

**AccuDiag™**  
**ENA Combined Screen**  
**(6 Antigen)**  
**ELISA Kit**

REF 2552-2



<b>Test</b>	<b>ENA Combined Screen ELISA</b>
<b>Method</b>	<b>Enzyme Linked Immunosorbent Assay</b>
<b>Principle</b>	<b>Sandwich Complex</b>
<b>Detection Range</b>	<b>Qualitative - Positive, Negative and Cut-off</b>
<b>Sample</b>	<b>10 µL serum</b>
<b>Total Time</b>	<b>~ 65 min.</b>
<b>Shelf Life</b>	<b>12 Months from the manufacturing date</b>
<b>Specificity</b>	<b>100%</b>
<b>Sensitivity</b>	<b>93%</b>

**INTENDED USE**

The Diagnostic Automation ENA Screen ELISA Test System is a qualitative screening assay designed to detect antibodies to extractable nuclear antigens (anti-ENA) in human sera. When performed according to the enclosed instructions, this test system is capable of detecting all anti-ENAs commonly tested for, such as those against Jo-1, Sm, Sm/RNP, SSA, SSB, and Scl-70. *This device is for in vitro diagnostic use.*

**SUMMARY AND EXPLANATION**

In recent years it has become clear that autoantibodies to a number of nuclear constituents have proven to be useful in the diagnosis of various connective tissue diseases. The Jo-1 autoantibody is one of a family of characteristic autoantibodies seen in myositis patients (19). They are all specifically found in patients with myositis, and are all associated with a high incidence of accompanying interstitial lung disease (10). Antibodies directed against the Sm marker are highly specific for patients with SLE and are considered a diagnostic criterion for SLE (1,2). The presence of high level RNP antibodies alone are considered diagnostic of mixed connective tissue disease (MCTD) and are usually associated with a more benign disease course (3), while patients with low levels of RNP antibodies, together with other autoantibodies, may be observed in the serum of patients with progressive systemic sclerosis, Sjögren's Syndrome, and rheumatoid arthritis. The presence of RNP antibodies in the serum of SLE patients is usually associated with a lower incidence of renal involvement and a more benign disease course. To the contrary, patients with Sm antibodies experience a higher frequency of renal and central nervous system complications (4). Autoantibodies directed against SSA and SSB may be observed in patients with SLE (5-6) and Sjögren's disease (7-9). SSA antibodies are frequently present in the serum of ANA negative SLE patients, such as subacute cutaneous lupus erythematosus (12), a lupus-like syndrome associated with a homozygous C2 deficiency (13), and in a subset of patients who lack anti-dsDNA antibodies (11). Scl-70 antibodies are highly specific for scleroderma (11). They are also observed in a minority of SLE patients. Scl-70 positive scleroderma patients tend to have a more severe disease course, more internal organ involvement

and diffuse rather than limited skin involvement (14). Scl-70 antibodies are rarely found in other autoimmune diseases, and thus, their detection in a patient with the recent onset of Raynaud's phenomenon is highly significant (15).

Until recently, autoantibodies were tested individually by indirect immunofluorescence. Ouchterlony gel diffusion, hemagglutination, radioimmunoassay, or enzyme-linked immunoassay, or enzyme-linked immunosorbent assay (ELISA). Unlike several other systems, the ELISA methodology offers sensitive, objective, and rapid evaluation of specimens, and therefore, is suitable for screening a large number of samples for antibodies to ENA.

Although the exact etiology of autoimmune diseases is unknown, and the specific role played by autoantibodies in the onset of various autoimmune connective tissue diseases is obscure, the association and frequency of detection of these antibodies, particularly those of the IgG class, by the DAI ENA Screen ELISA test system, offers an efficient test procedure for the laboratory workup of patients with suspected various connective tissue diseases.

