

INTRODUCTION AND BACKGROUND

Circulating antibodies against various intracellular antigens are characteristic for systemic, autoimmune-mediated rheumatic diseases. These comprise Systemic Lupus Erythematosus (SLE), Mixed Connective Tissue Disease (MCTD), Sjögren's Syndrome (SS), Progressive Systemic Sclerosis (PSS, Scleroderma) resp. CREST-Syndrome, Rheumatoid Arthritis (RA) and Polymyositis (PM).

Often, these disorders can be diagnosed by measuring their associated antibodies. 8 antigens specifically recognised by these antibodies are immobilised on the solid phase of the present test:

Line	Antigen	Source	Disease
A	dsDNA	plasmid	SLE
B	RNP (proteins A, C, 68kDa)	recombinant	MCTD, SLE, RA, PSS
C	Sm (proteins B, B', D)	bovine thymus	SLE
D	SS-A/Ro (60kDa-protein)	bovine thymus	SS, SLE
E	SS-B/La	recombinant	SS, SLE
F	Scl-70 (DNA-topoisomerase 1)	recombinant	PSS
G	CENP-B	recombinant	PSS (CREST)
H	Jo-1 (Histidyl-tRNA-synthetase)	recombinant	PM

This enzyme-linked immunosorbent assay (ELISA) is intended for the individual, qualitative determination of IgG antibodies in human serum, directed against one of the antigens quoted above. The test is fast (incubation time 30 / 30 / 30 minutes) and flexible (divisible solid phase for 1 - 12 analyses, ready-to-use reagents). A negative and a positive control check the assay performance.

WARNINGS AND PRECAUTIONS

The test kit is intended for in vitro use only. In the United States, this kit is intended for Research Use Only.

The sample buffer, calibrators and controls contain Na-azide as preservative. The wash buffer contains bromonitrodioxane and the conjugate methylisothiazolone / bromonitrodioxane as preservative. The substrate contains 3, 3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide (H₂O₂). The stop solution, 0,5 M sulfuric acid (H₂SO₄), is acidic and corrosive.

The above mentioned reagents may be toxic if ingested. Follow routine precautions for handling hazardous chemicals. Avoid all body contact, wear gloves and eye protection. If one of the reagents comes into contact with skin, wash thoroughly with water. Never pipette by mouth.

Na-Azide may react with lead and copper plumbing to form explosive metal azides. On disposal, flush with a large amount of water to prevent azide build-up.

The controls contain components of human origin. They have produced negative results when tested for anti-HIV 1/2, anti-hepatitis C virus and hepatitis B surface antigen. However, no known test can guarantee that products derived from human blood will not be infectious. They should therefore be handled as if capable of transmitting infectious agents, and discarded appropriately.

PRINCIPLE OF THE TEST

The following immunological reactions take place on the surface of the sensitised solid phase.

- 1st reaction: Antigen-specific antibodies present in the sample bind to the respective immobilised antigen, forming the antigen-antibody complex. Unbound sample components are washed away.
- 2nd reaction: A second antibody, directed at human IgG antibodies and labeled with horse-radish peroxidase (conjugate), binds to the complex. Excessive conjugate is washed away.
- 3rd reaction: The enzyme-labelled complex converts a substrate into a blue product. Samples containing antigen-specific IgG antibodies develop the blue colour, whereas samples without these antibodies remain colourless.

CONTENTS OF THE KIT

- a. **1 microtitre plate**, coated line by line with 8 individual autoantigens.
Packed in a foil laminate pouch with a desiccant bag. The plate consists of 12 strips, thus providing maximum flexibility and economy in use of the assay.
- b. **Sample buffer**, 20,0 mL, 10x-concentrate, yellow coloured. Contains Tris-buffered saline (TBS), bovine serum albumin (BSA), Tween and Na-azide.
- c. **Wash buffer**, 100 mL, 20x-concentrate, blue coloured.
Contains TBS, Tween and bromonitrodioxane.
- d. **Negative control and positive control** (= calibrator), 3,0 mL each, yellow coloured, ready-to-use (in vials with a green and a red lid, respectively). Contain TBS, BSA, Tween and Na-azide.
- e. 14 mL **anti-human IgG peroxidase conjugate**, ready-to-use, green coloured.
Buffered solution containing stabilising protein, methylisothiazolone and bromonitrodioxane.
- f. 14 mL **substrate solution**, ready-to-use, colourless.
Contains a buffered solution of TMB and hydrogen peroxide. Contained in a vial impermeable to light.
- g. 14 mL **stop solution** (0,5 M H₂SO₄), colourless, ready-to-use.
Caution: sulfuric acid is corrosive.
- h. Directions for use
- i. Lot-specific certificate of analysis

