E L I S A

for the determination
of autoantibodies against

SS-A / Ro

Directions for use

REF 1211FE00.FWD ∑ 12 x 8 determinations

IVD C E

2–8°C

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Contents

The product described here complies with the requirements of the IVD Directive 98/79/EG.

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1. Introduction and background

Sjögren's syndrome (SS) is a chronic inflammatory, autoimmune disorder of the exocrine glands. Especially the lacrimal and salivary glands are affected: lymphocytic infiltration causes swelling and progressive loss of function, often accompanied by development of the sicca syndrome (1). Systemic lupus erythematosus (SLE) is another autoimmune-mediated, chronic inflammatory illness, with variable clinical presentation, ranging from localised skin lesions to a destructive systemic disorder without cutaneous changes (2).

Autoantibodies directed against the subcellular ribonucleoprotein (RNP) particles SS-A / Ro (soluble substance A / patient Robair-antigen) and SS-B / La (soluble substance B / patient Lane-antigen) are regarded as specific serological marker for SS (prevalence about 65 and up to 90 %, respectively) (3); as well as for SLE (prevalence 5 - 50 %), in particular and with higher prevalence for some of its variants, e.g. subacute cutaneous LE, neonatal LE, ANA-negative SLE (4, 5).

The SS-A / Ro antigen consists of two distinct proteins, 52 and 60 kDa, complexed with one small, uridine-rich, specific RNA chain, either hY1, hY2, hY3 or hY5 (6). The physiological role of this complex is only in part resolved (7), as is the question of its primary location, nucleus or cytoplasma. The majority of SS-A / Ro-positive sera contains antibodies directed at both Ro60 and Ro52; nevertheless, the Ro60 protein is regarded as the most significant autoantigen (4, 8). Ro60 positive and simultaneously Ro52 negative sera mostly indicate SLE (9).

The present ELISA is intended for the quantitative or qualitative determination of IgG antibodies directed against Ro60 (isolated from bovine thymus) in human serum or plasma (cf. section 7). Ro 52 is excluded from the immobilised antigen preparation since some Jo-1 positive sera (primarily indicative for myositis) also cause positive signals on this substrate (10, 11). The test is fast (incubation time 30 / 30 / 30 minutes) and flexible (divisible solid phase, ready-to-use reagents). Six calibrators allow quantitative measurements; a negative and a positive control check the assay performance.

2. Warnings and precautions

The test kit is intended for in vitro diagnostic use only; not for internal or external use in humans or animals. It must be executed by trained personnel staff.

Do not use reagents beyond their expiration dates. Adherence to the protocol is strongly recommended.

The sample buffer, calibrators and controls contain Na-azide as antimicrobial agent. The wash buffer contains bromonitrodioxane and the conjugate methylisothiazolone / bromonitrodioxane as preservative. The substrate contains 3, 3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2). The stop solution, 0,2 M sulfuric acid (H_2SO_4), 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 or mucous membrane, wash thoroughly with water. Never pipette by mouth. Dispose in a manner complying with local/national regulations.

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 calibrators and controls contain components of human origin. They were tested for human immunodeficiency virus (HIV)-Ag, hepatitis B surface (HBs)-Ag and antibodies against HIV 1/2 and hepatitis C virus (HCV) and showed negative results; either in an FDA-approved or a CE-compliant test, according to European Directive 98/79/EC.

However, no test can guarantee that material of human origin is not actually infectious. The preparations should therefore be treated as potentially infectious and disposed of accordingly, as should the samples (and residues thereof); according to CDC (Center of Diseae Control, Atlanta, USA) or other local / national guidelines on laboratory safety and decontamination.

3. Principle of the test

The wells of the solid phase are coated with SS-A / Ro antigen, as described above. On this surface, the following immunological reactions take place:

1st reaction: SS-A / Ro-specific antibodies present in the sample bind to the immobilised antigen, forming the antigen-antibody complex. Then, non-bound sample components are washed away from the solid phase.

2nd reaction: A second antibody, directed at human IgG antibodies and conjugated with horse-radish peroxidase (HRP), is added. This conjugate binds to the complex. Then, excess conjugate is washed away from the solid phase.

3rd reaction: The enzyme-labelled complex converts a colourless substrate into a blue product. The degree of colour development reflects the concentration of SS-A / Ro IgG in the sample.

4. Contents of the kit

a. 1 microwell plate, coated with the SS-A / Ro antigen described above and hermetically packed in a foil laminate pouch together with a desiccant bag. The plate consists of 12 strips, each of which can be broken into 8 individual wells.

