DRG

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DRG® Anti-Prothrombin IgG/IgM (EIA-3612)

Revised 23 Nov. 2010 rm (Vers. 1.1) **RUO in the USA**

This kit is intended for Research Use Only.

Not for use in diagnostic procedures.

PRINCIPLE OF THE PROCEDURE

Anti-Prothrombin IgG/IgM is an indirect solid phase enzyme immunometric assay (ELISA). It is designed for the measurement of IgG or IgM autoantibodies directed against Prothrombin. The microplate is coated with highly purified prothrombin. The microplate 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 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 (or anti-human-IgM) horseradish peroxidase conjugate solution is pipetted into the wells of the microplate to recognise 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`-Tetramethylbenzidine) is dispensed into the wells. During 15 minutes of incubation the colour of the solutions change into blue. Adding 1 M hydrochloric acid as stop solution stops colour development. The solutions colour change into yellow. The amount of colour is directly proportional to the concentration of IgG resp. IgM 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.

CALIBRATION

Since no international reference preparation for Anti-Prothrombin autoantibodies is available, the assay system is calibrated in arbitrary units.

WARNINGS AND PRECAUTIONS

This kit is intended for Research Use Only. Not for use in diagnostic procedures.
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.

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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 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-Prothrombin 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 specimen need not be from persons 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 has 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.

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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 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 changes the tip between samples. 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 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 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... sample 1, 2 ...
C1: positive control
C2: negative control

2. For the determination of one class of autoantibodies pipette 100 µl of calibrators, controls and prediluted samples into the wells.
   For determination of both IgG and IgM autoantibodies calibrators, controls and samples have to be pipetted in two attempts.
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.
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-Prothrombin IgG and IgM 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

No	Position	OD 1	OD 2	Mean	Conc. 1	Conc. 2	Mean	decl.Conc	CV%
STA	A 1/B 1	0.050	0.050	0.050	0.1	0.1	0.1	0.0	0
STB	C 1/D 1	0.322	0.300	0.311	7.8	7.0	7.4	7.5	7
STC	E 1/F 1	0.520	0.519	0.520	15.2	15.2	15.2	15.0	0
STD	G 1/H 1	0.803	0.824	0.814	29.2	30.5	29.9	30.0	3
STE	A 2/B 2	1.226	1.191	1.209	61.6	58.2	59.9	60.0	3
STF	C 2/D 2	1.640	1.637	1.638	120.5	119.9	120.2	120.0	0

MATERIALS SUPPLIED

Package size **96 determ.**

Divisible microplate consisting of 12 modules of 8 wells each, coated with a mixture of highly purified prothrombin 1

Anti-prothrombin calibrators with IgG and IgM class anti-Prothrombin antibodies in a PBS/BSA matrix containing: 0;6.3;12.5;25;50;100 U/ml ..... 6 vials, 1.5 ml each

Anti-Prothrombin controls in a PBS/BSA matrix (positive and negative), for the respective concentrations see the enclosed package insert ..... 2 vials, 1.5 ml each

Anti-prothombin 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

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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:	0-100 U/ml
Sensitivity:	1 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

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