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Detection of antinuclear antibodies: recommendations from EFLM, EASI and ICAP

Citation for published version (APA):

Bonroy, C., Vercammen, M., Fierz, W., Andrade, L. E. C., Van Hoovels, L., Infantino, M., Fritzler, M. J., Bogdanos, D., Kozmar, A., Nespola, B., Broeders, S., Patel, D., Herold, M., Zheng, B., Chan, E. Y. T., Uibo, R., Haapala, A.-M., Musset, L., Sack, U., ... International Consensus on Antinuclear Antibody Patterns (ICAP) (2023). Detection of antinuclear antibodies: recommendations from EFLM, EASI and ICAP. *Clinical Chemistry and Laboratory Medicine*, *61*(7), 1167-1198. https://doi.org/10.1515/cclm-2023-0209

Document status and date:
Published: 27/06/2023

DOI:
10.1515/cclm-2023-0209

Document Version:
Publisher's PDF, also known as Version of record

Document license:
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Please check the document version of this publication:

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EFLM Paper

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Detection of antinuclear antibodies: recommendations from EFLM, EASI and ICAP

https://doi.org/10.1515/cclm-2023-0209
Received February 27, 2023; accepted February 27, 2023;
published online March 29, 2023

Abstract

Objectives: Antinuclear antibodies (ANA) are important for the diagnosis of various autoimmune diseases. ANA are

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usually detected by indirect immunofluorescence assay (IFA) using HEp-2 cells (HEp-2 IFA). There are many variables influencing HEp-2 IFA results, such as subjective visual reading, serum screening dilution, substrate manufacturing, microscope components and conjugate. Newer developments on ANA testing that offer novel features adopted by some clinical laboratories include automated computer-assisted diagnosis (CAD) systems and solid phase assays (SPA).

Methods: A group of experts reviewed current literature and established recommendations on methodological aspects of ANA testing. This process was supported by a two round Delphi exercise. International expert groups that participated in this initiative included (i) the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Working Group "Autoimmunity Testing"; (ii) the European Autoimmune Standardization Initiative (EASI); and (iii) the International Consensus on ANA Patterns (ICAP).

Results: In total, 35 recommendations/statements related to (i) ANA testing and reporting by HEp-2 IFA; (ii) HEp-2 IFA methodological aspects including substrate/conjugate selection and the application of CAD systems; (iii) quality assurance; (iv) HEp-2 IFA validation/verification approaches and (v) SPA were formulated. Globally, 95% of all submitted scores in the final Delphi round were above 6 (moderately agree, agree or strongly agree) and 85% above 7 (agree and strongly agree), indicating strong international support for the proposed recommendations.

Conclusions: These recommendations are an important step to achieve high quality ANA testing.

Keywords: antinuclear antibodies; HEp-2 indirect immunofluorescence; recommendations.

Introduction

Since their first description in 1958, the detection of antinuclear antibodies (ANA) has a central role in the diagnostic work-up of several systemic autoimmune rheumatic diseases, the so-called ANA-associated rheumatic diseases (AARD) (reviewed in [1]). ANA are also important in the diagnosis of juvenile idiopathic arthritis [2], autoimmune hepatitis [3] and primary biliary cholangitis [4]. Traditionally, they have been detected by indirect immunofluorescence assay (IFA) using rat liver substrate, or HEp-2 cells, the latter usually used. IFA is challenging to harmonize due to subjectivity and inter-observer variation (visual microscope reading and pattern recognition, nomenclature assignment) and a high number of technical variables impacting the results (e.g., serum screening dilution, substrate, secondary conjugate) [5].

At present, HEp-2 IFA analysis is increasingly supplemented with automated approaches such as automated slide processors, computer-aided diagnosis (CAD) IFA systems and solid phase assays (SPAs). All these newer approaches represent important evolutions within the ANA field and provide new opportunities, especially in the context of quality assurance.

For this work, we reviewed current publications and guidelines in the field of ANA testing with a focus on areas including preferred methodology (IFA vs. SPA), optimal screening dilution and titer reporting, IFA HEp-2 pattern reporting, substrate and conjugate selection, use of CAD platforms, quality assurance approaches and validation/verification. Based on this literature review, a set of recommendations on these topics was formulated and subjected to expert review and a Delphi exercise for further fine-tuning and documenting international support.