MATERIALS REQUIRED BUT NOT SUPPLIED

- a. Deionised or distilled water
- b. Graduated cylinders, 250 and 2000 mL
- c. Transfer tubes in the microplate format (recommended)
- d. Pipettes for 10 - 1000 µL (1- and multi-channel pipettes recommended)
- e. Microplate washer (optional)
- f. Microplate photometer fitted with a 450 nm filter
- g. ELISA evaluation program (recommended)

STORAGE OF THE KIT

Store the kit at 2-8°C. It is stable up to the expiry date stated on the label of the box. Do not use kit beyond its expiry date.

REAGENT AND SAMPLE PREPARATION / SPECIMEN REQUIREMENTS

Do not exchange or pool corresponding components from different kits, due to possibly different shipping or storage conditions.

- Before opening the pouch of the solid phase, it must have reached room temperature. Remove supernumerary microwell strips from the frame and immediately put them back into the pouch, together with the desiccant bag. Reseal the pouch hermetically with adhesive tape and keep it refrigerated for future use.
- Dilute the sample buffer 10x-concentrate (20,0 mL, yellow) with 180 mL deionised water. Mix thoroughly. The diluted buffer is stable for several weeks if stored refrigerated.
- Dilute the wash buffer 20x-concentrate (100 mL, blue) with 1900 mL deionised water. Mix thoroughly. The diluted buffer is stable for several weeks if stored refrigerated.
- Preparation of the samples: Handle patient specimens as if capable of transmitting infectious agents. Prepare sera using normal laboratory techniques and dilute them 1/100, e.g. 10 µL serum + 990 µL sample buffer. Mix thoroughly.

For rapid dispensing during the assay procedure, preparation of the controls and samples in microtitre transfer tubes is recommended. This allows the operation of a multichannel pipette during the assay procedure.

If samples are not assayed immediately, they should be stored at 2-8°C and assayed within 3 days. For longer storage, -20°C or lower temperature are recommended. Repeated freezing and thawing of sera should be avoided. Thawed samples must be mixed prior to diluting.

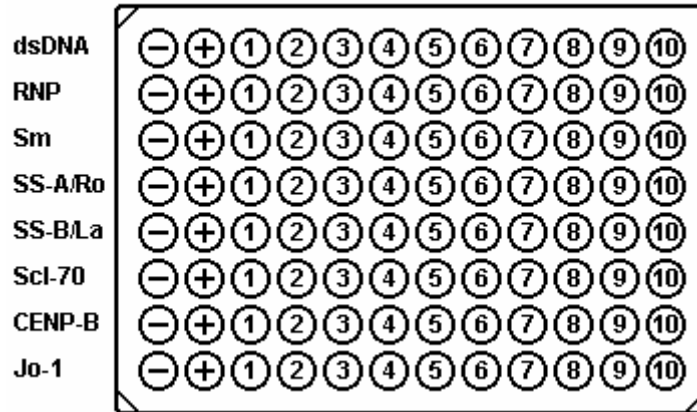
Specimen requirements: Highly lipemic, haemolysed or microbially contaminated sera may cause erroneous results and should be avoided.

ASSAY PROCEDURE

Before starting the assay, all components of the kit must have reached room temperature ($22 \pm 4^\circ\text{C}$).

To achieve best results, i.e. the maximum ratio between specific and background signal, careful washing is essential (steps a, c and e). It is crucially important to remove the wash solution completely. For that purpose, tap the plate firmly on several layers of absorbent tissue. Automated washers must be verified according to results obtained by manual washing.

- Immediately prior to use, wash the solid phase once: fill wells with 350 µL wash buffer each, soak for about 10 seconds in the wells and remove.
- Dispense the controls (3,0 mL each, ready-to-use, yellow) and the diluted samples (1 - 10) rapidly into the microwell strips as depicted below; 100 µL per well.



Incubate the plate for 30 minutes at room temperature (22 ± 4°C).

Note: A higher incubation temperature (up to 30°C) can be used if the substrate incubation is shortened (step f).

