Clinical optimization and multicenter validation of antigen-specific cut-off values on the INNO-LIA™ ANA Update for the detection of autoantibodies in connective tissue disorders

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Abstract

Objectives

The INNO-LIA™ ANA Update is a qualitative multiparameter line immunoassay for detection of autoantibodies to several different antigens associated with connective tissue disorders. We sought to optimize and validate the cut-off values for its antigen-specific components: SmB, SmD, RNP-70k, RNP-A, RNP-C, SSA/Ro52, SSA/Ro60, SSB/La, Cenp-B, Topo-I, Jo-1, ribosomal P, and histones. Our aim was to achieve 98% specificity for each of the markers, with respect to differential disease controls, while maintaining sensitivity.

Methods

For optimization, the cut-off value of the different antigen lines was fixed to achieve this specificity using an in-house set of 955 patient samples. Specificity was validated at multiple sites using a different set of 330 samples obtained from 158 apparently healthy blood donors, 100 patients with a variety of infections, 20 each with Wegener's granulomatosis, inflammatory bowel disease, and primary antiphospholipid syndrome, and 12 with psoriatic arthritis. Sensitivity was evaluated, using this optimized cut-off control, in 147 patients with scleroderma, 93 with Sjögren's disease, 40 with systemic lupus erythematosus, 40 with rheumatoid arthritis, 39 with mixed connective tissue disease, and 19 with polymyositis. Sensitivity and specificity of the INNO-LIA™ ANA Update were determined using the clinical diagnosis as reference.

Results

The optimized cut-off values resulted in a specificity 98% or more for all LIA™ markers except one (histones 97.8%) in the validation set of 330 samples. The sensitivity for each marker tested in 378 samples from the target patient groups was comparable to that reported in the literature.

Conclusion

The INNO-LIA™ ANA Update shows uniformly high specificities combined with sensitivities very similar to those of reference assays, in a single test format.

Key words

Receiver operator characteristics analysis, line immunoassay, antinuclear autoantibodies, specificity, sensitivity.

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Introduction

Autoantibodies directed against specific intracellular antigens are often associated with connective tissue disorders (CTD) (1,2). This has been shown for pathologies such as systemic lupus erythematosus (SLE), scleroderma (SSc), Sjögren's syndrome (SS), mixed connective tissue disease (MCTD), and polymyositis (PM) or dermatomyositis (DM). Serologic tests for the detection of these antinuclear autoantibodies (ANA) are valuable tools in aiding the clinician to correctly identify the different pathologies. The ANA described here also include antibodies to cytoplasmic antigens.

ANA are commonly detected by the indirect immunofluorescence method (IIF). The distinct fluorescence patterns obtained with IIF are indicative of the presence of different autoantibodies (2). However, correct interpretation of the fluorescence-staining pattern is difficult: it requires skilled technicians, and test results can vary depending on the cell type used as substrate. Furthermore, recent studies have reported that samples negative on IIF can show reactivity on other assays (3). Certain positive results on IIF are usually followed by a second test to determine specific reactivities of the ANA (4, 5). Such assays include immunoblotting, double immunodiffusion, countercurrent immunoelectrophoresis (CIE), enzyme-linked immunosorbent assays (ELISA), or line immunoassays (LIA).

Quality control programs examining the ability of immunoassays to detect ANA in CTD have mainly focussed on improving test sensitivity (avoiding false-negative results), although the specificity (avoiding false-positive results) of these assays must also be assessed in parallel. The latter is of major importance because in those cases when there is doubt about the clinical diagnosis, a false-positive result may contribute to uncertainty for the physician or even to diagnostic error in the case of disease-specific antigens. We previously described the use of a single multiparameter LIA™ to detect multiple antinuclear autoantibodies (6). The INNO-LIA™ ANA Update is a qualitative test detecting the presence of autoantibodies against SmB, SmD, RNP-70k, RNP-A, RNP-C, SSA/Ro52, SSA/Ro60, SSB/La, Cenp-B, Topo-I, Jo-1, ribosomal P, and histones. We sought to further optimize and validate the antigen-specific cut-off values for this test in order to achieve at least 98% specificity towards disease controls for each of the included markers, without compromising assay sensitivity.

Methods

Samples used for optimization

A total of 955 study samples were used to define disease and control groups for receiver operator characteristics (ROC) analysis. These samples were chosen based on the clinical diagnosis or on unequivocal IIF reactivity (Table I). Four disease groups (reactive for certain antigens) and corresponding control groups (not reactive for these antigens) were composed from these samples (Table II).

Samples used for validation

A set of samples, independent of those used for optimization, was selected for the validation of assay performance at the cut-off values obtained. To this end, assay specificity was validated using 330 samples obtained from 20 patients each with Wegener's granulomatosis (WG), inflammatory bowel disease (IBD), antiphospholipid syndrome (APS), 12 with psoriatic arthritis (PsA), 100 with an infection (including 10

Table I. Serum samples used for optimization.

