DRG

DRG® Anti-Centromer B (EIA-3569)

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Revised 31 May 2007 **RUO in the USA**

PRINCIPLE OF THE PROCEDURE

Anti-Centromer B is an indirect solid phase enzyme immunometric assay (ELISA). It is designed for the quantitative measurement of IgG class autoantibodies directed against Centromer B. 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"). 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 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`-Teramethyl-benzidine) is dispensed into the wells. During 15 minutes of incubation the color of the solutions change into blue. Adding 1 M hydrochloric acid as stop solution stops color development. 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

Progressive systemic scleroderma (PSS) is an autoimmune multisystemic disorder characterized by tight skin. PSS is a disorder of the vascular connective tissue leading to slowly progressive fibrosis and to sclerosis in the advanced phases of the disease. Aside from skin the gastrointestinal tract of the patients is the most affected organ. Moreover kidney, lung, heart and muscles are involved. Serologically PSS can be characterized by the detection of antinuclear antibodies. Up to 86% of the patients suffering from scleroderma exhibit autoantibodies against the Scl-70 antigen, i.e. Topoisomerase I. Anti-Scl-70 antibodies are mostly present in patients with the more diffuse manifestations of PSS (with lungs and joints being affected and fast progression tendency). CREST-Syndrome is a variant of PSS with a more protracted course of disease. However, prognosis is much better compared to patients exclusively suffering from PSS. The acronym CREST has been derived from the first letters of the five most important clinical manifestations: Calcinosis, Raynaud's phenomenon, esophageal involvement, sclerodactyly, teleangiectasia). In patients suffering from CREST syndrome circulating antibodies against the centromere protein B can be detected. Up to 70% of all CREST patients exhibit Anti-Centromere B autoantibodies. The determination of Anti-Centromere B autoantibodies is of prognostic significance in diagnosing Raynaud's phenomenon. Raynaud's phenomenon often comes out as the first symptom of scleroderma and precedes the additional manifestations by several years. Also in patients suffering from primary biliary cirrhosis (PBC) Anti-Centromere B antibodies can be found. PBC and CREST often overlap. Anti-Centromere B are found in about 10-20% of sera from patients with PBC preferentially identifying those patients with Raynaud's phenomenon and sclerodactyly. Along with other centromere proteins (e.g. CENP-A (19 kDa), CENP-C (140 kDa)) centromere protein B

(80 kDa) belongs to a protein complex of the chromosomal kinetochore, the attachment site of microtuble spindles during cell division.

NORMAL VALUES
In a normal range study with serum samples from healthy blood donors the following ranges have been established with the Anti-Centromer B test:

	Anti-Centromer B [U/ml]
normal:	< 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 establish its own normal and pathological ranges of serum Anti-Centromer B.

SPECIFICITY
The microplate is coated with recombinant Centromer B as antigen. The Anti-Centromer B test kits recognizes only autoantibodies specific to the concerning antigen. No cross reactivities to other autoantigens have been observed.

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

WARNINGS AND PRECAUTIONS
All reagents of this test kit are strictly intended for in vitro use only. In the United States, this kit is intended for Research 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, coated with recombinant Centromer B	1
Anti-Centromer B Calibrators in a serum/buffer matrix Containing: 0; 10, 30; 100; 300 U/ml	5 vials, 1.5 ml each
Anti-Centromer Controls in a serum/buffer 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:	10 - 300 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

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-Channel Dispenser
- Or repeatable pipet for 100 µl
- Data reduction software

SPECIMEN COLLECTION AND PREPARATION
For determination of Anti-Centromer B antibodies 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, 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 changes 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.

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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**	**P2**	**P..**			
B	**SA**	**SE**	**P2**	**P..**			
C	**SB**	**C1**	**P3**				SA – SE: standards A to E
D	**SB**	**C1**	**P3**				P1, P2.... patient sample 1,2 ...
E	**SC**	**C2**	**P4**				C1: positive control
F	**SC**	**C2**	**P4**				C2: negative control
G	**SD**	**P1**	**P5**				
H	**SD**	**P1**	**P5**				

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-Centromer B tests 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.

ASSAY CHARACTERISTICS

Sensitivity

The lower detection limit for Anti-Centromer B has been determined at 1.0 U/ml.

Parallelism
In dilution experiments sera with high antibody concentrations were diluted with sample buffer and assayed in the Centromer B kit. The assay shows linearity over the full measuring range.

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			Inter Assay		
Sample No.	Mean (U/ml)	CV (%)	Sample No.	Mean (U/ml)	CV (%)
1	15.2	5.4	1	16.4	5.4
2	122.0	4.4	2	125.6	5.0
3	220.0	4.7	3	225.4	4.2

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 three 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 three 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**

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