MWP	12x8

b. Sample buffer, 100 mL, ready-to-use, orange coloured. Contains Tris-buffered saline (TBS), bovine serum albumin (BSA), Tween and Na-azide.

BUF	SPL

c. Wash buffer, 100 mL, 10x-concentrate, blue coloured. Contains TBS, Tween and bromonitrodioxane.

BUF	WASH	10x

d. 6 calibrators, 2,0 mL each, 0 - 0,70 – 2,0 - 7,0 - 20 and 70 U SS-A / Ro IgG / mL, ready-to-use, gradually blue coloured. Contain TBS, BSA, Tween and Na-azide.

CAL	1-6

e. Negative and positive control, 2,0 mL each, ready-to-use, green and red coloured, respectively. Contain TBS, BSA, Tween and Na-azide.

CONTROL	–		CONTROL	+

f. Anti-human IgG HRP conjugate, 14 mL, ready-to-use, red coloured. Buffered solution containing stabilising protein, methylisothiazolone and bromonitrodioxane.

CONJ	IgG

g. Substrate solution, 14 mL, ready-to-use, colourless. Contains a buffered solution of TMB and H_2O_2. Contained in a vial impermeable to light.

SUBS	TMB

h. Stop solution (0,2 M H_2SO_4), 14 mL, colourless, ready-to-use. Caution: sulfuric acid is corrosive.

SOLN	STOP

i.   Directions for use

j.   Lot-specific certificate of analysis

5. Materials required but not supplied

a. Deionised or distilled water

b. Graduated cylinder, 1000 mL

c. Tubes for sample dilution (transfer tubes in the microwell plate format recommended)

d. Pipettes for 10, 100 and 1000 µL (1- and 8-channel pipettes recommended)

e. Microwell plate washer (optional)

f. Microwell plate photometer fitted with a 450 nm filter

g. ELISA evaluation program (recommended)

6. Storage of the kit

Store 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.

7. Reagent and sample preparation / specimen requirements

Do not exchange or pool corresponding components from different kits, due to possibly different shipping or storage conditions. If the kit is to be used for several tests, only the currently needed amount of reagents should be withdrawn. It is **crucially important** that no cross-contamination between the reagents occurs. Use only clean pipettes and do **not pour back** residues into the original flasks.

a. The solid phase must reach room temperature before opening the pouch. Remove the supernumerary microwells from the frame and immediately put them back into the pouch, together with the desiccant bag. Reseal the pouch hermetically and keep it refrigerated for future use.

b. Dilute the wash buffer 10x-concentrate (100 mL, blue) with 900 mL deionised water. Mix thoroughly. The diluted buffer is stable for several weeks if stored refrigerated (2 - 8°C).

c. Preparation of the samples: handle patient specimens as potentially infectious agents. Besides serum, EDTA-, citrate- or heparin-treated plasma is suitable sample material as well.

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

   Prepare samples using normal laboratory techniques. Turbid samples must first be clarified (centrifuged). The clarified or clear samples are mixed and then diluted 1/100, e.g. 10 µL serum or plasma + 990 µL sample buffer. Also mix the dilution.

   For rapid dispensing during the assay procedure, preparation of the calibrators, controls and samples in microwell transfer tubes is recommended. This allows the operation of an 8-channel 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 temperatures are recommended. Repeated freezing and thawing of samples should be avoided. Thawed samples must be mixed prior to diluting.

8. Assay procedure

8.1. Manual operation
Before starting the assay, all components of the kit must have reached room temperature (23 ± 3°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.

a. Immediately prior to use, wash the solid phase once: fill wells with 350 µL wash buffer each, let soak for about 10 seconds in the wells and remove.

b. Dispense the calibrators (2,0 mL each, ready-to-use, gradually blue), controls (2,0 mL each, ready-to-use, green and red) and the diluted samples rapidly into the microwells; 100 µL per well. Duplicate measurements are recommended.

   Incubate the plate for 30 minutes at room temperature (23 ± 3°C).

c. Wash the wells 4 times as in step a.

d. Rapidly (preferably using an 8-channel pipette) dispense the conjugate (14 mL, ready-to-use, red); 100 µL per well. Incubate the plate as in step b.

e. Repeat wash step c.

f. Rapidly (preferably using an 8-channel pipette) dispense the substrate solution (14 mL, ready-to-use, colourless, 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 an 8-channel pipette) dispense the stop solution (14 mL, ready-to-use, colourless. Caution: corrosive!); 100 µL per well. Use the same sequence as for the substrate. The colour changes from blue to yellow. Agitate the plate, preferably on an orbital shaker, for about 10 seconds.

h. Immediately read the absorbance in the microwell plate photometer at 450 nm.