Patient group	Number of samples
Systemic lupus erythematosus	234
Myositis	124
Sjögren's syndrome	29
Scleroderma	25
Univocal centromere IIF pattern	24
Rheumatoid arthritis	214
Arthritis without autoimmune CTD (non-RA)	94
Epstein-Barr virus-positive	112
Negative for antinuclear antibodies on IIF (non-ANA)	99
Total	955

Table II. Disease and control groups.

Antigens	Disease group* (positive samples)	Control group* (negative samples)
SmB, SmD, rRNP, Hist.	SLE (234)	All other groups (721)
RNP-70, RNP-A, RNP-C,	SLE, myositis, SS, SSc,	Non-ANA, EBV, non-RA
Ro52, Ro60, SSB	Centromere (436)	(305)
Cenp-B, Topo-1	Centromere and SSc (49)	All other groups (906)
Jo-1	Myositis (124)	All other groups (831)

* Number of samples in parenthesis

Table III. Overview of the samples included in the multicenter validation study.

Center	Type of sample	No. samples
1*	Systemic lupus erythrematosus (SLE)	40
	Scleroderma (SSc)	40
	Rheumatoid arthritis (RA)	40
	Mixed connective tissue disease (MCTD)	39
	Dermato/polymyositis (PM/DM)	19
	Primary Sjögren's syndrome (SS)	40
	Wegener's granulomatosis (WG)	20
	Inflammatory bowel disease (IBD) (Ulcerative colitis/Crohn's disease)	20
	Antiphospholipid syndrome (APS)	20
	Infectious diseases – European control samples	100
	Psoriatic arthritis (PsA)	12
	Apparently healthy blood donors (BD)	100
	TOTAL	490
2§	Limited SSc with pulmonary hypertension	10
	Diffuse SSc	11
	Diffuse SSc with pulmonary fibrosis	11
	Apparently healthy volunteers	10
	TOTAL	42
3	Primary SS¥	53
	Apparently healthy blood donors	48
	TOTAL	101
4§	Limited SSc#	56
	Diffuse SSc	17
	SSc	2
	TOTAL	75
TOTAL		708

* Diagnosis based on in-house criteria of investigator.
§ Diagnostic criteria unknown.
¥ The SS patients all met at least 4 of the 6 criteria described by Vitali *et al*. (21) including ocular symptoms, oral symptoms, positive Schirmer test, and anti-Ro/La autoantibodies.
In the category of the limited SSc patients, there are 14 subjects with overlap syndromes.

each with hepatitis B virus (HBV), hepatitis C virus (HCV), parvovirus, streptococcus, mycoplasma, herpes simplex, salmonella, rubella, cytomegalovirus (CMV), and EBV infections), and 158 samples from healthy controls (Table III).

The sensitivity of the INNO-LIA™ ANAUpdate (Innogenetics NV, Ghent, Belgium**)** was tested on another 378 patient samples, including 147 patients with SSc, 93 with SS, 40 patients with SLE, 40 with RA, 39 with MCTD, and 19 with polymyositis. Sensitivity and specificity of the INNO-LIA™ ANA Update were determined using the clinical diagnosis as reference (Table III).

INNO-LIA™ ANA Update

The INNO-LIA™ ANA Update is based on the principle of an enzyme immunoassay. Recombinant antigens (SmB, RNP-70k, RNP-A, RNP-C, SSA/Ro52, SSB/La, Cenp-B, Topo-I, Jo-1), synthetic peptides (SmD and ribosomal P), and natural proteins (SSA/Ro60, histones) are coated as discrete lines on a nylon membrane with a plastic backing. The synthetically modified SmD peptide included on the strip has been described previously (7). In addition to these autoantigens, one control line, which must be positive for the test to be valid, is coated on each strip. The assay was performed according to the manufacturer's instructions for all samples (6). In short, the test sample was incubated in a test trough together with a multiple antigen-coated strip. Binding of specific autoantibodies was visualized by counterstaining with a goat anti-human IgG labeled with alkaline phosphatase, followed by incubation with the chromogen nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP). Color development was stopped with sulfuric acid. An illustration of the INNO-LIA™ ANA Update strip is shown in Figure 1.

Interpretation of the test results

Together with the INNO-LIA™ ANA Update, a software module - the LIA-*Scan* ANAtest module - was developed for scanning and objective interpretation of the INNO-LIA™ ANA Update strips. By use of image processing algorithms, a specific scan value for each antigen line can be obtained. This scan value is then converted into a positive, negative, or equivocal test result. Relative scan values (scan value of the antigen line divided by the scan value of the control line multiplied by 100) are used to correct for strip development-dependent variation. As a very high correlation between the visual and the LIA-*Scan* interpretation was shown, they were considered to be equivalent. The results in the present studies were all interpreted using the LIA-*Scan*

