standards A to F

P1, P2... patient sample 1, 2 ...

C1: positive control

C2: negative control

2. Pipet **100 µl of calibrators, controls and prediluted patient samples** into the wells.
3. Incubate for **30 minutes** at room temperature (20 - 28 °C).
4. Discard the contents of the microwells and wash **3 times with 300 µl of wash solution**.
5. Dispense **100 µl of enzyme conjugate** solution into each well.
6. Incubate for **15 minutes** at room temperature.
7. Discard the contents of the microwells and wash **3 times with 300 µl of wash solution**.
8. Dispense **100 µl of TMB substrate solution** into each well.
9. Incubate for **15 minutes** at room temperature protected from light.
10. Add **100 µl of stop solution** to each well of the modules and leave untouched for 5 minutes.
11. Read the optical density at **450 nm** and calculate the results. Bi-chromatic measurement with reference at 600-650 nm is recommended.

**The developed color is stable for at least 30 minutes.
Read optical densities during this time.**

CALCULATION OF RESULTS

For the anti-Insulin test a 4-Parameter-Fit with lin-log co-ordinates for optical density and concentration is recommended. Smoothed Spline approximation and log-log co-ordinates are also suitable.

Recommended Lin-Log Plot

First calculate the averaged optical densities for each calibrator well. Use lin-log graph paper and plot the averaged optical density of each calibrator versus the concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

CALCULATION EXAMPLE

The figures below show typical results for anti-Insulin. These data are intended for illustration only and should not be used to calculate results from another run.

No	Position	OD 1	OD 2	Mean	Conc. 1	Conc. 2	Mean	decl.Conc.	CV %
STA	A 1/B 1	0.036	0.032	0.034	0.1	0.1	0.1	0.0	8
STB	C 1/D 1	0.354	0.345	0.349	6.3	6.1	6.2	6.3	2
STC	E 1/F 1	0.621	0.602	0.611	12.9	12.4	12.7	12.5	2
STD	G 1/H 1	0.984	1.005	0.994	25	25	25	25	1
STE	A 2/B 2	1.503	1.500	1.502	50	50	50	50	0
STF	C 2/D 2	2.057	2.035	2.046	102	99	100	100	1

ASSAY CHARACTERISTICS

Sensitivity

The lower detection limit for anti-Insulin has been determined at 0.5 U/ml.

Parallelism

In dilution experiments sera with high antibody concentrations were diluted with sample buffer and assayed in the anti-Insulin kit.

Sample No	Dilution	Observed [U/ml]	Expected [U/ml]	Observed/Expected
1	1:50	77.6		
	1:100	41.7	38.8	107 %
	1:200	21.1	19.4	109 %
	1:400	10.3	9.7	106 %
	1:800	4.7	4.9	96 %
2	1:100	100.7		
	1:200	50.7	50.4	101 %
	1:400	23.7	25.2	94 %
	1:800	11.1	12.6	88 %
	1:1600	5.3	6.3	84 %

Precision

Statistics were calculated for each of three samples from the results of 24 determinations in a single run for Intra-Assay precision. Run-to-run precision was calculated from the results of 5 different runs with 6 determinations each:

Intra-Assay		
Sample No	Mean [U/ml]	CV [%]
1	11.2	2.5
2	27.6	2.9
3	59.7	4.0

Inter-Assay		
Sample No	Mean [U/ml]	CV [%]
1	11.6	6.0
2	31.2	5.2
3	69.5	4.3

INCUBATION SCHEME

- ① Pipet **100 µl** calibrator, control or diluted patient sample
→ Incubate for **30 minutes** at room temperature
→ Discard the contents of the wells and wash 3 times with **300 µl** wash solution
- ② Pipet **100 µl** enzyme conjugate
→ Incubate for **15 minutes** at room temperature
→ Discard the contents of the wells and wash 3 times with **300 µl** wash solution
- ③ Pipet **100 µl** substrate solution
→ Incubate for **15 minutes** at room temperature
- ④ Add **100 µl** stop solution
→ Leave untouched for **5 minutes**
→ Read at **450 nm**