Refrigerate the remainder of the reagents (2 - 8°C) if they are to be used again.

8.2. Dynex DS2 automated ELISA system
This product has been validated for use with the Dynex DS2 automated ELISA system. A description of the program flow for the assay execution and evaluation can be provided as a pdf file. The parameters of this program are merely a proposal and may need to be adapted by the operator to the requirements of the actual assay. In general terms, we have attempted to stick as close as possible to the protocol of manual operation, as above. However, due to the necessarily elevated temperature within the DS2, the substrate incubation period had to be shortened.

Section 11.8. gives a performance comparison between manual assay operation and the DS2 ELISA system.

9. Evaluation and quality control

Quantitative evaluation: the data obtained are quantitatively evaluated with the standard curve, as shown below. However, the depicted curve can only serve as a model. It can not substitute the measurement of the calibrators, together with the controls and actual samples. The curve has been constructed with a conventional ELISA evaluation program, using a 4-parameter function. The Spline approximation is also appropriate.

Dynex DS2 --x----x--
manual op. --o----o--

mOD 450nm
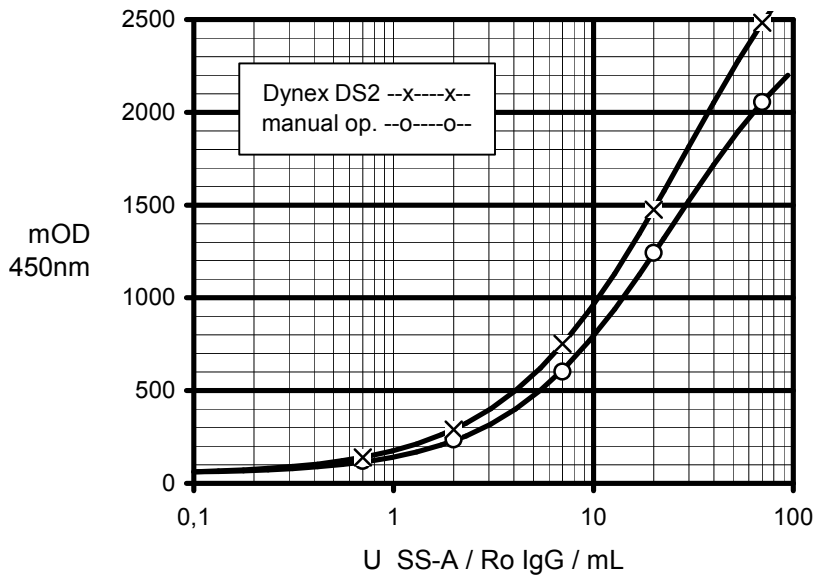
U SS-A / Ro IgG / mL

1211FE00.FED/StdKurveV0301K

If no computer-supported evaluation is possible, the standard curve may be drawn by hand. It allows transformation of the absorbance value of a sample into its concentration, i.e. into U SS-A / Ro IgG per mL sample.

Qualitative evaluation: the test may also be evaluated in a qualitative manner. This requires measurement of the positive control only. Nevertheless, measurement and examination of the negative control is recommended (see below: quality control).

In qualitative test evaluation, the absorbance of the samples is compared with the borderline absorbance (= cut-off). It is determined according to the following formula:

$$\text{absorbance}_{\text{borderline}} = \text{absorbance}_{\text{positive control}} \times \text{factor}$$

The factor depends on the kit lot and is quoted in the lot-specific certificate of analysis which is included with each test kit. Example:

$$\text{absorbance}_{\text{positive control}} = 1250 \text{ mOD}$$
$$\text{factor} = 0,35$$
$$\text{absorbance}_{\text{borderline}} = 1250 \text{ mOD} \times 0,35 = 438 \text{ mOD}$$

In order to gain an impression of how positive a particular sample is for SS-A / Ro-Ab IgG, one may calculate the ratio, according to the formula:

$$\text{ratio} = \text{absorbance}_{\text{sample}} / \text{absorbance}_{\text{borderline}}$$

Example:

absorbance_{borderline}	= 438 mOD
absorbance_{sample}	= 1480 mOD
ratio	= 1480 mOD / 438 mOD = 3,4

absorbance$_{borderline}$ = 438 mOD
absorbance$_{sample}$ = 1480 mOD
ratio = 1480 mOD / 438 mOD = 3,4

Quality control: the positive and negative control check the assay performance. Their authorised values and acceptable ranges, respectively, are quoted in the lot-specific certificate of analysis. Values of the controls must within the indicated ranges; otherwise, the results of the assay are invalidated.