Methods

Literature review and expert committees

A literature review was performed by XB, CB and MV. Searches were done in PubMed and through limo libis KU Leuven. References in the papers were checked and retrieved if relevant. Additional sources included publications from "Clinical and Laboratory Standards Institute" (CLSI), College of American Pathologists (CAP), ISO 15189, ISO 17025, Bureau International des Poids et Mesures (https://www.bipm.org), the World

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Health Organization (WHO), and the In-Vitro Diagnostic Medical Devices Regulation (EU) 2017/746 (IVDR). Finally, national guidelines/recommendations from France (COFRAC – Comité Français d'Accréditation), The Netherlands (NVKC – Nederlandse Vereniging voor Klinische Chemie en Laboratorium geneeskunde and CMI – College of Medical Immunologists) and Belgium were consulted.

Three groups of experts participated in this initiative: (i) the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Working Group "Autoimmunity Testing;" (ii) the European Autoimmune Standardization Initiative (EASI); and (iii) the International Consensus on ANA Patterns (ICAP), including members of its parent IUIS Subcommittee for the Standardization of Autoantibodies in Rheumatic and Related Diseases (a.k.a. Autoantibody Standardization Committee).

In this study, EASI experts from 18 countries (Austria, Belgium, Croatia, Estonia, Finland, France, Germany, Greece, Hungary, Israel, Italy, The Netherlands, Norway, Poland, Portugal, Spain, Sweden and Switzerland) and from Russia participated. For ICAP, experts from 10 countries (USA, Mexico, Canada, Brazil, Argentina, Chili, Uruguay, Japan, Korea, China and Hong Kong) participated.

Development of the recommendations/statements

A three-step process (see overview of the Delphi process in Supplementary Figure 1) was utilized to create the recommendations on the detection of ANA. First, based on the literature review, a draft manuscript was prepared and 36 preliminary recommendations/statements were formulated by the EFLM Working Group "Autoimmune Testing" and ratified by EFLM. The statements were graded as A [experimental data/literature source is available] or B [expert opinion]. Then the manuscript and the statements were subjected to open comments/suggestions and grading by EASI (EASI Europe) and ICAP on a scale of 1–5 (i.e., 1=no agreement to 5=full agreement) (performed by one representative per country [EASI]/geographical area [ICAP]). Finally, a Delphi exercise with closed voting followed. During this final round, scoring on a scale 1–9 [0=absolutely no agreement with the recommendation; 9=maximal support for the recommendation] and an option to skip the question based on inexperience (score 15) was performed by 149 experts (consisting of 98 participants engaged by EASI (median six members/country [range 1–7]) and 51 participants engaged by ICAP (median six members/geographical area [range 3–7])), affiliated with 133 centres (an overview of the geographical distribution of the participants is included in Supplementary Table 1). Median and % high scores at two different cut-offs (score ≥8 or ≥7 on a scale of 9) of all participants were calculated for each recommendation.

Clinical conditions associated with ANA

Systemic rheumatic diseases

ANA are important laboratory biomarkers to support the diagnosis and, in some cases, the prognosis of systemic lupus erythematosus (SLE), Sjögren Syndrome (SjS), systemic sclerosis (SSc), mixed connective tissue disease (MCTD) and idiopathic inflammatory myopathies (IIM). These diseases are commonly denoted as AARD (ANA-associated rheumatic diseases). ANA can be detected by indirect IFA using HEp-2 cell substrates, or alternatively SSA/Ro60-transfected HEp-2000 cells. Many target antigens of autoantibodies in AARD have been identified and specific SPAs to detect these antibodies are available (e.g., antibodies to double-stranded DNA [dsDNA], Sm, Rib-P in SLE, SSA/Ro60 and/or Ro52/TRIM21 and SSB/La in SjS, U1-RNP in MCTD, centromere protein B [CENP-B], topoisomerase-I [Scl-70], RNA polymerase III, fibrillarin, PM-Scl in SSc and Jo1, PL7, PL12, EJ, OJ, Mi2, MDA5, TIF1γ, NXP2, SAE, SRP, and HMGCR in IIM). Some specific autoantibodies are included in the classification criteria for SLE (anti-dsDNA, anti-Sm), SjS (anti-SSA/Ro), SSc (anti-topoisomerase-I/Scl70, anti-CENP-B, anti-RNA polymerase III), MCTD (anti-U1-RNP) and IIM (anti-Jo1) (reviewed in [1]). SPAs that detect antibodies to a mixture of autoantigens relevant for AARD and multiplexed assays that simultaneously detect and identify these different ANA reactivities are available.