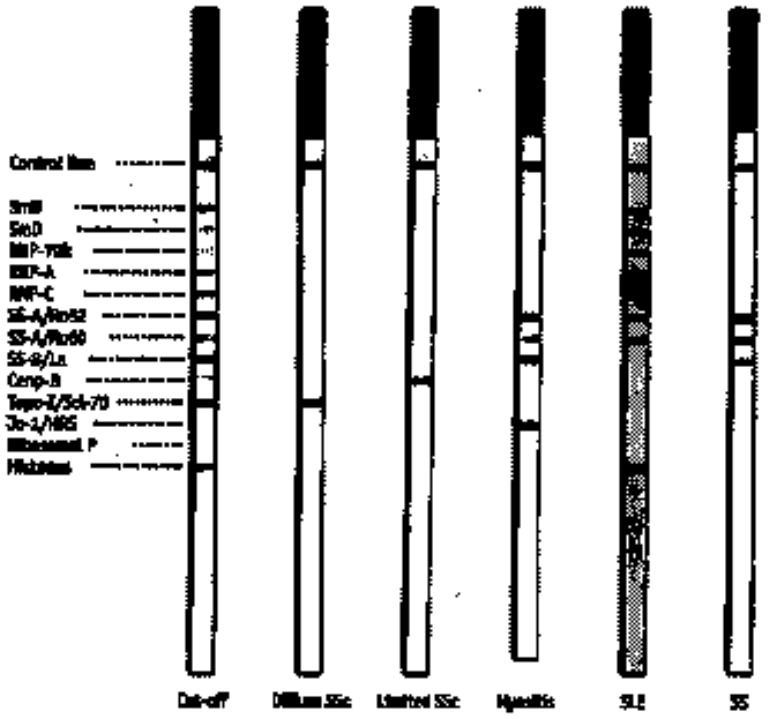


Fig. 1. Illustration of the INNO-LIA™ ANAUpdate strip. Typical examples of LIA™ reactivities.

ANA test module.

The INNO-LIA™ ANA Update uses a cut-off control that serves to determine whether a sample is positive, negative, or equivocal for an antigen line, this to compensate for the naturally occurring autoantibodies in normal individuals (8). This cut-off control is developed during the assay along with the patient sera (Fig. 1). The reactivity of the sample strips is then compared with this cut-off strip. A sample was considered positive for a specific antigen line when the relative scan value was greater than or equal to the relative scan value of the corresponding antigen line of the cut-off strip. Samples with antibody levels (as determined by scan values) below to -33% of the cut-off value were considered as equivocal. This corresponds to a band with intensity close to, but nevertheless below, the cut-off in visual reading. Equivocal results were considered negative for the specificity and sensitivity calculations. Although these samples are negative for a given antigen, the fact that an equivocal test result might, in some cases, have a clinical significance cannot be excluded considering the high specificity that is maintained for the INNO-LIA™ ANA Update. Samples with antibody levels below the equivocal region are called negative.

According to the guidelines of the European consensus workshop for detection of autoantibodies (9), anti-Sm activity can only be distinguished by the presence of anti-SmD-specific antibodies since anti-SmB positivity alone may be due to cross-reactivity with anti-RNP antibodies. Therefore, a sample was considered Sm-positive when at least the SmD-line was positive. Furthermore, according to internal guidelines, a sample was considered RNP-positive when at least two out of the three RNP-antigen lines were positive. A sample was considered anti-SSA positive when Ro52 and/or Ro60 were positive.

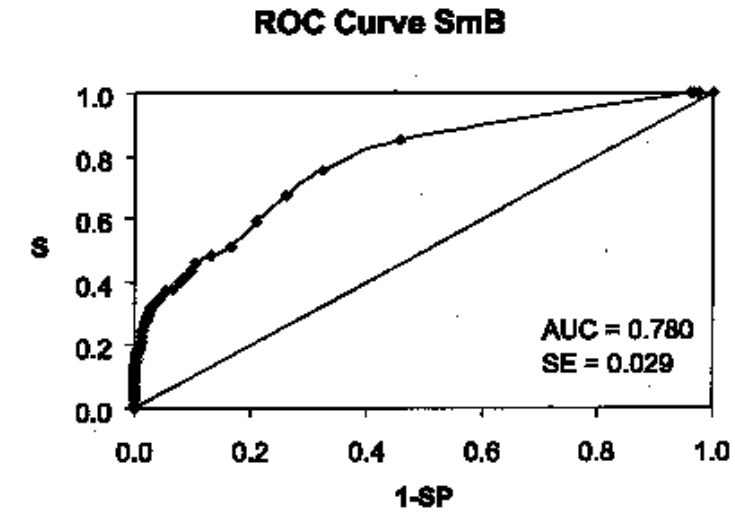
ROC analysis and composition of the cut-off control

The principles of ROC analysis for the selection of cut-off values for a diagnostic test have been reviewed by Zweig *et al.* (10) and Greiner *et al.* (11). A ROC curve was drawn for each antigen to calculate the theoretical cut-off value. To this end, disease and control groups were composed from the 955-patient serum samples, depending on the expected reactivity of the antigen, as deduced from the literature (Table I). To obtain the ROC curve, the INNO-LIA™ ANAUpdate strips were developed using the selected serum samples, and scanned. The scanned bitmap was further processed using mathematical techniques to obtain one single scan value for each line on the strip, representing the reactivity of that line. A specificity of at least 98% was chosen as the main criterion for the determination of the theoretical cut-off value.

