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Auto-immunity

CLOSE UP

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A.MENARINI diagnostics

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Aims & Scope
Autoimmunity Close Up is the new A. Menarini Diagnostics publication in the field of autoimmunity. The magazine establishes an interdisciplinary forum connecting experts involved in all aspects of the complex world of autoimmunity diagnosis.
Autoimmunity Close Up provides our customers and colleagues with important product information and updates, insights into issues of general interest in autoimmunity and the latest findings in autoimmune diseases.
The magazine encompasses a wide range of topics including connective tissue diseases, rheumatoid arthritis, antiphospholipid syndrome, celiac disease, IBD, vasculitis, autoimmune thyroid and liver diseases, as well as POCT, ITC & health economics.
Each issue features reviews, editorials, and interviews with leading scientists who actively participate in building the content.
If you have any questions or require further information about *Autoimmunity Close Up*, please contact your local A. Menarini Diagnostics Affiliate or Distributor.

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EDITOR'S NOTE

Is IFA still the Gold Standard in ANA testing?

In this issue of our company magazine "Autoimmunity Close Up", I would like to draw your attention to a topic which continues to be one of the most frequently discussed in congresses, fairs and exhibitions concerning autoimmunity: indirect immunofluorescence (IFA, also IIF).

In a 2011 statement, the American College of Rheumatology recommended that HEp-2 by IFA "should remain the gold standard for antinuclear antibody (ANA) testing". This is especially important when doing initial ANA and antibody screening for patients that may have some form of scleroderma.

Historically all ANA testing was done by IFA. Now, however, some ANA testing uses newer or less-expensive methods such as ELISA or Multiplex. ANA testing by ELISA or Multiplex is very accurate if the patient has one of the antibodies included in the testing panel. However, if the patient has an antibody that is not included in the testing panel, the ANA result itself will be falsely reported as negative, suggesting that the patient does not have an autoimmune disease.

Moreover the appearance of digital imaging systems has recently eliminated some drawbacks of the method, such as subjectivity and lack of an automated procedure, but there are still variabilities in the working protocols to prepare the slides. Therefore the importance of IFA has become controversial, with many people still highlighting its advantages, but others complaining about its drawbacks.

In this issue of Autoimmunity Close Up, **A. Menarini Diagnostics** is happy to have a prestigious contribution on this matter by Professor Isabel Abreu. Professor Abreu lives and works in Portugal and is very well known in the Autoimmunity field throughout Europe thanks to her experience in IFA. In her article, she analyzes the advantages and drawbacks of the IFA technology, and presents a clear conclusion.

Last April, Professor Abreu participated in a symposium organized by Menarini Diagnosticos in Figueira da Foz (Portugal) during the 10th Scientific Meeting of the Portuguese Society of Laboratory Medicine (SPML), which was a huge success, drawing approximately 350 participants. At the same event, Dr. João Pedro Ramos (Unilabs) spoke of the importance of quality control and workflow of IFA tests. To conclude the event, Daria Picchioni (Marketing Manager of Visia Imaging) presented the new **A. Menarini Diagnostics** all-in-one IFA system, Zenit PRO. Further information is included in the Company Pinboard section.

A preliminary evaluation study performed by Dr. Martina Fabris (ASUIUD, Udine, Italy) on Zenit PRO is also included in this issue of Autoimmunity Close Up. Dr. Fabris's suggestions and recommendations following her study were invaluable for **A. Menarini Diagnostics** in improving the

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system, which testifies to the Company's belief in the value of research and innovation to reach reliability and accuracy of results.

Always keeping in mind the importance of research and education, **A. Menarini Diagnostics** is also proud to participate as Gold Sponsor in the most significant Autoimmunity event in this year, the 11th International Autoimmunity Congress in Lisbon (May 16-20, 2018). That's one of the reasons why we decided to publish this special issue of Autoimmunity Close Up. Our booth at the Congress site (#12) will be totally devoted to new Autoimmune IFA systems. We invite everyone to visit us there and see our innovative systems and solutions.

Daria Franceschi
Corporate Product Specialist, Autoimmunity
A. Menarini Diagnostics

TECHNICAL INSIGHTS

Is there still a role for the Indirect Immunofluorescence (IIF) technique in the autoimmune serology laboratory?

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The diagnosis of autoimmune diseases is largely based on the combination of clinical, serological, and radiographic findings.