- c. Wash the wells 4 times as in step a.
- d. Rapidly (preferably using a multichannel pipette) dispense the conjugate (14 mL, ready-to-use, green); 100 µL per well. Incubate the plate as in step b.
- e. Repeat wash step c.
- f. Rapidly (preferably using a multichannel pipette) dispense the substrate solution (14 mL, ready-to-use, black vial); 100 µL per well. Incubate the plate as in step b. As the substrate is photosensitive, avoid intense light exposure (e.g. direct sunlight) during incubation.
- g. Rapidly (preferably using a multichannel pipette) dispense the stop solution (14 mL, ready-to-use, colourless); 100 µL per well. Use the same sequence as for the substrate. Agitate the plate, preferably on an orbital shaker, for about 10 seconds.
- h. Immediately read the absorbance at 450 nm.

Store the remainder of the reagents refrigerated if they are to be used again.

EVALUATION AND QUALITY CONTROL

The assay is evaluated in a qualitative manner. The absorbance of the samples is compared to the borderline absorbance (= cut-off absorbance), separately for each of the 8 parameters. The cut-off absorbance is determined by means of the positive control (= calibrator), according to the formula:

$$\text{Absorbance (borderline)} = \text{absorbance (positive control)} \times \text{factor}$$

The factor depends on the kit lot and is individually quoted for each parameter in the lot-specific certificate of analysis (included with each test kit).

Example:

absorbance (positive control)	=	1250 mOD
factor	=	0.35
absorbance (borderline)	=	1250 mOD x 0.35 = 438 mOD



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In order to gain an impression of the degree of a sample's reactivity, the ratio between sample and borderline absorbance is calculated:

$$\text{ratio} = \text{absorbance (sample)} / \text{absorbance (borderline)}$$

Example:

absorbance (borderline)	= 438 mOD
absorbance (sample)	= 1480 mOD
ratio	= 1480 mOD / 438 mOD = 3.4

Quality control

The positive control (calibrator) and negative control check the assay performance. Their acceptable ranges are quoted below. Values of the controls have to fall within the indicated ranges; otherwise, the results of the assay are invalidated.

	OD450nm
positive control (calibrator)	> 0.6
negative control	< 0.3

INTERPRETATION OF RESULTS / LIMITATIONS OF THE PROCEDURE

Based on the measurement of a blood donor collective of sera (n = 80), we suggest for the assessment of patient sera:

	ratio
normal (negative) range	< 0.80
cut-off	1.0
equivocal range	0.80 – 1.25
positive range	> 1.25

These specifications apply uniformly for all 8 parameters. However, they are given as an indication only; in order to check their accuracy, each analysis should include parallel samples of normal sera.

A negative test result indicates that the patient does not have an elevated level of IgG antibodies to the respective antigen. However, the presence of the corresponding systemic autoimmune disorder, as outlined in the beginning, can nevertheless not be excluded.

A positive result should be considered as an indication for the associated disease.

Specimens exhibiting results between the borderlines quoted above should be considered as equivocal and reported as such. It is recommended that a second sample be collected some days later and run in parallel with the first sample to document a possible change of antibody titer.

As with any serological test, the results should be interpreted in the light of the patient's symptoms and other diagnostic criteria.

PERFORMANCE CHARACTERISTICS

a. Standardisation

The test is standardised with a purified serum preparation containing IgG antibodies directed at each of the 8 antigens. It constitutes the stock material for both controls of the test. The proportion of the antibodies was adjusted in such a manner that the controls generate an approximately uniform signal on all 8 solid phases (lines of the microtitre plate). The stock preparation is calibrated against a set of gradually positive sera, solely reserved for this purpose. The degree of sample reactivity is expressed as ratio, as outlined above, separately for all 8 antigens.

b. Specificity

The test permits the specific and differentiated determination of human IgG antibodies, directed at 8 autoantigens. It has been validated (among other criteria) using human reference sera from the Center of Disease Control (CDC; Atlanta, USA) which are generally available.

The following results (ratio values) are typical (n = 3).