10. Interpretation of results / limitations of the procedure

Based on the measurement of a blood donor and a positive collective of sera (see below), we suggest for the assessment of patient sera:

	quantitative evaluation U SS-A / Ro IgG / mL sample	qualitative evaluation ratio
normal (negative) range	< 3,2	< 0,84
cut-off	4,0	1,00
equivocal range	3,2 - 5,0	0,84 - 1,19
positive range	> 5,0	> 1,19

These specifications 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 SS-A / Ro 60 kDa protein. Hence, SS is not very likely. Due to the lower prevalence of SS-A / Ro-Ab for SLE (< 50 %), this disease cannot be ruled out with similar confidence. If SLE is suspected, presence of e.g. dsDNA- or other antinuclear autoantibodies (ANA) should be examined. It should be noted that in SLE patients the titer of IgG autoantibodies may decrease in response to B cell depleting therapy (12).

A positive result should be interpreted primarily as an indication of SS or SLE, even though SS-A / Ro-Ab can occur with other autoimmune disorders as well (e.g. vasculitis, other collagenopathies). However, the test should be positive on at least two occasions, separated by several weeks. For confirmation and discrimination, further ANA should be determined.

Specimens exhibiting results within the borderline range quoted above should be considered as equivocal and reported as such. It is recommended that a second sample be collected two weeks 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.

11. Performance characteristics

11.1. Standardisation
The test is standardised with a purified serum preparation containing IgG antibodies specifically directed at the SS-A / Ro 60 kDa antigen. This preparation is calibrated against a set of gradually positive sera, solely reserved for this purpose. The degree of sample reactivity is measured in arbitrary units (U/mL) since no international standard is available.

11.2. Analytical specificity
The test allows the specific determination of human IgG antibodies directed against SS-A / Ro. It has been validated (among other parameters) by means of the commercially available human reference sera of the "Center of Disease Control" (CDC, Atlanta, USA). The following results are typical:

| serum	1	2	3	4	5	6	7	8	9	10
CDC-result	ds-DNA	SS-B /La	--	U1-RNP	Sm	--	SS-A /Ro	--	Scl-70	Jo-1
immune-fluorescence	homo-gen / rim	speck-led	speck-led	--	--	nuc-leolar	--	centro-mere	--	--
ELISA (U/mL)	1,1	19	37	1,1	5,0	0,6	>70	0,5	2,6	0,5

11.3. 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 U SS-A / Ro IgG per mL sample (n = 24). Recommended measuring range: 0,5 - 50 U / mL

11.4. Homogeneity of the solid phase
Measurement of the solid phase homogeneity is a regular QC part of each production lot. This is determined by 288-fold measurement of a positive but non-saturating sample on 3 selected plates. Acceptance criterion: mOD-coefficient of variation (cv) over the plates < 8%. The figure below shows a representative excerpt (solid phase lot no. 3004S) of such an analysis.