The following table summarizes the various autoantibodies noted above with respect to disease association:

**Table 1 (16)**

<b>Antibody</b>	<b>Disease State</b>	<b>Relative Frequency of Antibody Detection %</b>
Anti-Jo-1	Myositis	25-44% (19)
Anti-Sm	SLE	30*
Anti-RNP	MCTD,SLE	100** and >40, respectively
Anti-SSA (Ro)	SLE, Sjögren's	15 and 30-40, respectively
Anti-SSB (La)	SLE, Sjögren's	15 and 60-70, respectively
Anti-Scl-70	Systemic sclerosis	20-28*
* Highly Specific		
** *Highly specific when present alone at high titer		

**TEST PRINCIPLE**

The Diagnostic Automation ENA Screen ELISA test system is designed to detect IgG class antibodies to a variety of common nuclear antigens in human sera. Wells of plastic microwell strips are sensitized by passive adsorption with ENA antigen.

The test procedure involves three incubation steps:

1. Test sera (properly diluted) are incubated in antigen coated microwells. Any antigen specific antibody in the sample will bind to the immobilized antigen. The plate is washed to remove unbound antibody and other serum components.
2. Peroxidase Conjugated goat anti-human IgG is added to the wells and the plate is incubated. The Conjugate will react with ENA antibody immobilized on the solid phase in step 1. The wells are washed to remove unreacted Conjugate.
3. The microwells containing immobilized peroxidase Conjugate are incubated with peroxidase Substrate Solution. Hydrolysis of the Substrate by peroxidase produces a color change. After a period of time the reaction is stopped and the color intensity of the solution is measured photometrically. The color intensity of the solution depends upon the antibody concentration in the original test sample.

**SPECIMEN COLLECTION AND PREPARATION**

1. It is recommended that specimen collection be carried out in accordance with CLSI document M29: Protection of Laboratory Workers from Infectious Disease (Current Edition).
2. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, all blood derivatives should be considered potentially infectious.
3. Only freshly drawn and properly refrigerated sera obtained by approved aseptic venipuncture procedures should be used in this assay (17, 18). No anticoagulants or preservatives should be added. Avoid



using hemolyzed, lipemic, or bacterially contaminated sera.

4. Store sample at room temperature for no longer than 8 hours. If testing is not performed within 8 hours, sera may be stored between 2 and 8°C for no longer than 48 hours. If delay in testing is anticipated, store test sera at – 20°C or lower. Avoid multiple freeze/thaw cycles that may cause loss of antibody activity and give erroneous results.

configurations. Return unused strips to the resealable pouch with desiccant, seal, and return to storage between 2 and 8°C.

## MATERIALS AND COMPONENTS

### Materials provided with the test kits

Each kit contains the following components in sufficient quantities to perform the number of tests indicated on packaging label. Note: The following reactive reagents contain sodium azide as a preservative at a concentration of <0.1% (w/v).

1. **Plate:** 96 wells configured in twelve, 1x 8-well, strips coated with inactivated antigen. The strips are packaged in a strip holder and sealed in an envelope with desiccant.
2. **Conjugate:** Conjugated (horseradish peroxidase) goat anti-human IgG (Fc chain specific). One, 15 mL, white- capped bottle. Ready to use.
3. **Positive Control (Human Serum):** One, 0.35 mL, red-cap.
4. **Calibrator (Human Serum):** One, 0.5mL, blue-cap.
5. **Negative Control (Human Serum):** One, 0.35mL, green-cap.
6. **Sample Diluent:** One, 30mL, green-cap, bottle containing Tween-20, bovine serum albumin and phosphate-buffered-saline, (pH 7.2 ± 0.2). Ready to use. Note: Shake well before use. The Sample Diluent will change color when combined with serum.
7. **TMB:** One, 15 mL, amber-capped, amber bottle containing 3, 3', 5, 5' – tetramethylbenzidine (TMB). Ready to use.
8. **Stop Solution:** One, 15 mL, red-capped, bottle containing 1M H<sub>2</sub>SO<sub>4</sub> 0.7M HCl. Ready to use.
9. **Wash Buffer Concentrate (10X):** Dilute 1 part concentrate + 9 parts deionized or distilled water. One, 100mL, clear-capped, bottle containing a 10X concentrated phosphate-buffered-saline and Tween-20 solution (Blue solution). Note: 1X solution will have a pH of 7.2 ± 0.2.

The following components are not kit lot number dependent and may be used interchangeably with the ELISA assays: TMB, Stop Solution, and Wash Buffer.

### Note: Kit also contains

1. Component list containing lot specific information is inside the kit box.
2. Package insert providing instructions for use.