Commercially available samples were selected to compose the cut-off control, based on the theoretical values calculated by ROC analysis. Selection criteria were mono-specificity for one antigen - if possible - and reactivity (evaluated by the scan value) higher than the theoretical cut-off reactivity obtained from the ROC curves, in a normal dilution of 1/200.

Different dilutions from these samples were analyzed on the INNO-LIA™ ANA Update strips. The dilution most closely matching the theoretical cut-off scan value was selected for each sample. Obtaining the theoretical cut-off values, as determined by ROC curves, was not feasible due to the unavailability of mono-specific samples (i.e., some of the samples were positive for different antigens) and the non-linearity between reactivity, as expressed by the scan value, as well as dilution factors. For this reason, a stepwise or iterative approach was followed to find the best compromise between multi-antigen sera availability and approximating the theoretically proposed cut-off value as much as possible. To start, a first composition of the cut-off control was proposed, and the individual samples mixed into the composite cut-off control were tested on the INNO-LIA™ ANA Update strip. The scan values for each antigen for each individual cut-off control were then regressed to best match the theoretical cut-off values.

Multi-linear regression resulted in regression coefficients from which the new dilution factor for each serum was calculated. The cut-off control was then recomposed of the individual samples



Cutoff	%SP	%S
49	98.6	25
50	98.9	25
51	98.9	23
52	98.9	21
53	99	21

Fig. 2. ROC curve for SmB. ROC curve for SmB showing the sensitivity (S) and specificity (SP) for different cut-off values (u).

at the new appropriate dilutions. In some instances, the first choice of the dilution factor deviated substantially from the "ideal" dilution factor, as multiple linear regression did not always give the optimal result in a first calculation due to non-linearity. In these cases, the dilution factor obtained from the fit was used as a closer approximation to the "ideal" dilution factor and the entire process was repeated. In a few steps, the optimal choice of dilution factors was obtained.

This new cut-off control sample was then developed on the INNO-LIA™ ANAUpdate strips, and experimentally obtained scan-values were compared to the theoretical cut-off values. The best compromise then had to be challenged in an independent validation step to evaluate whether this cut-off control was acceptable in terms of sensitivity and specificity.

Results

Optimization (design of cut-off control)

A ROC curve was drawn for each individual antigen to determine the theoretical cut-off value, with a minimum specificity of 98% as the major criterion. In most cases, the cut-off value finally obtained was higher than the theoretical cut-off value resulting in an even higher theoretical specificity than the proposed 98%. The sensitivity remained acceptable as compared to values reported in the literature (2).

An example of the ROC curve for SmB is shown in Figure 2. A theoretical cut-off scan value of 50 was chosen, corresponding to a specificity of 98.9%. In the same manner, a theoretical cut-off scan value of 30 was chosen for SmD. Two of the eight commercial samples used to compose the cut-off control were reactive on SmB and SmD. First, a dilution was chosen approximating the respective theoretical cut-off values. As both samples reacted on multiple antigens (one sample was also reactive on RNP-A, RNP-C, and histones), the theoretical values could never be obtained exactly and a compromise had to be accepted. One sample, at a dilution of 1/200, resulted in a scan value of 78.3 for SmB and 16.6 for SmD; the other sample, in a dilution of 1/5000, resulted in a scan value of 26.6 for SmB and 25.7 for SmD. Multilinear regression was applied to calculate new dilution factors for both samples in order to approximate the theoretical cut-off values of each reactive line as closely as possible. The dilutions calculated in this manner for the two plasma samples were 1/427 and 1/7398, respectively. A similar approach was taken to determine the optimal dilutions for each of the 8 samples so that the best possible approximation of the respective theoretical cut-off values for each antigen line was obtained. The cut-off control, comprising the 8 samples in the appropriate dilution, was then analyzed on the INNO-LIA™ ANA Update to experimentally determine the true scan values of the cut-off control. In the case of SmB, the experimental cut-off value obtained was 67.9; for SmD, 25.6.

Comparison of positive reactivity in the different study groups for the ideal theoretical situation and the situation where the new cut-off control was used indicated that the target specificity was indeed maintained, but that some compromises had to be made in terms of sensitivity (data not shown).

Validation

After optimization of the composite cut-off serum, the performance of the INNO-LIA™ ANA Update was challenged on an independent set of samples in terms of specificity and sensitivity.