Recent literature (mainly reviews) is cited here for detailed information on ANA detection by HEp-2 IFA and SPA in AARD [1, 6]. In short, ANA detection by HEp-2 IFA at a 1:80 screening dilution is highly sensitive but suffers from low specificity. Because of its high sensitivity, HEp-2 IFA has been considered the gold standard for ANA detection [7, 8] and recently included as entry criterion for SLE classification [9]. The performance characteristics of SPAs differ between the different commercial assays. SPAs that screen for a set of AARD-associated antibodies (either by a screening assay or by a multiplexed assay) generally have a higher specificity but lower sensitivity than HEp-2 IFA (reviewed in [1], see [10] for recent meta-analysis). The performance of HEp-2 IFA and SPA is disease-dependent (e.g., IFA shows higher sensitivity than SPA for SSc but not for SjS) [11–13] (reviewed in [1]). An important characteristic of ANA testing is that, overall, the chance (likelihood) for disease increases with increasing antibody levels for both HEp-2 IFA and SPA [14–17] (reviewed in [1]). As there is no single HEp-2 IFA or SPA assay that has both high sensitivity and specificity, combining IFA with SPA confers the highest clinical utility: double positivity has the highest likelihood ratio (LR) for AARD and HEp-2 IFA may detect antibodies that are missed by SPA (e.g., in SSc) and, conversely SPA may identify antibodies that are missed by IFA (e.g., in SjS) (reviewed in [1]).

Juvenile idiopathic arthritis

In a recently published international consensus from the Pediatric Rheumatology International Trials Organization, new classification criteria for juvenile idiopathic arthritis (JIA) have been proposed [2]. In this consensus, early-onset ANA-positive JIA is defined by early-onset (≤6 years of age) arthritis for ≥6 weeks, and the presence of two positive ANA tests with a titer ≥1:160 (tested by HEp-2 IFA) at least 3 months apart [2]. Exclusions are systemic JIA, rheumatoid factor (RF)-positive

arthritis and enthesitis/spondylitis-related JIA [2]. The ≥1:160 cut-off was based on previous studies [19, 20].

Young age at onset of arthritis and ANA positivity by IFA (not by ELISA) are well-recognized predictors of uveitis (ocular complications) in JIA [21–24]. In recent population-based Nordic studies [21, 22], cutoff values for ANA as a marker for uveitis in JIA varied between 1:80 and 1:320, depending on the participating center. Nordal et al. [24] reported that the predictive value of ANA for uveitis increased with increasing titer, but the sensitivity decreased (e.g., 1:80 has high sensitivity whereas 1:320 has high specificity). More research is required to achieve clarity on ANA testing protocols in JIA [25].

Summary of current consensus/position statements for JIA

- For classification of early-onset ANA-positive JIA, ANA by HEp-2 IFA (cutoff ≥1:160) should be used, according to the international consensus from the Pediatric Rheumatology International Trials Organization.
- For prediction of uveitis in JIA, ANA by IFA should be used. There is no consensus cutoff, but the higher the titer, the higher the predictive value and the lower the sensitivity.

Autoimmune hepatitis

Several guidelines on the diagnosis of autoimmune hepatitis (AIH) have been formulated by international expert groups. An overview of these guidelines follows.

In 1999, the International Autoimmune Hepatitis Group (IAIHG) reported criteria for diagnosis of autoimmune hepatitis in which it was stated that seropositivity for ANA, smooth muscle antibodies (SMA) or anti-liver-kidney microsomes-1 (LKM-1) at titers >1:80 contribute to a definite diagnosis of AIH (a titer 1:40 contributes to a probable diagnosis) [26]. In children, lower titers (particularly for LKM-1) may be significant [26]. The authors stated that the antibodies should be determined by IFA on rodent tissues or, for ANA, on HEp-2 cells [26]. In 2008, the IAIHG issued simplified criteria for the diagnosis of AIH [26]. In these criteria, ANA or SMA with titer ≥1:40 contribute one point and ANA or SMA with titer ≥1:80 or LKM with titer ≥1:40 or SLA/LP (soluble liver antigen/liver-pancreas antibody) positivity (by ELISA SPA, not detectable on IFA) contribute two points [27]. The authors recognized that ANA screening by HEp-2 IFA may give higher titers than screening on tissue sections and stated that if results from HEp-2 cells are used, the titers should be halved [27].