Serum	1	2	3	4	5	6	7	8	9	10
CDC- result	dsDNA	SS-B/La	--	U1-RNP	Sm	--	SS-A/Ro	--	Scl-70	Jo-1
Immun-Fluorescence	homogen /rim	speckled	speckled	--	--	nucleolar	--	centro-mere	--	--
dsDNA	4.5	0.2	0.2	0.2	0.4	0.2	0.5	0.2	0.4	0.2
RNP	0.8	0.2	6.0	5.0	5.6	0.6	0.3	0.2	0.3	0.2
Sm	1.5	0.1	1.6	0.1	4.6	0.2	0.2	0.1	0.1	0.1
SS-A/Ro	0.4	3.0	4.2	0.3	1.0	0.2	6.1	0.2	0.6	0.2
SS-B/La	0.2	5.8	4.2	0.1	0.2	0.4	0.2	0.1	0.3	0.1
Scl-70	0.3	0.1	0.1	0.1	0.4	0.5	0.1	0.1	4.1	0.1
CENP-B	0.2	0.2	0.2	0.2	0.3	0.2	0.2	5.0	0.3	0.2
Jo-1	0.2	0.1	0.2	0.1	0.2	0.2	0.2	0.1	0.2	5.4

c. Detection limit (analytical sensitivity)

The detection limit is defined as that concentration of analyte that corresponds to the mean absorbance of sample buffer plus 3-fold standard deviation (s). It was determined as < 0.2 (ratio; n = 12); this applies for all 8 parameters. Recommended measuring range: 0.2 < ratio < 7 (for all 8 parameters).

d. Frequency distribution of the autoantibodies (IgG)

In a sera collective of 79 blood donors, equally distributed by sex and age, the following distribution of the analyte was determined (ratio values):

Parameter	Mean	Mean + 2s	Median	95%-Percentile
dsDNA	0.32	0.55	0.29	0.51
RNP	0.37	0.87	0.30	0.74
Sm	0.13	0.16	0.12	0.16
SS-A/Ro	0.24	0.33	0.23	0.33
SS-B/La	0.19	0.36	0.16	0.30
Scl-70	0.23	0.59	0.17	0.47
CENP-B	0.23	0.34	0.21	0.36
Jo-1	0.17	0.24	0.16	0.23

e. Precision:

For the assessment of the test precision, the variability of results under the following conditions was determined:

1. within 1 assay and between 3 assays,
2. between 3 operators and
3. between 2 kit lots.

Ratio and coefficient of variability (cv) values are given as mean of all 8 antigens.

i. Intra- and inter-assay variability

n = 3 and 9, respectively

Sample	Ratio	Variability (CV %)	
		intra-assay	inter-assay
1	1.1	1.3	2.2
2	1.7	1.2	2.1
3	2.5	1.0	1.5

ii. Operator to operator variability

n = 2

Sample	Ratio	Variability (CV %)
1	1.1	2.2
2	1.8	1.7
3	2.7	1.6

iii. Variability between 2 kit lots

(n = 2)



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Sample	Ratio	Variability (CV %)
1	1.1	4.6
2	1.8	2.8
3	2.8	5.2

WARRANTY

DRG guarantees that the product delivered has been thoroughly tested to ensure that its properties specified herein are fulfilled. No further warranties are given.

The performance data presented here were obtained using the procedure indicated. Any modification in the procedure may affect the results in which case DRG disclaims all warranties whether expressed, implied or statutory. Moreover, DRG accepts no liability for any damage, whether direct, indirect or consequential, which results from inappropriate use or storage of the product.

SUMMARY FLOW CHART

- a. Dilute the sample buffer 10x-concentrate (20,0 mL, yellow) with water and mix.
- b. Dilute the sera 1/100 in sample buffer and mix.
- c. Dilute the wash buffer 20x-concentrate (100 mL, blue) with water and mix.
- d. Wash the wells once with 350 µL wash buffer each.
Dispense 8 * 100 µL of the controls (3,0 mL, ready-to-use, yellow) and of the diluted samples into the wells of 1 column each.
Incubate for 30 minutes at room temperature (22 ± 4°C).
- e. Wash the wells 4 times with 350 µL wash buffer each.
- f. Dispense 100 µL of the conjugate (14 mL, ready-to-use, green) into the wells.
Incubate as in step d.
- g. Repeat washing step e.
- h. Dispense 100 µL of the substrate solution (14 mL, ready-to-use, black vial) per well.
Incubate as in step d.
Then, add 100 µL stop solution (14 mL, ready-to-use, colourless) per well and agitate the plate briefly.
- i. Immediately measure the absorbance at 450 nm.
- j. **Evaluation:**
Determine the borderline absorbance by multiplying the absorbance of the positive control with the factor quoted in the certificate of analysis. Then, calculate the ratio of the samples by dividing their absorbance by the borderline absorbance.