### Materials required but not provided

1. ELISA microwell reader capable of reading at a wavelength of 450nm.
2. Pipettes capable of accurately delivering 10 to 200µL.
3. Multichannel pipette capable of accurately delivering (50-200µL).
4. Reagent reservoirs for multichannel pipettes.
5. Wash bottle or microwell washing system.
6. Distilled or deionized water.
7. One liter graduated cylinder.
8. Serological pipettes.
9. Disposable pipette tips. Paper towels.
10. Laboratory timer to monitor incubation steps.
11. Disposal basin and disinfectant. (example: 10% household bleach - 0.5% sodium hypochlorite).

## ASSAY PROCEDURE

1. Remove the individual components from storage and allow them to warm to room temperature (20-25°C).
2. Determine the number of microwells needed. Allow six Control/Calibrator determinations (one Blank, one Negative Control, three Calibrators and one Positive Control) per run. A Reagent Blank should be run on each assay. Check software and reader requirements for the correct Controls/Calibrator

EXAMPLE PLATE SET-UP		
	1	2
A	Blank	Patient 3
B	Neg. Control	Patient 4
C	Calibrator	Etc.
D	Calibrator	
E	Calibrator	
F	Pos. Control	
G	Patient 1	
H	Patient 2	

3. Prepare a 1:21 dilution (e.g.: 10µL of serum + 200µL of Sample Diluent) of the Negative Control, Calibrator, Positive Control, and each patient serum. The Sample Diluent will undergo a color change confirming that the specimen has been combined with the diluent.
4. To individual wells, add 100µL of each diluted control, calibrator and sample. Ensure that the samples are properly mixed. Use a different pipette tip for each sample.
5. Add 100µL of Sample Diluent to well A1 as a reagent blank. Check software and reader requirements for the correct reagent blank well configuration.
6. Incubate the plate at room temperature (20-25°C) for 25± 5 minutes.
7. Wash the microwell strips 5X.

### A. Manual Wash Procedure:

- a. Vigorously shake out the liquid from the wells.
- b. Fill each microwell with Wash Buffer. Make sure no air bubbles are trapped in the wells.
- c. Repeat steps a. and b. for a total of 5 washes.
- d. Shake out the wash solution from all the wells. Invert the plate over a paper towel and tap firmly to remove any residual wash solution from the wells. Visually inspect the plate to ensure that no residual wash solution remains. Collect wash solution in a disposable basin and treat with 0.5% sodium hypochlorite (bleach) at the end of the days run.

### B. Automated Wash Procedure:

If using an automated microwell wash system, set the dispensing volume to 300-350µL/well. Set the wash cycle for 5 washes with no delay between washes. If necessary, the microwell plate may be removed from the washer, inverted over a paper towel and tapped firmly to remove any residual wash solution from the microwells.

8. Add 100µL of the Conjugate to each well, including reagent blank well, at the same rate and in the same order as the specimens were added.
9. Incubate the plate at room temperature (20-25°C) for 25 ± 5 minutes
10. Wash the microwells by following the procedure as described in step 7.
11. Add 100µL of TMB to each well, including reagent blank well, at the same rate and in the same order as the specimens were added.
12. Incubate the plate at room temperature (20-25°C) for 10-15 minutes.
13. Stop the reaction by adding 50µL of Stop Solution to each well, including reagent blank well, at the same rate and in the same order as the TMB was added. Positive samples will turn from blue to yellow. After adding the Stop Solution, tap the plate several times to ensure that the samples are thoroughly mixed.
14. Set the microwell reader to read at a wavelength of 450nm and measure the optical density (OD) of each well against the reagent blank. The plate should be read within 30 minutes after the addition of the Stop Solution.

## RESULTS

The Calibrator within this Test System has been assigned both a Correction Factor for the generation of Index Values and a Calibrator Value for the generation of Unit Values. Based upon testing of normal and disease-state specimens, a maximum normal Unit Value has been determined by the manufacturer and correlated to the Calibrator.

### A. Calculations:

#### 1. Correction Factor

The manufacturer determined a Cutoff OD Value for positive samples and correlated it to the Calibrator. The Correction Factor (CF) allows for the determination of the Cutoff Value for positive samples. It will also correct for slight day-to-day variations in test results. The Correction Factor is determined for each lot of components and is printed on the Component Label located in the Test System box.