In 2004, a consensus statement from the committee for autoimmune serology of IAIHG stated that the basic technique for routine testing of autoantibodies relevant to AIH is IFA on a freshly removed rodent multi-organ substrate panel (including kidney, liver and stomach) [3]. This allows detection of ANA, SMA, anti-LKM-1, anti-mitochondrial (AMA) antibodies and antibodies to liver cytosol type 1 (anti-LC1) [3]. The starting dilution proposed is 1:10, but a clinically significant level of positivity is considered 1:40 for adults [3]. For subjects <18 years old positivity at dilutions of 1:20 for ANA and SMA and 1:10 for anti-LKM is considered clinically relevant [3]. In case ANA are detected, the pattern can be further defined by IFA on HEp-2 cells as these cells have more prominent nuclei [3].

The use of rodent substrate for screening for AIH was further emphasized by the EASL practice guidelines on autoimmune hepatitis [28] and by the ESPGHAN Hepatology Committee position statement on diagnosis and management of pediatric autoimmune liver disease [29]. The authors of the ESPGHAN position statement concluded that IFA on rodent tissue not only aids in the diagnosis of AIH but also allows the differentiation in AIH-1 (characterized by ANA and SMA) and AIH-2 (characterized by anti-LKM-1 and anti-LC1) [29]. The authors also stressed that a dilution ≥1:40 should be considered positive in adults and a dilution ≥1:20 should be considered positive in children [29]. The ESPGHAN position statement did not recommend the use of HEp-2 cells to screen for ANA in AIH because of the relatively high positivity rate in the normal population and in infections, especially in children [29]. HEp-2 cells (with their prominent nuclei), however, were recommended to define the ANA IFA pattern when a positive ANA is detected on rodent tissue [29].

Summary of current consensus/position statements (or criteria) for AIH

- In case of suspicion of autoimmune hepatitis (abnormal liver tests, elevated IgG, interface hepatitis), antibodies should be detected by IFA on rodent tissue (liver, kidney, stomach), which allows detection of ANA, SMA, anti-LKM-1 and anti-LC1.
- In case of positive ANA, the pattern should be defined by IFA on HEp-2 cells.
- According to international guidelines and position statements, the threshold for positivity should be ≥1:40 for adults and ≥1:20 for children on rodent tissue.

Primary biliary cholangitis

European Association for the Study of the Liver (EASL) recommends to screen for anti-mitochondrial antibodies (AMA) (1:40) and for primary biliary cholangitis (PBC)-specific ANA by IFA in patients with chronic intrahepatic cholestasis (elevated ALP, γGT, conjugated bilirubin, elevated IgM) [4]. AMA are directed against the E2-subunits of the 2-oxo-acid dehydrogenases (PDC-E2, OGDC-E2, BCOADC-E2) and are positive in >90% of patients with PBC. Although IFA on triple-tissue (liver, kidney, stomach) is considered the reference method for detection of AMA, a

recent study suggested that IFA on rodent triple-tissues might miss some antibodies [30]. AMA can also be detected by HEp-2 IFA, however, the AMA-like cytoplasmic reticular pattern (anti-cellular [AC]-21 in the ICAP classification) cannot be considered specific and an antigen-specific immunoassay is necessary for confirmation [30]. For AMA detection, Florin et al. [31] reported a good agreement between IFA on triple-tissues and dot blot but not between HEp-2 IFA and dot blot. The typical AMA-like pattern (AC-21) on HEp-2 cells was only observed in 25% of the AMA-positive samples by triple substrate and/or dot blot [31]. Additional studies are needed to document the performance of HEp-2 IFA vs. triple substrate IFA for screening for AMA.

PBC-specific ANA include anti-Sp100 (multiple nuclear dots [AC-6] on HEp-2 IFA) and gp210 (punctate nuclear envelope [AC-12] on HEp-2 IFA). They can be found in a subset of patients (including AMA-negative PBC sera) [4]. Antigen-specific assays (e.g., ELISA or dot/line blots) are available to test for these antibodies.