plate row	1 1	n/2 1	n 1	1 2	n/2 2	n 2	1 3	n/2 3	n 3	1 4	n/2 4	n 4	mean	cv %
line a	1382	1301	1345	1347	1288	1297	1326	1302	1291	1282	1297	1254	1309	2,7
line b	1389	1309	1331	1350	1313	1336	1380	1325	1323	1347	1322	1395	1343	2,2
line c	1362	1307	1314	1359	1306	1312	1342	1305	1302	1319	1313	1322	1322	1,6
line d	1358	1309	1328	1371	1317	1317	1363	1333	1280	1322	1305	1308	1326	2,0
line e	1399	1353	1335	1357	1336	1332	1355	1336	1316	1342	1338	1337	1345	1,5
line f	1391	1314	1353	1365	1322	1338	1365	1351	1340	1357	1352	1336	1349	1,5
line g	1403	1342	1344	1378	1362	1344	1371	1347	1345	1368	1374	1340	1360	1,4
line h	1331	1293	1338	1329	1292	1284	1332	1265	1244	1253	1259	1257	1290	2,7
mean	1377	1316	1336	1357	1317	1320	1354	1321	1305	1324	1320	1319	**1330**	
cv %	1,8	1,6	0,9	1,1	1,8	1,6	1,4	2,2	2,6	3,0	2,7	3,5		**2,5**
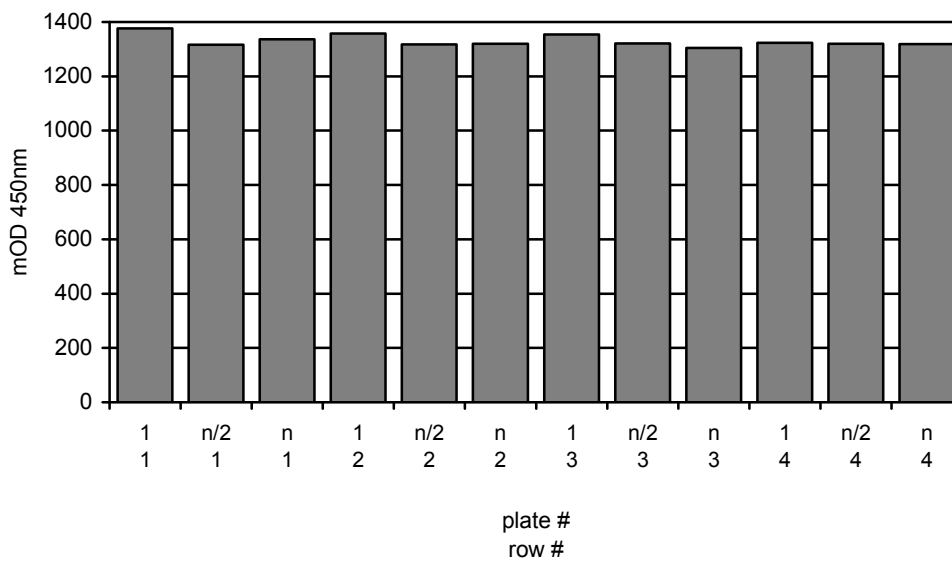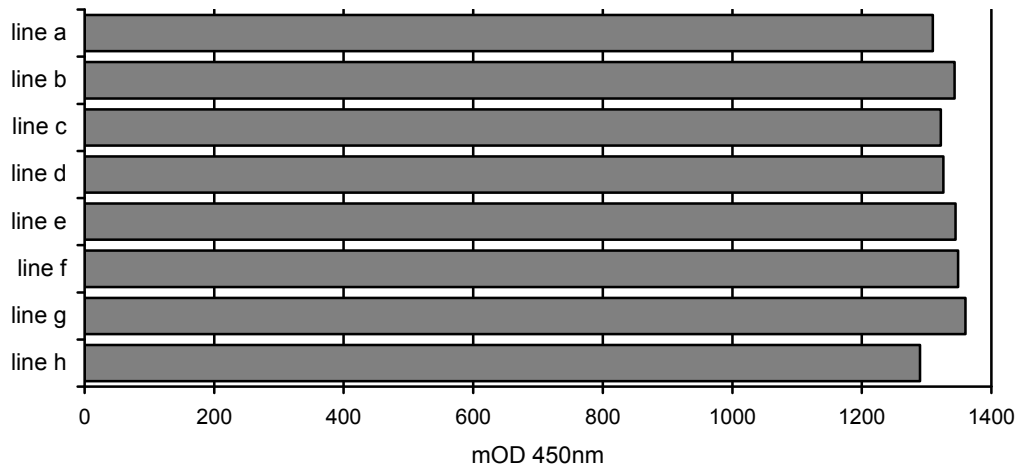
11.5. Linearity

In order to assess the dose-response relationship of the test, positive sera were measured in serial 2-fold dilution. Acceptance criterion: linear regression of 4 successive dilutions must yield a correlation factor > 0,98. A typical result is depicted below.
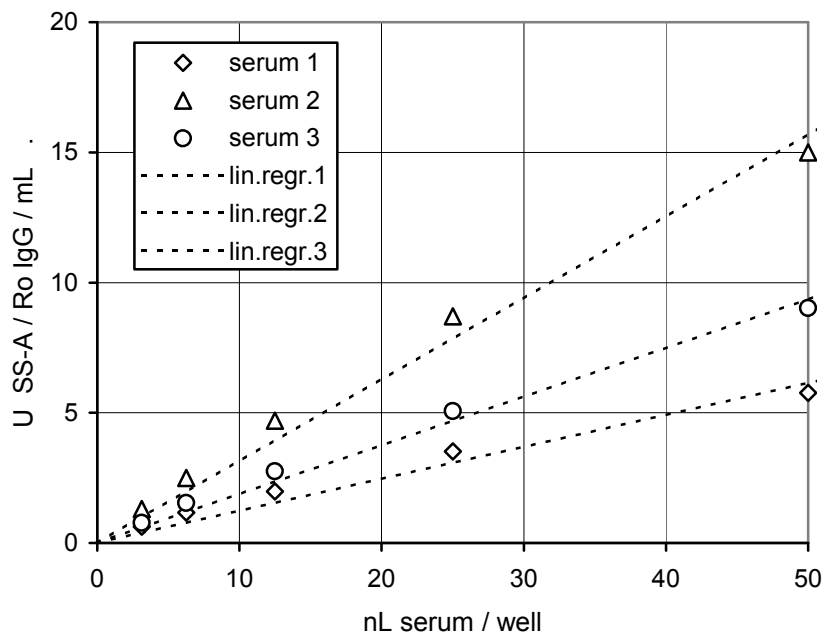
11.6. Precision