#### 2. Cutoff OD Value

To obtain the cutoff OD value, multiply the CF by the mean OD of the Calibrator determined above.

$$(CF \times \text{mean OD of Calibrator} = \text{cutoff OD value})$$

#### 3. Index Values or OD Ratios

Calculate the Index Value or OD Ratio for each specimen by dividing its OD value by the cutoff OD from step 2.

<b>Mean OD of Calibrator</b>	=	0.793
<b>Correction Factor (CF)</b>	=	0.25
<b>Cut off OD</b>	=	0.793 x 0.25 = 0.198
<b>Unknown Specimen OD</b>	=	0.432
<b>Specimen Index Value or OD Ratio</b>	=	0.432 / 0.198 = 2.18

### B. Interpretation:

Index Values or OD ratios are interpreted as follows:

	Index Value or OD Ratio
Negative Specimens	≤ 0.90
Equivocal Specimens	0.91 to 1.09
Positive Specimens	≥ 1.10

- An OD ratio of less than or equal to 0.90 is interpreted as negative for anti-ENA IgG antibodies.
- An OD ratio greater than or equal to 1.10 is interpreted as positive for anti-ENA IgG antibodies.
- Retest specimens with OD ratio values in the equivocal range (0.91 – 1.09) in duplicate. Report any two of the three results which agree. Test repeatedly equivocal specimens using an alternate serologic procedure and/or re-evaluate by drawing another sample one to three weeks later.

## PERFORMANCE CHARACTERISTICS

### Comparative Study

In a clinical investigation conducted by Diagnostic Automation, Inc. 176 serum specimens were tested using DAI ENA Screen ELISA test system and various commercial ELISA test system. Specificity was evaluated using 61 asymptomatic normal specimens from southeastern United States, and sensitivity was evaluated using 115 disease-state sera from northeastern United States. The results of the study are summarized in Table 1 through 4 below:

Commercial ELISA Test kit	Diagnostic Automation ENA Screen ELISA				Total
	+	-	Equivocal <sup>a</sup>		
+	0	20 <sup>b</sup>	0		20
-	0 <sup>c</sup>	41	0		41
Equivocal <sup>a</sup>	0	0	0		0
<b>Total</b>	<b>0</b>	<b>61</b>	<b>0</b>		<b>61</b>

<sup>a</sup>Equivocal samples were excluded from calculations.

<sup>b</sup>All twenty specimens were confirmed ANA negative.

<sup>c</sup>Represents discrepant specimens.

Relative Specificity = 41/41 = 100 %

Commercial ELISA Test kit	Diagnostic Automation ENA Screen ELISA				Total
	+	-	Equivocal <sup>a</sup>		
+	77	6 <sup>b</sup>	0		83
-	3	29	0		32
Equivocal <sup>a</sup>	0	0	0		0
<b>Total</b>	<b>80</b>	<b>35</b>	<b>0</b>		<b>115</b>

<sup>a</sup>Equivocal samples were excluded from calculations.

<sup>b</sup>Represents Discrepant Specimens (See Table 3).

Relative Sensitivity = 77/83 = 93%

Sample #	DAI ELISA Results	Other ELISA Results	Resolution *
78	0.444/-	RNP +	Confirmed RNP Neg.
83	0.562/-	Jo-1 +	Unresolved
85	0.569/-	SSA +	Confirmed SSA Neg.
87	0.597/-	SSA +	Confirmed SSA Neg.
89	0.687/-	RNP +	Confirmed RNP Neg.
95	0.777/-	Sm,RNP,SSA +	Confirmed ENA Neg.

\*Confirmed using one or all of the following test systems:

- Diagnostic Automation, Inc. Hep-2 IFA Test System.
- Diagnostic Automation, Inc. Poly-ENA Gel Immunodiffusion Test System.
- Diagnostic Automation, Inc. Autoantibody ENA Profile-6 ELISA Test System.

### 2. Reproducibility

Reproducibility was evaluated as outlined in document number EP5-T2; Evaluation of Precision Performance of Clinical Chemistry Devices- Second Edition, as published by National Committee for Clinical Chemistry Laboratory Standards (NCCLS).