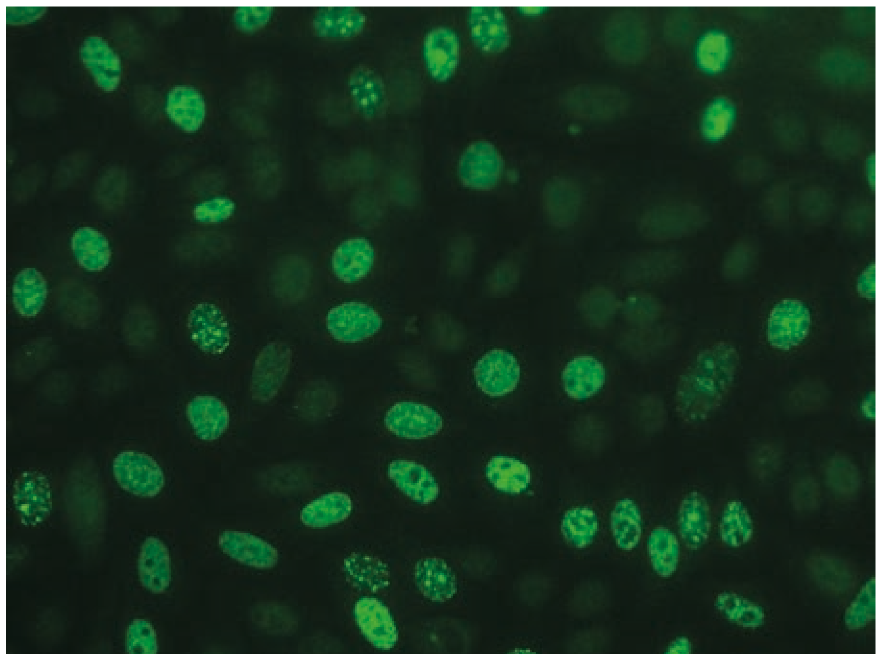
Testing for autoantibodies (AAbs) is an essential step in the serological diagnosis of autoimmune diseases, in particular systemic autoimmune rheumatic diseases (SARDs)[1, 2]. The detection of AAbs that target intracellular antigens, commonly termed anti-nuclear antibodies (ANAs), is a serological hallmark in the diagnosis of SARDs[3].

In the early 1940s, Coons et al. conceptualized and developed immunofluorescent (IF) techniques for labeling antibodies[4,5]. In 1950, Coons and Kaplan described the improvement of an IF method for the detection of antigens in tissue cells[6].

Seven years later (1957), Friou and Holborrow et al. first described an indirect immunofluorescence (IIF) assay for the detection of anti-nuclear antibodies (ANAs)[7,8], which has since become the most widely used test for diagnosis of connective tissue diseases[9]. Initially, different substrates were tried, but later on tissue sections using rat liver or a composite multiblock substrate of rodent (rat/mouse) liver, kidney and stomach became the standard substrate to detect AAbs against cellular antigens[9].

In 1975, Hahon et al. introduced HEp-2 cells (an epithelial cell line derived from an human laryngeal carcinoma), which increased the sensitivity of the test. HEp-2 cells have replaced the frozen sections of organs[10]. Nuclear patterns of fluorescence in rodent substrates were difficult to discern, some ANAs directed against subcellular structures such nucleoli were difficult to identify, and Abs against cell cycle-dependent antigens (Ags) exhibit no immunofluorescence pattern. However, they may be of significance in the diagnosis of some autoimmune disorders, like proliferating cell nuclear antigen (PCNA) in the diagnosis of systemic lupus erythematosus (Fig. 1).

Figure 1: PCNA fluorescent pattern in HEp-2 cells



Other example of AAbs which are also not detected in rodent tissues, but have diagnostic or prognostic utility are the anti-nucleolar Abs in systemic sclerosis[11]. Different specificities show different staining patterns. PM/Scl gives a homogeneous nucleolar pattern, U3-RNP (Fibrillarin)

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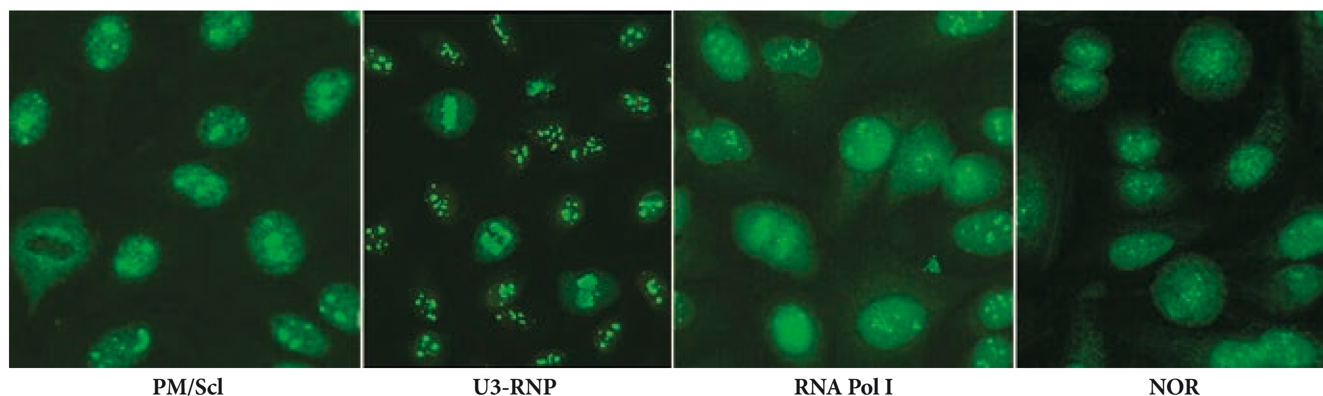
Figure 2: Nucleolar fluorescent pattern in HEp-2 cells



PM/Scl	U3-RNP	RNA Pol I	NOR

Table 1: Advantages of HEp-2 cells over rodent tissue

1.	Higher sensitivity (greater Ag expression)
2.	Human origin ensures better specificity
3.	Higher cell division rates, so cell cycle dependent Abs are easily identified
4.	Nucleus are much larger, visible; and complex nucleolar detail can be seen
5.	Antigens distribution is uniform, not obscuring intercellular matrix

a clumpy nucleolar, RNA polymerase I a speckled, and NOR a punctuate pattern (Fig. 2).

