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Anti-
Parietal Cell

ORG 531

96 Tests

Immunometric Enzyme Immunoassay for the quantitative determination of anti-Parietal Cell (H+/K+-ATPase)-Autoantibodies

Instruction for use

CONTENTS

CONTENTS 2
WARNINGS AND PRECAUTIONS 3
MATERIALS SUPPLIED 3
CONTROLS 4
TECHNICAL DATA 4
PRINCIPLE OF THE PROCEDURE 4
CLINICAL RELEVANCE 5
NORMAL VALUES 6
SPECIFICITY 6
CALIBRATION 6
REFERENCES 6
MATERIALS REQUIRED 7
SPECIMEN COLLECTION AND PREPARATION 8
PREPARATION AND STORAGE OF REAGENTS 8
NOTES ON TECHNIQUE 8
IMMUNOASSAY PROCEDURE 9
CALCULATION OF RESULTS 10
CALCULATION EXAMPLE 10
ASSAY CHARACTERISTICS 11
INCUBATION SCHEME 11

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 thorougly 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, coated with highly purified pig H+/K+-ATPase, α- and β-subunits	1
Anti-Parietal Cell calibrators in a PBS/BSA matrix containing: 0; 6.3; 12.5; 25; 50 and 100 U/ml (A - F)	6 vials, 1.5 ml each
Anti-Parietal Cell controls in a PBS/BSA matrix (positive and negative) for the respective concentrations see the enclosed package insert	2 vials, 1.5 ml each
sample buffer, yellow, Concentrate	1 vial, 20 ml
enzyme conjugate solution, (light red) containing polyclonal rabbit anti-h-IgG-IgG; labelled with horseradish peroxidase	1 vial, 15 ml
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-Parietal Cell (H+/K+-ATPase) is an indirect solid phase enzyme immunometric assay (ELISA). It is designed for the quantitative measurement of IgG class autoantibodies directed against the α- and β-subunits of the Parietal Cell H+/K+-ATPase. Preparations of highly purified pig Parietal Cell H+/K+-ATPase are coated on microplates.

The microplates can be divided into 12 modules of 8 wells each or can be used completely for 96 determinations. Each well can be separated from the module ("break-away").

The binding of present autoantibodies, 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 recognise IgG class autoantibodies bound to the immobilized antigens. After a 15 minutes incubation any excess 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`-Teramethyl-benzidine) is dis-pensed into the wells. During 15 minutes of incubation the colour of the solutions change into blue. Colour development is stopped by adding 1 M hydrochloric acid as stop solution. The solutions colour change into yellow. The amount of colour is directly proportional to the concentration of IgG antibodies 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.

CLINICAL RELEVANCE

Circulating autoantibodies to gastric parietal cells have been first detected in patients with pernicious anemia by the complement fixation test, described by Irvine et al. 1962 and following with an immunofluorescence test described by Taylor et al. 1962. The responsible parietal cell autoantigen was localised to the secretory canaliculi of gastric parietal cells and to gastric microsomes. Further biochemical and molecular investigations identified the responsible antigens as a- and b-subunit of the gastric H/K ATPase.

The gastric H/K ATPase (EC 3.6.1.3) is a hydrogen transporting enzyme, responsible for the acidifi-cation of the stomach lumen (Rabon and Reuben, 1990). It belongs to the family of electroneutral P-type ATPases which also include the Na/K and the Ca ATPases (Pederson and Carfoli, 1987). This parietal cell antigen consists of two subunits, an 8-10 transmem-brane catalytic α-subunit of 1033 amino acids and a heavily glycosilated β-subunit with a 294 amino acid core. This H/K ATPase shows a high degree of conversation in the amino acid sequence across species (van Driel and Callaghan, 1995).