Briefly, six specimens were tested; two strong positive samples, two moderately positive specimens, and two negative specimens. Each sample was tested in duplicate, two times per day (AM and PM), on each day. The results have been summarized in Table 4 below:



**Table 4. Summary of Reproducibility testing**

I D	Mean Ratio	Swr <sup>a</sup>	St <sup>c</sup>	Days Tested	Percent CV	Total Observations
1	12.16	0.58	1.12	20	9.17	80
2	9.72	0.78	1.05	20	10.80	80
3	4.48	0.54	0.56	20	12.44	80
4	4.21	0.53	0.73	19	17.39	76
5	0.41	0.07	0.15	19	N/A	76
6	0.20	0.08	0.10	18	N/A	72

<sup>a</sup>Point estimate of within run precision standard deviation.

<sup>b</sup>Point estimate of total precision standard deviation.

### 3. Cross Reactivity

Specimens negative for ANA HEP-2 IFA and positive for IgG antibody to various antigens such as EBV-VCA-, EBNA, HSV-1, HSV-2, CMV, Rubella, and/or Toxo, were tested for potential cross-reactivity using the Diagnostic Automation, Inc. Ana Screen ELISA test system. All samples were negative on the ELISA, indicating that the potential for cross reactivity with such antibodies is not likely, and therefore should not interfere with the results obtained.

### LIMITATIONS OF PROCEDURE

1. The ENA Screen ELISA test is a diagnostic aid and by itself is not diagnostic. Test results should be interpreted in conjunction with the clinical evaluation and the results of other diagnostic procedures.
2. Positive antibodies to ENA may be found in apparently healthy people. It is therefore imperative that the results be interpreted in conjunction with the patient's clinical picture by a medical authority.
3. The DAI, Inc. ENA Screen ELISA test system will not identify the specific type of anti-ENA present in a positive specimen. Positive specimens should be tested for individual autoantibodies using the DAI, Inc. ENA Profile-6 ELISA test system.

### EXPECTED VALUES

The expected value for a normal patient is a negative result. The number of reactivities, and the degree of reactivity is dependent upon parameters such as population type being tested, treatment, etc. Each laboratory should establish their own expected values based upon the specimens typically being tested.

With respect to disease-state and percent reactivity, Table 1 in the Significance and Background section of this package insert shows the relative frequency of autoantibody activity for various rheumatic disorders.

### QUALITY CONTROL

1. Each time the assay is run the Calibrator must be run in triplicate. A reagent blank, Negative Control, and Positive Control must also be included in each assay.
2. Calculate the mean of the three Calibrator wells. If any of the three values differ by more than 15% from the mean, discard that value and calculate the mean using the remaining two wells.
3. The mean OD value for the Calibrator and the OD values for the Positive and Negative Controls should fall within the following ranges:

	OD Range
Negative Control	≤ 0.250
Calibrator	≥ 0.300
Positive Control	≥ 0.500

- a. The OD of the Negative Control divided by the mean OD of the Calibrator should be ≤ 0.9.
  - b. The OD of the Positive Control divided by the mean OD of the Calibrator should be ≥ 1.25.
  - c. If the above conditions are not met the test should be considered invalid and should be repeated.
4. The Positive Control and Negative Control are intended to monitor for substantial reagent failure and will not ensure precision at the assay cut-off.
  5. Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.
  6. Refer to NCCLS document C24: Statistical Quality Control for Quantitative Measurements for guidance on appropriate QC practices.