Because both the mitotic phase and the metaphase of the cell cycle are identifiable in HEp-2 cells, information regarding the patterns of the chromosomes is also available. HEp-2 cells combine a good sensitivity with the detection of a wide range of nuclear, nucleoli, cytoplasmic, mitotic spindle apparatus and cell cycle-related autoantibodies.

The advantages of HEp-2 cells over rodent tissue are described in Table 1.

The HEp-2 cell is a native protein array with hundreds of antigens, providing an ideal substrate for the detection of ANA (Figs. 3, 4)[12]. Different Abs give rise to characteristic staining patterns on the cells, depending on the cellular location and properties of antigenic target. To interpret the pattern of fluorescence in HEp-2 cells we have to look at both the resting cells (in interphase) and the dividing cells (in mitosis). It is therefore important to have several cells in different phases of mitosis. The test is highly sensitive, but for the same reason specificity is limited. However, the HEp-2 ANA test has also some disadvantages, which are described in Table 2.

To eliminate the disadvantages of the

Figure 3: ANA in rat liver section

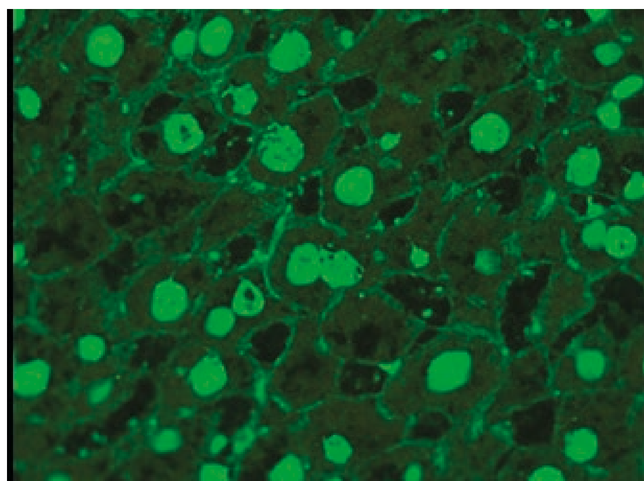


Figure 4: ANA on HEp-2 cells

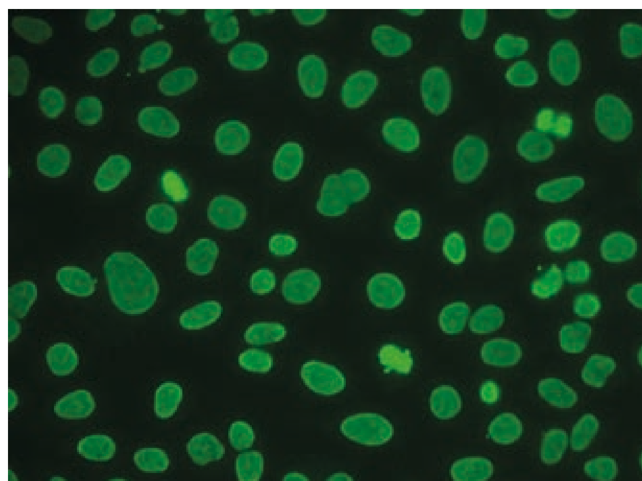


Table 2: Disadvantages of HEp-2 ANA test (adapted from[3])

1.	Subjectivity
2.	Time consuming
3.	Poorly standardized across manufacturers
4.	Requires training and expertise
5.	Low sensitivity for certain clinically important autoantibodies (i.e., Jo-1, ribosomal P, SS-A/Ro60, Ro52/TRIM21)
6.	Low specificity (high false positive rate)

IIF method, many attempts have been made to find a reliable substitution to the IIF assay. Therefore, in some laboratories with high workload the IIF has been replaced by novel techniques based on solid phase assays (SPA) (e.g., ELISA, dot/line immunoassay, and addressable bead/microarray assays)[12, 14-18].

However, high rates of false-negative findings have been reported for these techniques[12, 18]. Addressing this issue, the American College of Rheumatology (ACR) task force confirmed IIF as the gold standard for ANA testing[12].

To overcome the drawbacks of the IIF method in the screening of antibodies against cellular antigens (AC/ANA), automated systems are being introduced on the market to eliminate errors due to subjectivity and manual preparation, thereby reducing the intra-inter laboratory variability. The new all-in-one platforms are powerful tools and represent the future of the autoimmune serology laboratory.