Summary of current recommendations/literature for PBC

- In case of a clinical suspicion of PBC, anti-mitochondrial antibodies should be screened for by IFA on triple-substrate. Anti-PDH-E2 antibodies can be confirmed by antigen-specific assays.
- The value and performance of HEp-2 IFA for detection of AMA needs further study.
- PBC-specific ANA can be screened for by HEp-2 IFA and confirmed by antigen-specific assays for anti-sp100 and anti-gp210.

ANA in the general/healthy population

To better interpret ANA test results, it is important to recognize that ANA are frequently found in the general population. Hereunder is a selection of studies that describe ANA prevalence (assessed by HEp-2 IFA) in the general population in different geographies.

- In a cross-sectional study in China, the prevalence of ANA (by HEp-2 IFA) in the general population (20,970 individuals aged 2–88 years [mean age 32]) was 13.98% when a 1:100 cutoff was used and 5.92% when a 1:320 cutoff was used [32]. The positivity rate was higher in females/girls than in males/boys and in older people than in younger people (with a higher prevalence at the age of 20–30 years old) [32].
- In a cross-sectional study in the USA, the prevalence of ANA (by HEp-2 IFA) in the general population [4,754 individuals aged ≥4 years] was 13.8% when a 1:80 cutoff was used [33]. ANA prevalence increased with age and ANA were more prevalent among females than males [33].
- In a cross-sectional study in Germany [34], the prevalence of ANA (by semi-automated HEp-2 IFA system) in the general population [1,199 individuals aged ≥20 years] was 33% when a cutoff of 1:80 was used. The majority (29%) of the ANA were weak positive (titer 1:80 or 1:160). ANA positivity was more common among women than among men [34]. Comparable to the results obtained in Germany, the prevalence of ANA (by semi-automated HEp-2 IFA) in 279 Belgian blood donors (median age [range] 46 [18–68]) at a 1:80 cutoff was 34% [16].
- In a cross-sectional study in Brazil, the prevalence of ANA (by HEp-2 IFA) in a cohort of 918 healthy individuals was 12.9% at a 1:80 cut-off [35]. The majority (53.4%) had titer ≤1:160 in contrast to only 10.8% in a cohort of AARD. The IFA pattern also differed between the healthy and the AARD cohort. The dense fine speckled nuclear pattern (DFS, AC-2) was present in 33.1% of the ANA-positive healthy individuals and in none of the AARD patients. In contrast, the homogeneous nuclear pattern (AC-1), centromere pattern (AC-3), and the coarse speckled nuclear pattern (AC-5) were exclusively observed in the AARD patients.
- A meta-analysis on ANA studies performed between 1997 and 2013, reported a specificity (determined in healthy controls) of 91.3% (CI 86.1–94.7%) at a 1:80 dilution and of 79.2% (CI 72.3–84.8%) at a 1:40 dilution [36].

Some studies specifically focused on children. Sperotto et al. [37] reported that 12% of 261 healthy Italian children (aged 8–13 years) were ANA-positive (by HEp-2 IFA), Hilario et al. [38] that 12.7% of Brazilian healthy children and adolescents were ANA positive (by HEp-2 IFA) and Somers et al. [39] that up to 15% of children of the general pediatric population (aged 9–17 years) of Mexico City (n=114) were ANA positive, with a higher rate of positivity among females. All three studies applied a 1:80 cutoff. In 207 children from Thailand, ANA (by HEp-2 IFA) positivity was found in 15% using a 1:40 cutoff [40]. In all these studies, positive ANAs were mainly at low antibody levels. Sperotto et al. [37] found that the ANA prevalence and titer increased during puberty, especially in females. An older American study reported an ANA positivity of only 0.4% in 241 children (aged 4 months–16 years) [41].

ANA can be found in a substantial part of apparently healthy individuals (children, adults, elderly; increasing in prevalence with age) and this should be taken into account when interpreting test results.