Specificity: The specificity of the INNO-LIA™ ANA Update was calculated based on the results of samples from 158 apparently healthy volunteers, 100 patients with infections, and 72 with a variety of disorders including WG, IBD, APS, and PsA(Table III). The optimized cut-off values of the INNO-LIA™ ANA Update resulted in very high specificity in samples obtained from apparently healthy blood donors and other controls described in Table II (99.7% on average), as well as in those from patients with infectious diseases (99.4% on average) or rheumatologic disorders (98.5% on average) (data not shown). The overall specificity of all LIA™ markers was above 98%, except for histones and isolated RNP-C which had a specificity of 97.8% and 97.6%, respectively. For 11 out of the 13 markers, specificity exceeded 99% (Table

Table IV. Prevalence of autoantibodies detected by INNO-LIA™ ANA Update in control groups – specificity calculation.

	Individual reactivity (n = 330)	% specificity
Sm	1	99.7
SmB	*2*	*99.4*
SmD	*1*	*99.7*
RNP	1	99.7
RNP-70k	*3*	*99.1*
RNP-A	*6*	*98.2*
RNP-C	*8*	*97.6*
SSA/Ro52	3	99.1
SSA/Ro60	6	98.2
SSB/La	1	99.7
Cenp-B	1	99.7
Topo-I/Scl-70	0	100
Jo-1/HRS	1	99.7
Ribosomal P	1	99.7
Histones	7	97.8

Table V. Sensitivity (%) of autoantibody detection by INNO-LIA™ ANAUpdate in different autoimmune pathologies.

	SLE (n = 40)	MCTD (n = 39)	SS (n = 93)	Scleroderma (n = 147)*	PM/DM (n = 19)	RA (n = 40)
Sm	5§	12.5	1 (0-2)¥	0	0	0
SmB	*17.5*	*43.5*	*0*	*3 (0-5)*	*0*	*2.5*
SmD	*5*	*12.5*	*1 (0-2)*	*0*	*0*	*0*
RNP	20	(87)#	0	3 (0-5)	0	2.5
RNP-70k	*15*	*(72)*	*1 (0-2)*	*4 (0-6)*	*0*	*5*
RNP-A	*20*	*(87)*	*1 (0-2)*	*4 (0-6*	*0*	*2.5*
RNP-C	*12.5*	*(74)*	*1 (0-2)*	*3 (0-6)*	*0*	*5*
SSA/Ro52	12.5	5	75 (58-89)	6 (0-9)	31.5	2.5
SSA/Ro60	20	2.5	72 (55-85)	3 (0-5)	10.5	2.5
SSB/La	5	2.5	65 (50-75)	1 (0-3)	16	2.5
Cenp-B	5	0	7 (2-13)	35 (19-60)	0	0
Topo-I/Scl-70	0	0	1 (0-2)	15 (12-25)	0	0
Jo-1/HRS	0	0	1 (0-3)	2 (0-4)	5	0
Ribosomal P	2.5	0	1 (0-2)	0.6 (0-1)	0	0
Histones	20	5	4 (3-6)	4 (0-5)	0	8

* 13 out of the 147 included SSc patients are known to have overlap with other autoimmune pathologies responsible for many of the autoantibody reactivities
§ Gray zones indicate markers expected to be positive in this pathology (2).
¥ Values between brackets are the minimum and maximum per center.
The samples were selected using in-house criteria of the Investigators. One of the inclusion criteria of the 39 MCTD samples was RNPpositivity. The sensitivity of RNPin MCTD on the LIAwas therefore biased.

IV).

Sensitivity: The validation of the sensitivity of the INNO-LIA™ ANA Update was based on the results of samples obtained from 378 patients with a variety of CTD (Table III). The individual results per marker are shown in Table V. The range by center is indicated if more than one center was involved. The autoantibody profile expected for each clinical diagnosis, as reported in the literature (2), is marked in gray.

Comparison of the INNO-LIA™ ANA Update with routinely used assays

The INNO-LIA™ ANAUpdate results were compared to those obtained with reference techniques in the different centers. These results are summarized in Tables VI and VII.

Comparison with IIF. The IIF patterns of 142 samples were compared to INNO-LIA™ ANA Update results in center 1 using an anti-IgG-specific conjugate (Table VI). In 23% (7/30) of the IIF-positive cases with a homogeneous pattern, the LIA™ showed a histone reactivity. This rather low figure was not surprising since a homogeneous pattern is also associated with anti-DNA positivity, which cannot be detected by the LIA™. For the other samples analyzed by IIF, a comparable result was found with LIA™.

Anti-SSA. Of the 47 samples tested on LIA™ and Shield ELISA(Axis-Shield Diagnostics Ltd., Dundee, UK) for anti-SSA, 35 were negative and 7 were positive in both tests; a discrepant result was found for 5 samples (Table VII). Of these five samples, one was Ro60-positive on LIA™, while negative on the SSAELISA. Although both the Shield ELISA and the LIA™ use natural Ro60, it remains unknown why the Shield ELISA did not detect the sample. The other four samples were positive on ELISA and negative on LIA™. Three samples were not retested because they reacted only weakly positive on ELISA. The remaining sample tested negative on both Ro52 and Ro60 with LIA™, while positive on anti-SSA ELISA. This sample was retested on blot, but the SSA reactivity could not be confirmed with this technique, which was in concordance with the LIA™ results.