In conclusion, the IIF assay is highly sensitive and cost-effective. A broad spectrum of Abs can be analyzed simultaneously. It is a discovery tool for new antibodies, and there are still some Abs that are detected exclusively by IIF. Both IIF on HEp-2 cells and SPAs have their individual advantages and limitations[3]. To overcome the limitations, we use ANA screening by IIF in combination with an SPA, which adds value to ANA detection.

Is there still a role for the Indirect Immunofluorescence technique (IIF) in the autoimmune serology laboratory? Yes, absolutely.

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RESEARCH UPDATES

Performance evaluation of the Zenit PRO, a new automated system for indirect immunofluorescence: a preliminary study

Martina Fabris

Laboratory of Autoimmunology, Institute of Clinical Pathology, University Hospital of Udine, Italy

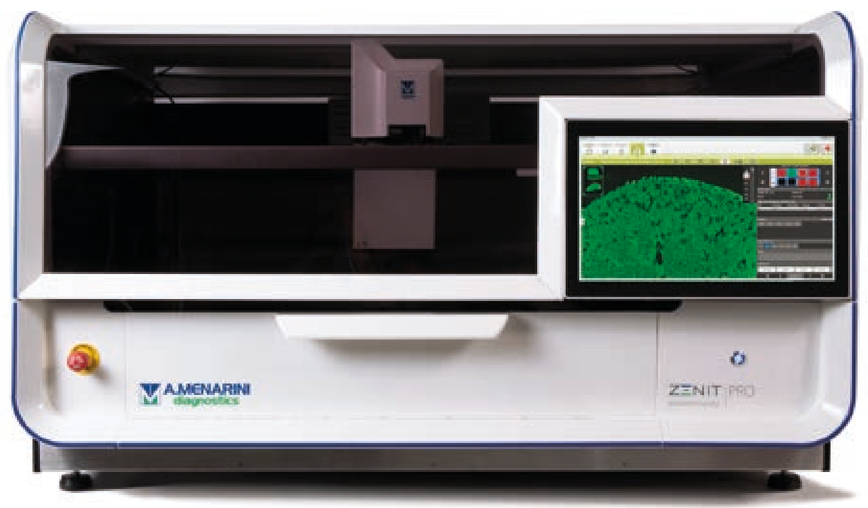
Presented at the 11th International Congress on Autoimmunity **Lisbon, Portugal, 16-20 May 2018**

Aim of the study

The newly developed Zenit PRO system (**A. Menarini Diagnostics**) is a fully automated instrument performing indirect immunofluorescence (IIF) assays that streamlines the complete IIF protocol, from slide processing to reading and interpretation of results (Fig. 1).

The aim of this study was a preliminary evaluation of Zenit PRO anti-nuclear antibody (ANA) testing by IIF on HEp-2 cells on a series of routine samples to set the negative/positive cut-offs and to evaluate operating mode, execution time and analytical performance.

Figure 1: The Zenit PRO IIF automatic analyser



Methods

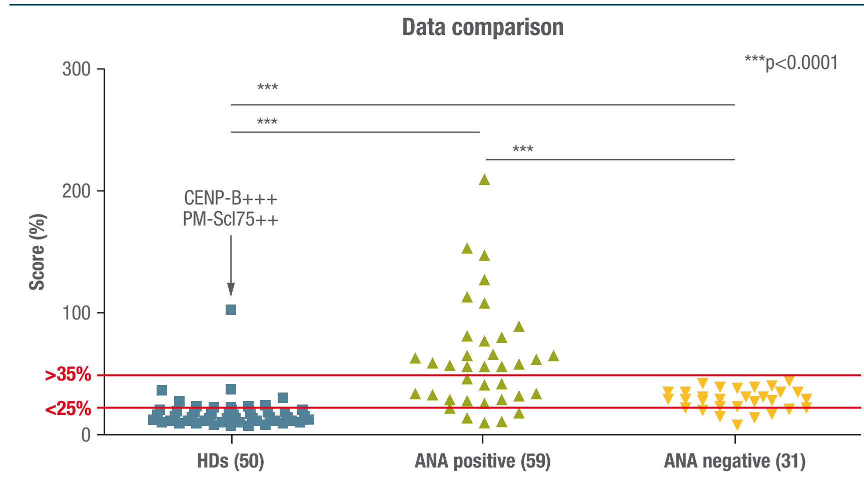
We selected 64 ANA-positive patients with nuclear or cytoplasmic patterns at different titres (from 1:80 to >1:5120), among those 32 with definite diagnoses of autoimmune diseases, either systemic and organ-specific [10 systemic lupus erythematosus (SLE), 9 systemic sclerosis (SSC), 6 Sjögren's syndrome (SjS), 1 undifferentiated connective tissue disease (UCTD), 1 polymyositis, 5 primary biliary cholangitis (PBC)]. In particular, 59 ANA-positive patients (mean age 55±14; 50F/9M) with coverage of all the major nuclear patterns at different titres and five ANA-positive patients (mean age 62±11; 4M/1F) with cytoplasmic patterns (three mitochondria-like, one diffuse fine speckled, one Golgi-like) at different titres (from 1:80 to 1:640).