For the assessment of the test precision, the variability of results under the following conditions was determined: a. within 1 assay and between 3 assays, b. between 3 operators and c. between 2 kit lots.

a. Intra- and inter-assay variability (n = 24 and 72, respectively)

sample	mean U/mL	variability (cv, %) intra-assay	inter-assay
1	5,4	1,5	2,9
2	11	1,2	3,2
3	22	1,7	2,4

b. Operator to operator variability (n = 12)

sample	mean U/mL	variability (cv, %)
1	5,6	2,4
2	11	1,3
3	22	1,8

c. Variability between 2 kit lots (n = 6)

| sample	mean U/mL	variability (cv, %)
1	5,6	2,2
2	11	3,8
3	23	2,6

11.7. Frequency distribution of SS-A / Ro IgG

This was analysed in a sera collective of blood donors, equally distributed by sex and age, and a collective of sera found positive for SS-A / Ro autoantibodies according to a CE-compliant reference ELISA or that were clinically defined. The following distribution of the analyte was observed:

blood donor sera			positive sera		
n:	160		n:	17	
mean:	0,94	U/mL	mean:	550	U/mL
mean + s:	2,0	U/mL	mean - s:	51	U/mL
mean + 2s:	3,1	U/mL	mean - 2s:	< 0	U/mL
median:	0,82	U/mL	median:	300	U/mL
95th percentile:	1,4	U/mL	5th percentile:	29	U/mL

ROC-analysis of these data was used to determine the cut-off as 4,0 U SS-A / Ro IgG / mL (13). The data presented here suggest a diagnostic specificity and sensitivity of the ELISA of about 99 and 100 %, respectively. These values apply for the measured sera only; other collectives may yield different results. In view of the low number of positive sera, particular caution is required when interpreting test sensitivity.

blood donor sera

positive sera

1211FE00.FED/HäufgPlotV0301K

- 13 -
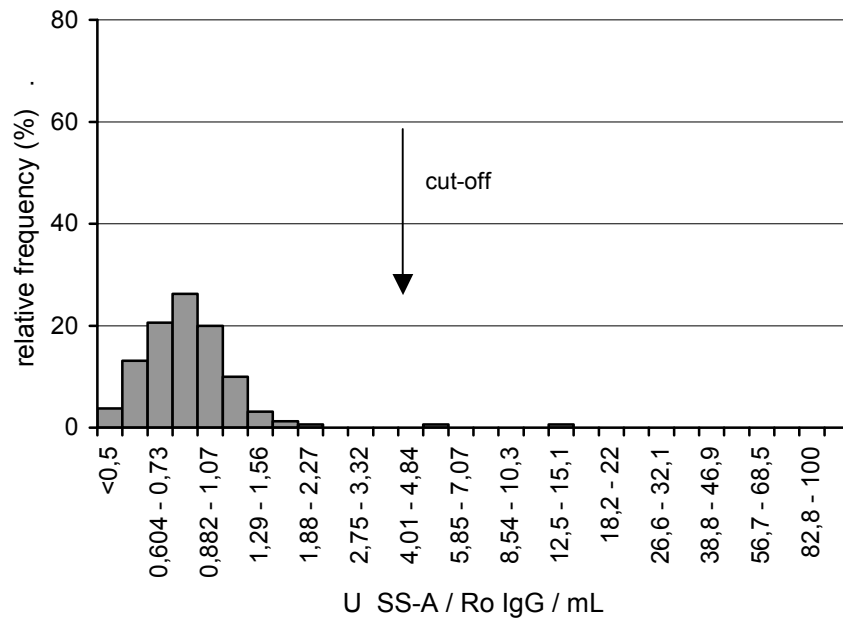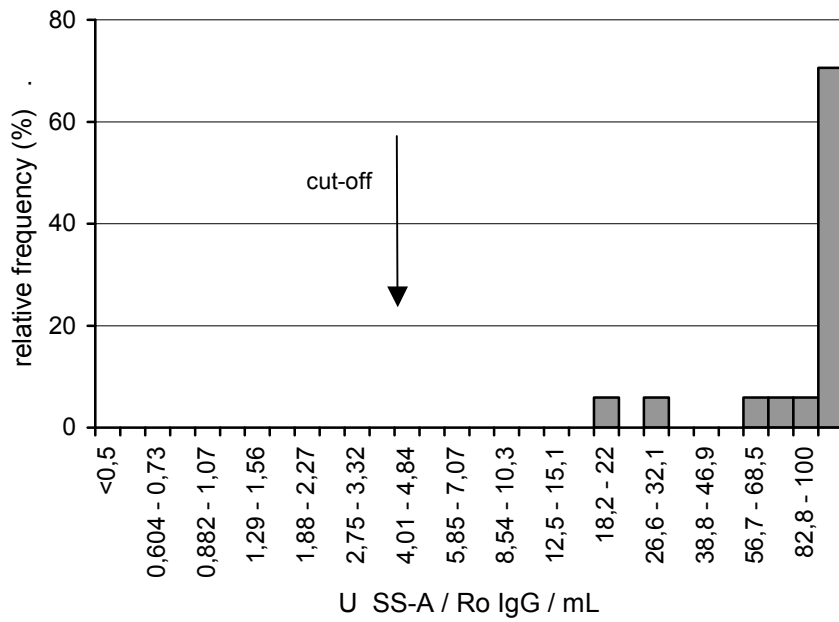
11.8. Manual operation vs. Dynex DS2 automated ELISA system