Out of 75 scleroderma patients tested on LIA™ and CIE, 2 samples tested positive and 66 negative on both assays, while a discrepant result was observed in 7 cases (Table VII). Of these 7 samples, 2 were positive on LIA™ (Ro52 as well as Ro60) while negative on CIE. Three CIE negative samples

Table VI. Comparison of INNO LIA-ANA™ Update with IIF.

IIF pattern	N	INNO-LIA™ ANAUpdate			
		Pos	Eq*	Neg	Remarks (specific reactivity)
Homogeneous	30	19	3	8	7 are histone-positive
Speckled	25	21	2	2	Sm, RNP, SSA, and SSB
Centromere	17	15	1	1	14 are Cenp-B-positive
Pos (pattern unknown)	17	15	0	2	14 are RNP-positive
Negative	53	8	4	41	
Total	142				

*Equivocal.

Table VII. Comparison of INNO-LIA™ ANAUpdate with reference assays.

Antigen (reference method)	Samples	Concordant/Discordant
SSA(ELISA)	SLE, RA, SSc	42/5*
SSA(CIE)	Scleroderma	68/7
SSB (ELISA)	SLE, RA, SSc	44/3
Scl-70 (blot)	SLE, RA, SSc	44/3§
Scl-70 (CIE)	Scleroderma	28/3
Scl-70 (CIE)	Scleroderma	73/2
Sm (hemagglutination)	SLE, RA, SSc	39/6¥
RNP(hemagglutination)	SLE, RA, SSc, MCTD, myositis	93/9#
RNP(CIE)	Scleroderma	70/5
Centromere (IIF)	Scleroderma	65/10
Ro52 (ELISA)	SS	53/0
Ro60 (ELISA)	SS	43/10$
SSA(CIE)	SS	51/2
SSB (ELISA)	SS	47/6£
SSB (CIE)	SS	47/6

The INNO-LIA™ ANAUpdate result was confirmed:
* in one sample by immunoblot; § in 2 samples upon retest; ¥ in 2 samples by immunoblot; # in 4 samples by immunoblot; $ in 7 samples by CIE; £ in 1 sample by CIE.

were only positive for Ro52, while negative for Ro60 on LIA™. These results confirm the findings of Peene *et al.* (12) showing that positive CIE results depend on anti-Ro60 reactivity and are independent of anti-Ro52 reactivity. Two samples were positive on CIE and negative on LIA™.

Anti-SSB. Forty-seven samples were tested on both LIA™ and Shield ELISA for anti-SSB (Axis-Shield Diagnostics Ltd., Dundee, UK); a discrepant result was found in 3 cases (Table VII). There were 2 samples which tested positive on LIA™ for anti-SSB which were negative on ELISA. In our experience, the recombinant SSB of the LIA™ is more sensitive than the natural SSB of the commercial ELISA. The other sample was borderline positive on ELISAwhile negative on LIA™.

Anti-Scl-70/Topo-I. Out of the 47 samples tested on immunoblot and LIA™ for anti-Scl-70, 3 samples were positive and 41 were negative in both tests; a discrepant result was found in 3 cases (Table VII). One sample tested positive on LIA™ for anti-Scl-70/Topo-I while negative on blot. Upon retesting, the Scl-70 immunoblot confirmed the LIA™ result. The two other samples were initially positive on immunoblot for anti-Scl-70, while negative on LIA™ Topo-I. Upon retesting, one sample was found to be negative for anti-Scl-70 blot (initially false-reactive on blot), while one sample was confirmed to be anti-Scl-70 positive on immunoblot.

Compared to the Scl-70 data available from 31 scleroderma patients from center 2, obtained at the study center by CIE, LIA™ identified 3 more anti-Topo-I-positive samples (Table VII). When anti-Scl-70 CIE and INNO-LIA™ ANA Update were mutually compared in center 4 on 75 scleroderma patient samples, there were 2 that tested positive for anti-Topo-I on the LIA™ but were negative with the reference test.

Anti-Sm. Forty-five samples were tested on LIA™ and hemagglutination for anti-Sm (Table VII). Six samples tested positive for anti-SmB on LIA™, while negative with the hemagglutionation technique. Upon retesting with the Sm immunoblot, 2 were confirmed to be anti-SmB positive. One sample could not be retested, and 3 others tested negative.

Anti-RNP. Out of the 102 samples tested by hemagglutination and LIA™ for anti-RNP; a discrepant result was found in 9 cases (Table VII). Four tested negative by the hemagglutination test, but were positive on the LIA™. In two of these, the LIA™ result was confirmed by immunoblot. Five samples tested positive on the hemagglutination test while being negative on the LIA™. Two were confirmed anti-RNP-negative on immunoblot.

Out of 75 scleroderma samples tested on LIA™ and CIE for RNP, 5 discrepant cases were found (Table VII). Two of them were negative on all 3 LIA™ RNP markers and one was RNP-70 positive only, while CIE was positive. In contrast, there were 2 samples that tested positive on two or more RNP lines, while CIE tested negative.