Thirty-one ANA-negative patients and 50 age/sex matched blood donors (HDs) were selected as a control series. We carried out three complete sessions on three different days. Eight positive samples (two homogeneous, two centromere, two fine speckled, two coarse speckled) at high (1:1280) and low (1:160) titre, were chosen for between-run (five runs in total) and within-run repeatability tests and titrations. The Zenit PRO expresses the fluorescence intensity index score as a percentage (0 to 100% of the sensor saturation), so we compared % versus standard titrations observed in the same sera using the in-house automatic method for IIF HEp-2 analysis (Inova Diagnostics, CA).

Results

Overall, ANA-positive samples disclosed higher % scores than the HDs (p<0.0001)

Figure 2: Raw data comparison between the fluorescence index scores obtained by the Zenit PRO in the HDs, ANA-positive and ANA-negative samples

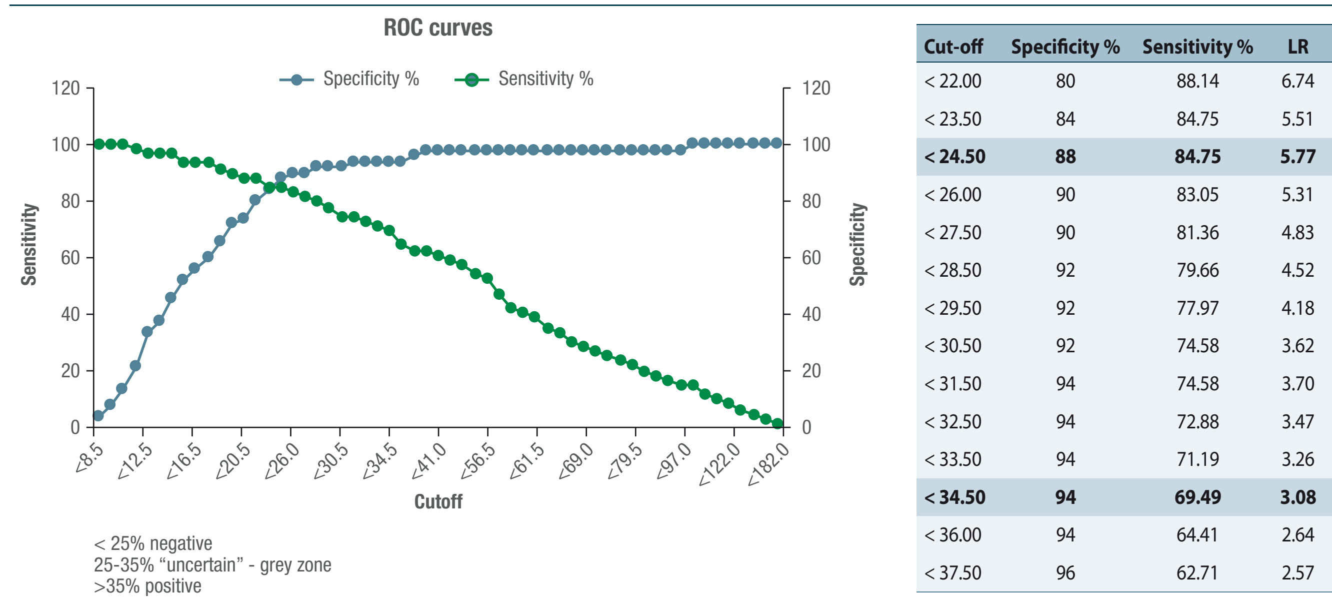


and ANA-negative samples (p<0.0001; Fig. 2). Of note, the only high positive sample among HDs finally disclosed high titre anti-centromere antibodies and anti-PM-Scl75 antibodies when tested by line blot (Euroimmun, Germany). When comparing ANA-positive samples versus HDs, the ROC curve analysis (Fig. 3) identified the fluorescence intensity index score <25% as the negative cut-off with a sensitivity of 85% and a specificity of 88% (LR 5.8), a grey zone between 25% and 35% and a positive cut-off >35%, with a sensitivity of 70% and a specificity of 94% (LR 3). When these cut-offs were applied to the three series (Fig. 2), all the samples reported as ANA-negative using the in-house IIF HEp-2 analytical method, remained below 25% or in the grey zone, with a moderate-to-good final concordance between the two automatic IIF analysers (overall concordance 0.779; K of Cohen 0.563), that appeared in line with previous experiences as regards automated ANA IIF methods comparison[1,2]. As shown in Table 1, the agreement between the two ANA IIF automatic methods increased from 50-75% for low-positive ANA samples (1:80 to 1:160) to 96-100% for high positive ANA samples (1:320 to ≥1:1280).

As concerns the cytoplasmic patterns, the overall concordance was 83.3% (4/5); the only discordant result was at low titre 1:80 (diffuse fine speckled with anti-SRP antibodies).