ANA in non-autoimmune individuals

Since the ANA test is expected to be requested for symptomatic but not for healthy individuals, it is also important to understand the results of the ANA test in non-autoimmune individuals. Several studies have addressed this point. A 12% frequency of positive HEp-2 IFA tests at 1:80 dilution was reported in patients with multiple medical problems, including hypertension, diabetes mellitus, gout and dyslipidemia [42]. Individuals with solid and hematological cancers have been reported to present a 20% frequency of positive HEp-2 IFA test, with titer predominantly below 1:320 [43]. A recent study compared the results of the HEp-2 IFA test in 558 individuals with non-autoimmune diseases, 194 AARD patients and 1,217 healthy individuals. The frequency of positive results was higher in non-autoimmune (18.3%) than in healthy individuals (12.3%). The distribution of patterns was similar in non-autoimmune vs. healthy individuals, with predominance of nuclear fine speckled (AC-4) and dense fine speckled (AC-2) patterns, and absence of nuclear homogeneous (AC-1), centromere (AC-3), and nuclear coarse speckled (AC-5) patterns. Of interest, very low titer (1:80 and 1:160) ANA were more frequent in healthy than in non-autoimmune individuals, whereas titer 1:320 was more common in non-autoimmune than in healthy individuals [44].

ANA by HEp-2 IFA: reporting of results

The term ANA holds a conundrum in that this historical term does not accurately reflect that the test also reveals autoantibodies to cell compartments other than the nucleus. In this context, many specialists consider that the term 'antinuclear antibody' is no longer appropriate [8, 45], and the alternative term "anti-cell antibodies" has been proposed, with the HEp-2 IFA test being the tool for determination of anti-cell antibodies [8, 45].

ANA by HEp-2 IFA test results should report the antibody titer and the ANA pattern [8, 46]. In line with the recently published ICAP guidelines the pattern should preferentially be reported according to the ICAP nomenclature (www.anapatterns.org) [45, 46].

> ANA by HEp-2 IFA should report the antibody titer and the pattern.

Establishment of a reference range and importance of ANA titer

Existing recommendations

Recommendations for establishing reference intervals have been proposed by The Clinical and Laboratory Standards Institute (CLSI) and by European Autoimmunity Standardization Initiative (EASI) in conjunction with International Union of Immunology Societies (IUIS). CLSI stated that "*the laboratory should establish a range of reference values for 95% of the non-rheumatic disease population with representative patients from age groups younger than 40 years and older than 40 years*" [47, 48]. The EASI/IUIS [8] recommendations stated that "*An abnormal ANA should be the titer above the 95th percentile of a healthy control population*". Both CLSI and EASI/IUIS recommend that the reference ranges should be established locally [8, 47, 48]. EASI/IUIS stated that "*a screening dilution of 1:160 on HEp-2(000) substrates is often suitable for ANA detection in adults*" [8]. According to a recent survey of ICAP approximately 80% of laboratories use a 1:80 screening dilution [46]. An EASI survey reported that the 1:80 screening dilution was used in 60.5% of laboratories [49].

Thus, the CLSI–EASI/IUIS recommended that the 95% specificity be determined, either in healthy (EASI/IUIS) or diseased controls [47, 48]. A higher degree of positivity is expected in pathological diseased controls (who might have non-AARD, immune mediated or inflammatory conditions) than in healthy controls. In fact, it has been confirmed that a cohort non-autoimmune patients have a higher frequency of positive HEp-2 IFA results than the normal population [44]. Furthermore, CLSI stressed the importance of age (higher prevalence of ANA in elderly population >65 years (18%) compared to the younger population (4%)) [50], of gender (a balance of men and women should be included in the reference population) and of the ANA pattern [35, 47, 50]. Moreover, CLSI also recognized that higher titers may denote a higher probability for an AARD or of positive test results for antibodies to dsDNA or extractable nuclear antigens and it was suggested to define different levels of positive results [48]. This is in line with the concept to define and report test result (interval)-specific LR (see below) [1].

Wener et al. [36] suggested that in order to harmonize ANA assays, laboratories should adjust their assays so that about 10% of a healthy control population has a positive ANA when tested at a serum dilution of 1:80.

> Taking into account the antibody level helps to interpret ANA test results.

Recent insights

AARD are rare. The prevalence of SLE is estimated to be 47/100,000 [51], of IIM 14/100,000 [52], of SSc 30.7/100,000 [53] and of JIA 19.4/100,000 (girls)/11/100,000 (boys) [54]. Thus, in the setting of a low pre-test probability many false positive ANA results are to be expected if a 95% specificity cutoff is applied (more false positive results than true positive results).