Variability: Using specimen of one and the same kit lot, the variability of assay results were compared between manual operation and the Dynex DS2 automated ELISA system:

	manual operation	Dynex DS2
intra-assay variability (n = 16)	mean cv = 2,6 %	mean cv = 2,6 %
inter-assay variability (n = 48)	mean cv = 2,6 %	mean cv = 3,5 %

Standard curve: depicted in section 9

Correlation:

y = 1,075 x
r = 0,997
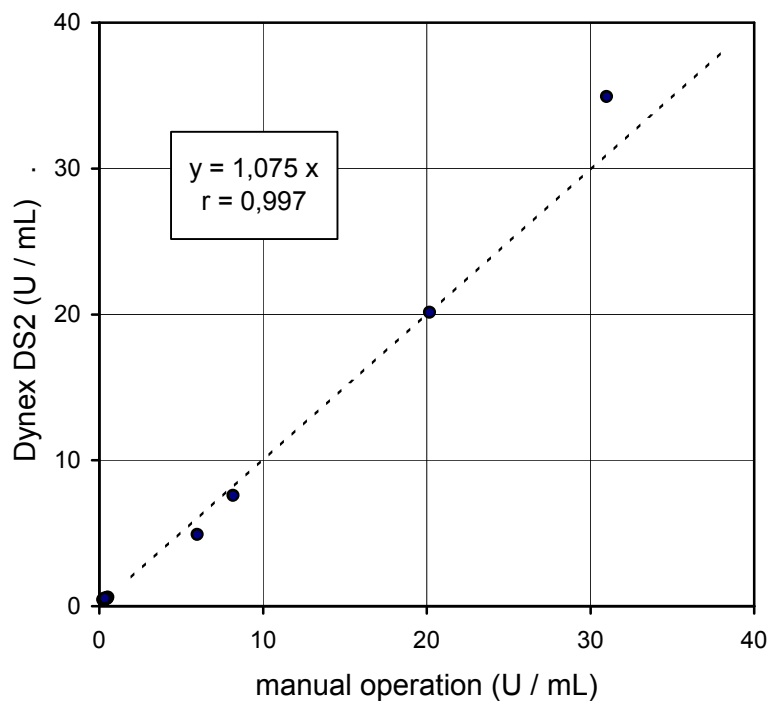
12. Warranty

Steffens biotechnische Analysen GmbH (SBA) 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 SBA disclaims all warranties whether expressed, implied or statutory. Moreover, SBA accepts no liability for any damage, whether direct, indirect or consequential, which results from inappropriate use or storage of the product.
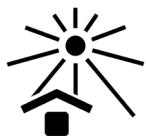
13. Symbols

| REF | Article code |

| LOT | Batch code |

| Σ | Contains x determinations |

| IVD | For *in vitro* diagnostic use |

| CE | Conformité Européenne |

| | Store shielded from sunlight |
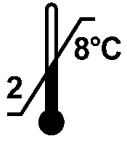
2 8°C (thermometer symbol)	Store at 2 - 8°C
(hourglass symbol)	Expiration date
i (book symbol)	Read "Directions for Use"
⚠ (warning triangle)	Warning
☣ (biohazard symbol)	Biological risk
(factory symbol)	Manufactured by