Pernicious anemia is the most common cause of vitamin B12 deficiency in Western populations. Longitudinal studies suggest, that pernicious anemia is the end stage of type A chronic atrophic gas-tritis (Irvine et al. 1974), a disease characterised by pathological lesions of the fundus and body of the stomach, including gastric mucosal atrophy, selective loss of parietal and chief cells from the gastric mucosa and submucosal lymphocytic infiltrates (Whittingham and Macckay, 1985).

Pernicious anemia is predominately a disease of middle age northern white Europeans and females show a higher incidence than males. Patients with pernicious anemia appear pale, physically tired and mentally depressed. Pernicious anemia associates with a number of other diseases and these are predominantly organ specific autoimmune diseases of endocrine glands, in which autoantibodies to other tissue specific antigens are also present. The specific diseases include Hashimoto's thyroiditis, diabetes melitus Type 1 and primary Addison's disease (Whittingham and Macckay, 1985). Late stages of pernicious anemia may also be associated with peripheral neuropathy and subacute combined degeneration of the spinal cord due to vitamin B12 deficiency.

Autoantibodies against the H/K ATPase can be detected in 80-90% of pernicious anemia patients, by indirect immunofluorescence and they are also detected in 2-5% of the healthy adult population. ELISA test systems show a sensitivity of about 80% and specificity of about 90%. There is an age related increase in the presence of parietal cell autoantibodies in the adult population. A study of the relationship between parietal cell autoantibody and gastric mucosal morphology, indicates these parietal cell positive individuals in a random population may indeed have early type A gastritis (Uibo et al., 1984). Higher prevalence rates (20-30%) of parietal cell autoantibodies have been noted in patients with autoimmune endocrine disorders such as thyrotoxicosis, Hashimoto's thyroiditis and insu-lin dependent diabetes (Whittingham and Macckay, 1985). Histological examinations of gastric biopsies reveals that the majority of parietal cell autoantibody positive individuals also have a type A gastric lesion (Varis et al. 1979).

NORMAL VALUES

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

	anti-Parietal Cell-Ab
normal:	< 10 U/ml
elevated:	≥ 10 U/ml

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 of serum anti-Parietal Cell antibodies.

SPECIFICITY

The microplate is coated with highly purified H+/K+-ATPase from pig Parietal Cells. The test kit is specific only for autoantibodies against the Parietal Cell antigen.

CALIBRATION

Since no international reference preparation for Anti-Parietal Cell 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

- destilled 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-Parietal Cell serum or plasma are the preferred sample matrixes.

All serum and plasma samples are prediluted 1 : 100 with sample buffer. Therefore 10 µl of sample may be diluted with 1000 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, exept 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 destilled 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 accomodate 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 Anti-Parietal Cell a 4-Parameter-Fit with lin-log co-ordinates for optical density and concentration is the data reduction method of choice. 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-Parietal Cell. 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	0.016	0.016	0.016	0.00	0.00	0.00	0.0	0.0
STB	C 1/D 1	0.332	0.335	0.334	6.45	6.40	6.43	6.3	0.6
STC	E 1/F 1	0.548	0.558	0.553	12.0	12.2	12.1	12.5	1.3
STD	G 1/H 1	0.934	0.956	0.945	25.6	24.8	25.2	25.0	1.6
STE	A 2/B 2	1.410	1.386	1.398	51.0	50.0	50.5	50.0	1.2
STF	C 2/D 2	1.823	1.840	1.832	98.3	101.1	99.7	100.0	1.8

ASSAY CHARACTERISTICS

Sensitivity

The lower detection limit for Anti-Parietal Cell has been determined at 0.5 U/ml.

Precision

Statistics for Coefficients of variation (CV) 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	12.5	3.5
2	22.5	2.8
3	75.0	3.2

Inter-Assay		
Sample No	Mean [U/ml]	CV [%]
1	12.0	4.2
2	20.5	3.7
3	85.9	2.6

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** substarte solution
   → Incubate for **15 minutes** at room temperature
4. Add **100 µl** stop solution
   → Leave untouched for **5 minutes**
   → Read at **450 nm**