As illustrated in Table 2A, between-run repeatability tests disclosed quite good performances in all the major ANA patterns, either at high or at low titre, with a mean CV of 18±6%. Even better results were obtained by the within-run repeatability tests (Table

Figure 3: ROC curve analysis and cut-off settings



Cut-off	Specificity %	Sensitivity %	LR
< 22.00	80	88.14	6.74
< 23.50	84	84.75	5.51
< 24.50	**88**	**84.75**	**5.77**
< 26.00	90	83.05	5.31
< 27.50	90	81.36	4.83
< 28.50	92	79.66	4.52
< 29.50	92	77.97	4.18
< 30.50	92	74.58	3.62
< 31.50	94	74.58	3.70
< 32.50	94	72.88	3.47
< 33.50	94	71.19	3.26
< 34.50	**94**	**69.49**	**3.08**
< 36.00	94	64.41	2.64
< 37.50	96	62.71	2.57

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Table 1: Comparison between ANA results and interpretation by the in-house IIF method and the Zenit PRO. ANA-positive samples (nuclear patterns) are displayed by increasing ANA titre (from 1:80 to 1:5120). Samples with low titre (1:80 to 1:160) showed 50% to 75% of concordance, while high positive ANA samples (1:320 to ≥1:1280) revealed very high concordance (96%-100%). Legend: pos= positive; neg= negative; unc: uncertain (grey zone)

Patient ID	Age	Sex	ANA pattern	Titre	ANA specificity	Diagnosis	ANA results in-house method	Zenit PRO index score	Zenit PRO interpretation
5569348604	50	F	DFS70-like	80	DFS70+		Pos	28%	Unc
5566647704	36	F	Nuclear Matrix	80			Pos	15%	Neg
5570797303	66	F	Homogeneous	80			Pos	12%	Neg
5567962906	74	F	Homogeneous	80	anti-dsDNA+++	SLE	Pos	42%	Pos
5548116907	29	F	Homogeneous	80	SSA-Ro52 / dsDNA	SLE	Pos	19%	Neg
5562825707	30	M	Coarse Speckled	80	Sm/RNP+		Pos	59%	Pos
5569375506	21	F	DFS70-like	160	DFS70+		Pos	20%	Neg
5567457703	51	F	DFS70-like	160	DFS70++ / SSA-Ro60+		Pos	32%	Unc
5570945205	67	M	Mitotic Fuse and Homog.	160			Pos	18%	Neg
5568824703	50	F	Nuclear Matrix	160			Pos	58%	Pos
5569442603	56	F	Multiple Nuc. dots	160	SP100++	PBC	Pos	23%	Neg
5568597703	56	F	Multiple Nuc. dots	160	PML++	PBC	Pos	25%	Unc
5570856103	39	F	Homogeneous	160			Pos	29%	Unc
5569544103	70	F	Homogeneous	160			Pos	27%	Unc
5545609707	78	M	Homo and cyto. diffuse fine sp.	160	OJ+		Pos	35%	Unc
5559429704	74	M	Homog. and nucleolar	160			Pos	47%	Pos
5548557603	55	F	Homog. and nucleolar	160			Pos	30%	Unc
5566787603	55	F	Fine speckled	160	SSA-Ro60++	SjS	Pos	43%	Pos
5569472904	63	F	Fine speckled	160	SSA-Ro60+++		Pos	15%	Neg
5569934405	65	M	Fine speckled	160			Pos	11%	Neg
5571915007	52	F	Fine speckled	160	SSA-Ro60+++	SjS	Pos	66%	Pos
5570371503	54	F	DFS70-like	320	DFS70+++		Pos	47%	Pos
5567404005	55	F	DFS70-like	320	DFS70+++		Pos	56%	Pos
5567791804	34	F	DFS70-like	320	DFS70+++		Pos	75%	Pos
5568612505	61	F	DFS70-like	320	DFS70+++		Pos	40%	Pos
5568504904	75	F	Homogeneous	320			Pos	37%	Pos
5570150604	43	M	Homogeneous	320	dsDNA++	SLE	Pos	35%	Unc
5571155406	53	F	Homogeneous	320	SSA-Ro60++		Pos	35%	Unc
5569373001	17	F	Homogeneous	320			Pos	33%	Unc