14. References

1. Franco, H. L., et al.: Autoantibodies directed against sicca syndrome antigens in the neonatal lupus syndrome. J Am Acad Dermatol 4 (1981), 67 - 72

2. Tan, E. M., et al.: The 1982 revised criteria for the classifiction of systemic lupus erythematosus. Arthritis Rheum 25 (1982), 1271 - 1277

3. Harley, J. B.: Autoantibodies in Sjögren's syndrome. J Autoimmun 2 (1989), 383 - 394

4. Messinger, M.: Autoantikörper bei systemischen entzündlich-rheumatischen Erkrankungen (Kollagenosen). In: L. Thomas (ed.): Labor und Diagnose (2005), TH-Books-Verlags-Gesellschaft, Frankfurt/Main, 1139 - 1161

5. Maddison, P. J., et al.: Serological findings in patients with "ANA-negative" systemic lupus erythematosus. Medicine 60 (1981), 87 - 94

6. Reichlin, M., Scofield, R. H.: SS-A (Ro) autoantibodies. In: Shoenfield, Y., et al. (eds.): Autoantibodies (2007), Elsevier Science, Amsterdam, 225 - 230

7. Chen, X., Wolin, S. L.: The Ro 60 kDa autoantigen: insights into cellular function and role in autoimmunity. J Mol Med 82 (2004), 232 - 239

8. Steiner, G.: Autoantikörperdiagnostik in der Rheumatologie. In: Thumb, N., et al. (eds.): Praktische Rheumatologie (2001), Springer-Verlag, Wien, 91 - 104

9. Ben Chetrit, E., et al.: Dissociation of immune responses to the SS-A (Ro) 52-kd and 60-kd polypeptides in systemic lupus erythematosus and Sjogren's syndrome. Arthritis Rheum. 33 (1990), 349 - 355

10. Rutjes, S. A., et al.: Anti-Ro52 antibodies frequently co-occur with anti-Jo-1 antibodies in sera from patients with idiopathic inflammatory myopathy. Clin Exp Immunol 109 (1997), 32 - 40

11. Frank, M. B., et al.: The association of anti-Ro52 autoantibodies with myositis and scleroderma autoantibodies. J Autoimmunity 12 (1999), 137 - 142

12. Persson, B., et al.: Disappearance and Reappearance of IgG, IgA and IgM Autoantibody Isotypes and Immune Complexes in Rituximab-Treated SLE Patients. Annals of the Rheumatic Diseases 72 (2013), A34

13. Sommer, R., Eitelberger, F.: Wertigkeit der Gliadin-Antikörper im Serum zur Diagnose der Zöliakie. Wien Klin Wochenschr 104/4 (1992), 86 - 92

15. Summary flow chart

a. Dilute the samples 1/100 in sample buffer (100 mL, ready-to-use, orange) and mix.

b. Dilute the wash buffer 10x-concentrate (100 mL, blue) with water and mix.

c. Wash the wells once with 350 µL wash buffer each. Dispense 100 µL of the calibrators (2,0 mL each, ready-to-use, gradually blue) and controls (2,0 mL each, ready-to-use, green and red) and of the diluted samples into the wells of the solid phase. Duplicate measurements are recommended. Incubate for 30 minutes at room temperature (23 ± 3°C).

d. Wash the wells 4 times with 350 µL wash buffer each.

e. Dispense 100 µL of the conjugate (14 mL, ready-to-use, red) into the wells. Incubate as in step c.

f. Repeat washing step d.

g. Dispense 100 µL of the substrate solution (14 mL, ready-to-use, black vial) per well. Incubate as in step c. Then, add 100 µL stop solution (14 mL, ready-to-use, colourless) per well and agitate the plate briefly.

h. Immediately measure the absorbance at 450 nm.

i. Quantitative evaluation: determine the standard curve and, using this curve, transform the absorbance of the samples into their respective antibody concentration (U/mL).

j. Qualitative evaluation: determine the borderline absorbance by multiplying the absorbance of the positive control with the factor shown in the certificate of analysis. Then, calculate the ratio of the samples by dividing their absorbance by the borderline absorbance.