Anti-Centromere. There were 10 discrepant results out of a total of 75 scleroderma samples tested for anti-centromere Ab in center 4 between IIF and INNO-LIA™ ANA Update (Table VII). Four samples tested Cenp-B-positive on LIA™ while negative in IIF, 6 were positive in IIF while negative on LIA™. The latter 6 samples probably recognized another centromere epitope than Cenp-B, which would explain a negative result on LIA™. The same holds true for the centromere results presented in Table VI. The reason why

Table VIII. INNO-LIA™ ANAUpdate for 10 CDC samples*.

CDC sample Nr Characterization (test)§	1 homogeneous (IIF)	2 SSB/La (WB)	3 speckled (IIF)	4 U1-RNP (WB)	5 Sm (WB)	6 Nucleolar (IIF)	7 SSA/Ro (WB)	8 Centromere (IIF)	9 Scl-70 (WB)	10 Jo-1 (WB)
SM	+	-	-	-	+	-	-	-	-	-
SMB	+	-	+	-	+	-	-	-	-	-
SmD	+	-	-	-	+	-	-	-	-	-
RNP	-	-	Eq	+	+	-	-	-	-	-
RNP-70k	-	-	+	+	Eq	-	-	-	-	-
RNP-A	-	-	Eq	+	+	-	-	-	-	-
RNP-C	+	-	-	-	+	-	-	-	-	-
SSA	-	+	+	-	-	-	+	-	-	+
SSA/Ro52	-	Eq¥	-	-	-	-	Eq	-	-	+
SSA/Ro60	-	+	+	-	-	-	+	-	-	-
SSB/La	-	+	+	-	-	-	-	-	-	-
Cenp-B	-	-	-	-	-	-	-	+	-	-
Topo-I/Scl-70	-	-	-	-	-	-	-	-	+	-
Jo-1/HRS	-	-	-	-	-	-	-	-	-	+
Ribosomal P	-	-	-	-	-	-	-	-	-	-
Histones	+	-	-	-	Eq	-	-	-	-	-

* Expected reactivities are shown in dark gray; expected weak reactivities are shown in light gray

§ IIF, indirect immunofluorescence; WB, Western Blot

¥ Equivocal: Samples with antibody levels (as determined by scan values) from below to -33% of the cut-off value were considered as equivocal. This corresponds to a band with intensity close to, but nevertheless below, the cut-off in visual reading. Although these samples are negative for a given antigen, the fact that an equivocal test result might, in some cases, have a clinical significance cannot be excluded considering the high specificity that is maintained for the INNO-LIA™ ANAUpdate.

LIA™ picked up 4 more samples compared to IIF for anti-centromere is currently unknown.

Anti-SSA/SSB. In center 3, comparisons between the INNO-LIA™ ANA Update, ELISA, and CIE were made for anti-Ro60, Ro52, and SSB in 53 SS patients (Table VII). The comparison between Ro52 ELISA and LIA yielded no discrepant results. In 7 out of the 10 samples for which the LIA™ tested positive and ELISA negative for anti-Ro60, SSACIE also gave a positive result. One sample was found to be negative on LIA™ upon retesting, which was confirmed by the ELISA result. The remaining two samples yielded positive results with natural Ro60 and negative ones with recombinant material, suggesting that they contained an epitope which is only recognized by the natural Ro60 material.

Six discrepant results were found between INNO-LIA™ ANA Update and both the SSB ELISA and CIE. One sample had an equivocal INNO-LIA™ ANA Update result, which was confirmed by a negative CIE; the SSB ELISA was borderline-positive. For 3 out of 5 anti-SSB discrepancies where LIA tested positive and ELISA negative, the SSB CIE also gave a negative result. However, 2 out of the 3 samples had an OD value just below the cut-off and were thus borderline-negative. The other two samples were positive on CIE.

Using McNemar's test for comparison of dependent proportions, the sensitivity of Ro60 on INNO-LIA™ ANA Update was found to be significantly better as compared to ELISA ($p = 0.002$), while the sensitivity of Ro52 and SSB was not statistically different between LIA™ and ELISA ($p = 1$ and 0.103 respectively). For the comparison between CIE and LIA™, the sensitivity of anti-SSB on INNO-LIA™ ANA Update was significantly better compared to CIE ($p = 0.014$), while there was no significant difference in sensitivity between LIA™ and CIE for anti-Ro ($p=0.157$).

Human Reference Sera

Rigorously defined reference sera are the most commonly used standards for autoantibody determinations. The human reference sera from the Centers for Disease Control and Prevention (CDC) have been widely used as standards since they were introduced in 1982 (13), and the methods used to define and establish the standards have been published (14). A consensus was established regarding the specific autoantibody profiles of the reference sera using IIF and Western blot (14).