### PRECAUTIONS

1. For *In Vitro* Diagnostic Use.
2. Normal precautions exercised in handling laboratory reagents should be followed. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing, gloves, and eye/face protection. Do not breathe vapor. Dispose of waste observing all local, state, and federal laws.
3. The wells of the ELISA plate do not contain viable organisms. However, the strips should be considered **POTENTIALLY BIOHAZARDOUS MATERIALS** and handled accordingly.
4. The human serum controls are **POTENTIALLY BIOHAZARDOUS MATERIALS**. Source materials from which these products were derived were found negative for HIV-1 antigen, HBsAg, and for antibodies against HCV and HIV by approved test methods. However, since no test method can offer complete assurance that infectious agents are absent, these products should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories": current edition; and OSHA's Standard for Bloodborne Pathogens (20).
5. Adherence to the specified time and temperature of incubations is essential for accurate results. **All reagents must be allowed to reach room temperature (20-25°C) before starting the assay.** Return unused reagents to refrigerated temperature immediately after use.
6. Improper washing could cause false positive or false negative results. Be sure to minimize the amount of any residual wash solution; (e.g., by blotting or aspiration) before adding Conjugate or Substrate. Do not allow the wells to dry out between incubations.
7. The Sample diluent, controls, wash buffer, and conjugate contain sodium azide at a concentration of 0.1% (w/v). Sodium azide has been reported to form lead or copper azides in laboratory plumbing which may cause explosions on hammering. To prevent, rinse sink thoroughly with water after disposing of solution containing sodium azide.
8. The Stop Solution is TOXIC. Causes burns. Toxic by inhalation, in contact with skin and if swallowed. In case of accident or if you feel unwell, seek medical advice immediately.
9. The TMB Solution is HARMFUL. Irritating to eyes, respiratory system and skin.
10. The Wash Buffer concentrate is an IRRITANT. Irritating to eyes, respiratory system and skin.
11. Wipe bottom of plate free of residual liquid and/or fingerprints that can alter optical density (OD) readings.
12. Dilution or adulteration of these reagents may generate erroneous results.
13. Reagents from other sources or manufacturers should not be used.

14. TMB Solution should be colorless, very pale yellow, very pale green, or very pale blue when used. Contamination of the TMB with conjugate or other oxidants will cause the solution to change color prematurely. Do not use the TMB if it is noticeably blue in color.
15. Never pipette by mouth. Avoid contact of reagents and patient specimens with skin and mucous membranes.
16. Avoid microbial contamination of reagents. Incorrect results may occur.
17. Cross contamination of reagents and/or samples could cause erroneous results
18. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
19. Avoid splashing or generation of aerosols.
20. Do not expose reagents to strong light during storage or incubation.
21. Allowing the microwell strips and holder to equilibrate to room temperature prior to opening the protective envelope will protect the wells from condensation.
22. Wash solution should be collected in a disposal basin. Treat the waste solution with 10% household bleach (0.5% sodium hypochlorite). Avoid exposure of reagents to bleach fumes.
23. Caution: Liquid waste at acid pH should be neutralized before adding to bleach solution.
24. Do not use ELISA plate if the indicator strip on the desiccant pouch has turned from blue to pink.
25. Do not allow the conjugate to come in contact with containers or instruments that may have previously contained a solution utilizing sodium azide as a preservative. Residual amounts of sodium azide may destroy the conjugate's enzymatic activity.
26. Do not expose any of the reactive reagents to bleach-containing solutions or to any strong odors from bleach-containing solutions. Trace amounts of bleach (sodium hypochlorite) may destroy the biological activity of many of the reactive reagents within this kit.

**STORAGE**

1. Store the **unopened kit** between 2 and 8°C.
2. **Coated microwell strips:** Store between 2 and 8°C. Extra strips should be immediately resealed with desiccant and returned to proper storage. Strips are stable for 60 days after the envelope has been opened and properly resealed and the indicator strip on the desiccant pouch remains blue.
3. **Conjugate:** Store between 2 and 8°C. **DO NOT FREEZE**.
4. **Calibrator, Positive Control and Negative Control:** Store between 2 and 8°C.
5. **TMB:** Store between 2 and 8°C.
6. **Wash Buffer concentrate (10X):** Store between 2 and 25°C. Diluted **wash buffer (1X)** is stable at room temperature (20 to 25° C) for up to 7 days or for 30 days between 2 and 8°C.
7. **Sample Diluent:** Store between 2 and 8°C.
8. **Stop Solution:** Store between 2 and 25°C.

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 <b>ISO 13485</b> <b>ISO 9001</b>	
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<b>REF 2552-2</b>	<b>AccuDiag™ - ENA Combined  Screen (6 Antigen)  ELISA</b>
<b>EC REP</b>	<b>CEpartner4U, Esdoornlaan 13,  3951DB Maarn. The Netherlands.  <a href="http://www.cepartner4u.eu">www.cepartner4u.eu</a></b>
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