Table 1 Continued

Patient ID	Age	Sex	ANA pattern	Titre	ANA specificity	Diagnosis	ANA results in-house method	Zenit PRO index score	Zenit PRO interpretation
5569536403	31	F	Homogeneous	320			Pos	57%	Pos
5570388704	51	F	Fine speckled	320	SSA-Ro60+++		Pos	64%	Pos
5563277005	62	F	Coarse Speckled	320	SSA-Ro60+++/Sm/RNP+	UCTD	Pos	34%	Unc
5568098708	38	F	Homogeneous	640	dsDNA++	SLE	Pos	148%	Pos
5570450604	53	F	Homogeneous	640	SCL70+++	SSC	Pos	57%	Pos
5567973207	65	F	Homogeneous	640	SSA-Ro60+++	SjS	Pos	90%	Pos
5569079306	45	F	Homogeneous	640	dsDNA+++	SLE	Pos	210%	Pos
5568303503	46	F	Homogeneous	640			Pos	58%	Pos
5568573405	70	F	Homogeneous	640			Pos	60%	Pos
5559434806	47	F	Homogeneous	640	SCL70+++	SSC	Pos	81%	Pos
5552921708	62	F	Homogeneous	640	SCL70+++	SSC	Pos	23%	Neg
5532668303	50	M	Homogeneous	640	SCL70+++	SSC	Pos	57%	Pos
5532253606	69	F	Homogeneous	640	SCL70+++	SSC	Pos	82%	Pos
5563085904	70	F	Coarse Speckled	640	Sm/RNP+++		Pos	154%	Pos
5556853206	56	F	Centromere and fine speckled	640	CENP-B+++ / SSA-Ro52+	SSC	Pos	30%	Unc
5569494005	62	F	Centromere	1280	CENP-B+++	SSC	Pos	116%	Pos
5570420906	55	F	Centromere	1280	CENP-B+++	SSC	Pos	76%	Pos
5569340804	51	F	DFS70-like and few dots	1280	DFS70+++ / SP100+++		Pos	109%	Pos
5555702503	49	F	Nucleolar	1280	PM-Scl100+		Pos	63%	Pos
5545732305	41	F	Nucleolar	1280	dsDNA+		Pos	71%	Pos
5571349706	68	M	Homogeneous	1280	dsDNA+++	SLE	Pos	67%	Pos
5570426103	64	F	Homogeneous	1280		SLE	Pos	66%	Pos
5558073804	37	F	Homogeneous	1280	Nucelosome+++/ Histone++	SLE	Pos	78%	Pos
5568908104	69	F	Fine speckled	1280	SSA+++ / SSB+++	SjS	Pos	109%	Pos
5561229703	67	F	Centromere	2560		SjS	Pos	58%	Pos
5570407406	50	F	Homogeneous	2560	SSA+++	SjS	Pos	91%	Pos
5566259607	66	F	Homogeneous	2560	dsDNA+++	SLE	Pos	63%	Pos
5569975504	79	F	Mitotic Fuse and fine speckled	2560	SSA+++		Pos	128%	Pos
5569076504	69	M	Centromere	5120		SSC	Pos	142%	Pos
5568095106	76	F	Homogeneous	5120	dsDNA+++	SLE	Pos	114%	Pos

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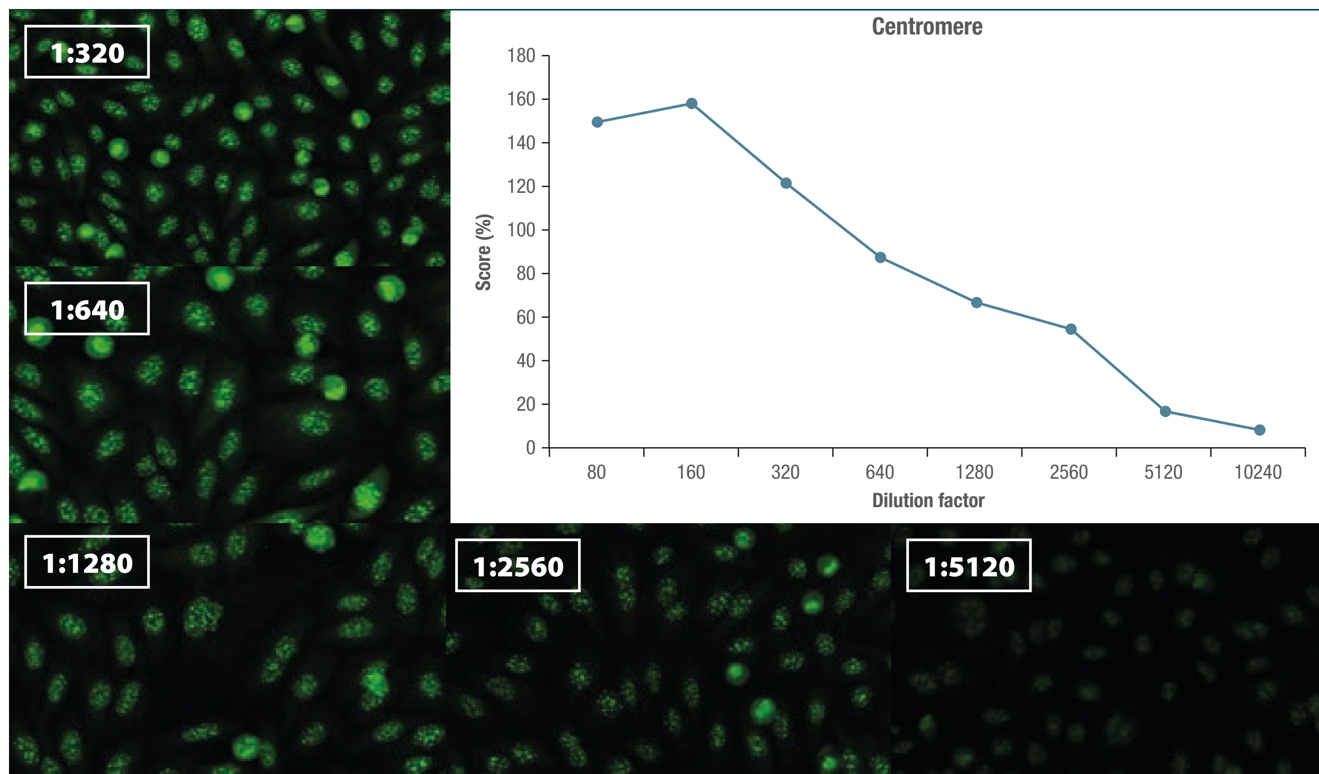
Table 2: Raw data of the between-run repeatability tests (A) and of the within-run repeatability tests (B)