In a last validation step, the CDC reference sera were analyzed on the INNO-LIA™ ANA Update. Table VIII shows the results obtained with the CDC sera, with the expected reactivities indicated in gray (14). These results showed that the INNO-LIA™ ANA Update detect-

ed all expected reactivities. In 2 cases, the reactivities were equivocal on LIA™ (CDC-5 for histones and CDC-3 for RNP). Of the 3 expected weak reactivities (light gray), 2 were positive on LIA™ (CDC-1 for Sm and CDC-2 for SSA) and 1 was negative (CDC-3 for Sm). Alternatively, the INNO-LIA™ ANAUpdate detected more reactivities than what is observed by IIF or Western blot (CDC-5 for RNP and CDC-10 for SSA/Ro52) (14).

Discussion

In order to optimize and validate the cut-off values for the INNO-LIA™ ANA Update assay, a new cut-off control sample was composed from commercially available samples in such a manner as to ensure the closest approximation to the optimal cut-off values as determined by ROC analysis. These optimal values were determined in order to achieve 98% specificity. The optimized cut-off values of the INNO-LIA™ ANA Update resulted in very high specificity as judged on samples obtained from control subjects, as well as on those from patients with infectious diseases or non-autoimmune rheumatologic disorders. A number of RA patients were anti-histone-positive. This is not uncommon, as antibodies to histones have been reported in as much as 80% of RA patients using an ELISA to detect H1, H2A, H2B, H3, and H4 histones (15).

The patterns of sensitivity for the different samples from the target patient groups corresponded well with the values reported in the literature and reflect the considerable degree of variability observed in previous studies (2). The observed variability may be explained by differences in the selection of patients between studies (e.g. primary versus secondary SS, disease activity, disease stage, etc.) and shows that comparison of data with results presented in the literature is difficult, suggesting that different assays are best compared on the same set of patient samples. Variability versus other assays can be due to the difference in strategy for determining the cut-off values between the different tests. It also has to be noted that as hemagglutination detects both IgM and IgG, these results cannot directly be compared with those obtained by other tests.

Overall, the results of the external studies have shown that the natural Ro60 of the INNO-LIA™ ANA Update is more sensitive than the recombinant material used for immunoblotting probably because conformational epitopes which are thought to be more frequently recognized by anti-Ro60 antibodies are destroyed during the preparation of the protein for immunoblotting. On the other hand, recombinant SSB as used in the INNO-LIA™ ANA Update seems to be more sensitive than natural SSB.

Five SS patients showed Cenp-B reactivity; others were only SSA and SSB positive. These observations are in accordance with a previous study describing SS patients with centromere antibodies showing some specific clinical SS symptoms (16). These patients are often not tested for anti-Cenp-B (although an anti-centromere pattern can be observed when IIF is performed), illustrating the advantage of the INNO-LIA™ ANA Update, which simultaneously tests a full panel of antigens on the same strip. Two of the SLE patients were also found to be reactive with Cenp-B. These observations confirm the recent findings that Cenp-B reactivity is not exclusively associated with scleroderma (17).

The high percentage of anti-SmB-positive MCTD samples was rather striking but can be explained in view of cross-reactivity. Indeed, it is known that anti-RNP-positive samples can cross-react with SmB (9) and, therefore, a sample is considered Sm-positive only in case of SmD positivity. As one of the inclusion criteria of the 39 MCTD samples was anti-RNP positivity, a comparison of the sensitivity of the locally used anti-RNP test with the INNO-LIA™ ANAUpdate would be biased, because both tests were not performed in parallel. It should also be noted that the diagnosis of MCTD is somewhat controversial. In some cases it represents an undifferentiated CTD that later evolves into a well known autoimmune pathology (e.g. SLE or scleroderma), with a high titer of anti-RNPantibodies (18).

The prevalence of anti-Jo-1 antibodies was rather low in the polymyositis group (5%) compared to that described in other polymyositis populations (18%) (19). In contrast, one SS patient was found to be anti-Jo-1 positive on LIA™, which was confirmed on the Jo-1 immunoblot. This patient was also anti-SSA- and SSB-positive when tested in both LIA™ and ELISA with recombinant material.

Noteworthy too is the SSA/Ro52 reactivity of the reference sample CDC-10. This result confirms the findings of Rutjes *et al.* (20) and Meheus *et al.* (6) who described that anti-Ro52 and anti-Jo-1 antibodies frequently co-occur. This was not observed by Smolen *et al*. (14), probably because immunoblotting may not distinguish between these two antigens as they co-migrate on SDS-polyacrylamide gel. Two Ro52 monospecific samples were found in this study, which confirms earlier findings (12).

In conclusion, using the optimized cut-off control, the INNO-LIA™ ANAUpdate assay shows uniformly high specificity mostly exceeding 98% in combination with a sensitivity that is similar to what has been described using other assays. This, together with the fact that the INNO-LIA™ ANA Update allows for simultaneous detection of ANA, makes it a valid complement for reference techniques as confirmation of results determined by IIF ANAor other assays, as well as a valuable assay for screening. The use of this test for early diagnosis of autoimmune CTD should be studied further, since the most relevant time point to study ANAis when a diagnosis has not yet been established. In such cases, positive serologic finding will be of great importance for further diagnostic work-up.

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