A - Between-run									
Sample ID	Pattern	Run 1	Run 2	Run 3	Run 4	Run 5	Mean	SD	CV%
5569076504	CENP +++	142	113	92	118	132	119	17.11	14.33%
5571349706	Homog +++	67	59	76	82	71	71	7.82	11.02%
5568908104	Fine Sp+++	109	203	176	144	142	155	32.10	20.73%
5563085904	Coarse Sp+++	154	132	107	158	155	141	19.45	13.77%
5562825707	Coarse Sp+	59	30	54	48	50	48	9.85	20.43%
5570150604	Homog +	35	34	56	71	40	47	14.27	30.24%
5570388704	Fine Sp+	64	80	52	50	55	60	11.00	18.27%
5569494005	CENP +	116	76	116	116	116	108	16.00	14.81%
								Mean	18%
								SD	6%

B - Within-run												
Sample ID	Pattern	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Mean	SD	CV%
5569076504	CENP +++	138	182	178	162	161	169	177	186	169	15.5	0.09
5571349706	Homog +++	111	75	96	108	101	102	109	95	100	11.6	0.12
5568908104	Fine Sp+++	144	152	146	140	162	136	142	179	150	14.1	0.09
5563085904	Coarse Sp+++	158	172	174	201	167	187	190	192	180	14.6	0.08
											Mean	10%
											SD	1%

Figure 4: Titration performance in a sample with high titre centromere pattern



2B), which revealed a mean CV around 10% for all the patterns investigated. Also the titrations run very well, as we observed a linear response along titrations for all the different patterns (see Figure 4 for the centromere sample).

Each session (comprising about 150 samples) was run completely (from slide processing to reading) in about four hours. The touch-screen monitor incorporated in the processing unit provides an intuitive and simple interface, displaying high resolution images with >3000 cells per well, and offering the possibility to navigate inside the well as a virtual microscope. In addition, a mitotic gallery is always available to discriminate critical cases.

Conclusions

The Zenit PRO automatic IIF analyser gave the impression of being a highly promising instrument. It showed good agreement with the in-house automatic method and good analytical performances. Its unique features (end-to-end management of the overall IIF analytical process) will allow maximizing the "walk-away" time and improve the standardization of the entire process.

Consolidation of the preliminarily identified negative/positive cut-offs is underway using larger series as is the optimization of the software for pattern recognition and other IIF substrate automatic analyses. This will finally close the gap in standardization by reducing significantly the variability of subjective interpretation.

References

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COMPANY PINBOARD

Latest Marketing & Scientific Events

SPML Congress, Figueira da Foz (Portugal)

In mid April 2018, about 350 participants attended an Autoimmunity Symposium organized by **A. Menarini Diagnosticos** Portugal during the 10th SPML Meeting of Laboratory Medicine, held in Figueira da Foz.
Prof. Abreu (Nova University, Lisbon), Dr. Ramos (Unilabs) and Dr. Daria Picchioni (Visia Imaging) presented and discussed the following topics:

- Is there still a role for IIF in the Autoimmune Serology Laboratory?
- Laboratory challenges: traceability, quality control and workflow
- An all-in-one workstation for IIF automated procedures.

The speakers with part of the Menarini Diagnosticos team at the SPLM meeting

2018 International Congress on Autoimmunity (16th-20th May 2018)

The 11th International Congress on Autoimmunity will take place in Lisbon, Portugal at the Lisbon Congress Center.
Organized by Professor Yehuda Shoenfeld, the Congress will see the participation of the main international leaders in Autoimmune diseases.
Participants are welcome to take advantage of the following contributions provided by **A. Menarini Diagnostics**, a Gold Sponsor of the meeting:

- The A. Menarini Diagnostics 64 sqm booth will display the latest technical achievements in IFA. Visitors will have the opportunity to stop by and discuss specific topics;
- A Parallel Session (PL28) in Auditorium I (18th May, 14:00-16:00) "Dilemmas in the diagnosis of autoimmune diseases, detection and standardization", will feature Nicola Bizzaro (Italy), Xavier Bossuyt (Belgium) and Guy Serre (France) as Chairmen
- A Short Oral Discussion (SO15, 19th May, 13:15-13:20) "Zenit PRO, a fully automated indirect immune fluorescence analyser: a preliminary evaluation of the analytical performance" by Dr. Martina Fabris (Italy).

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