As reinforced by two recent meta-analyses [10, 12], there is no single HEp-2 IFA or SPA cutoff that is associated with both a high sensitivity and a high specificity. A low cutoff value (e.g., 1:80) is associated with a high sensitivity but low specificity [10, 12], whereas a high cutoff (e.g., 1:320) is associated with a higher specificity but a lower sensitivity for AARD. Various studies have shown that the likelihood for AARD increases with increasing antibody titers (reviewed in [1]). This is found for manual [14, 15, 55, 56] as well as for automated HEp-2 IFA [16, 17, 57]. For example, Op de Beeck et al. [15] reported that the titer-specific LR for SLE was 0.05, 0.6, 0.5, 6, 7, 19, 13.3, 19 for results <1:40, 1:40, 1:80, 1:160, 1:320, 1:640, ≥1:1,280 and positivity (>1:40) on SSA/Ro60-transfected cells (HEp-2000), respectively [15]. These data not only show that the titer-specific LR increase with increasing antibody level, but also that a test result <1:40 has a lower LR than the LR associated with a 1:40 or 1:80 test result. Thus, a <1:40 test result has a low LR and a high negative predictive value. Such results are useful to exclude disease. It is clear that the higher the antibody titer, the higher the LR and positive predictive value are for an AARD [58]. Moreover, this is partially pattern-dependent [58]. Of note, the DFS pattern can be found at high titer [35] and monospecific anti-DFS70 is considered not to be associated with an AARD (for recent review and metanalysis see [59]).

Thus, a dichotomous interpretation (positive vs. negative) of ANA test results is an oversimplification as the information intrinsic in the antibody level is lost. This can be overcome by reporting titer-specific (or test result interval-specific) LR, which is an elegant way to convey clinically relevant information inherent to the test result [56, 60, 61]. The LR is the fraction of the individuals with a particular test result (this can be any test result or test result interval) divided by the fraction of controls (individuals that do not have the disease of interest) with such test result. We refer to a recent review for detailed background information on understanding and interpreting test result specific LR in ANA testing [1]. Many laboratory professionals and clinicians are not yet familiar with the concept of LR as the accuracy of a diagnostic test in scientific articles, systematic reviews, and test inserts is traditionally reported in terms of sensitivity, specificity and predictive values. In fact, the Bayesian approach to calculate post-test probabilities is considered impractical and difficult. The study of Vermeersch and Bossuyt [60], based on a 3-item questionnaire amongst 172 clinicians, showed that the use of a graphic representation of the post-test probability as a function of the pre-test probability could overcome this hurdle. They showed that most clinicians (81%) grossly overestimated the probability of disease when the diagnostic accuracy information was given as sensitivity and specificity. If the diagnostic accuracy information was provided as a LR in nontechnical terms, the number of correct answers increased significantly from 8 to 34%. With a graphic approach, 73% correct answers were obtained [60].

Recommendation

Given the (very) low prevalence of AARD, a 95% specificity threshold has limitations and a 97.5% and 99% specificity threshold would be more relevant, especially in the context of a low pre-test probability.

In order to convey clinically relevant information inherent to the antibody level, we recommend reporting the ANA titer and providing titer – or test (light intensity unit) result (interval) – specific LR for AARD.

In order to determine these LR, disease controls and healthy controls (with the same age and sex distribution as the patients) should be used. By preference this should be done in multicenter studies by laboratory medicine specialists in collaboration with clinicians and *in vitro* device (IVD) manufacturers. The laboratory can then locally verify these titer-specific LR. Including diseased controls is important.

Over the last years, Food and Drug Administration (FDA)- and/or CE-approved HEp-2 IFA kits and automated, closed commercial IFA systems with associated reagents have been introduced. Such commercial kits and systems have the potential to reduce the variation between laboratories that use instruments/reagents from the same manufacturer. For an example of assigning test result specific LR for automated HEp-2 IFA, see [57].

- For many patterns, the likelihood for AARD increases with antibody titer.
- For most patterns, there is no single HEp-2 IFA titer that is associated with both optimal sensitivity and optimal specificity for AARD.
- Reporting titer-specific or test result-specific likelihood ratios (LR) for AARD improves clinical interpretation of HEp-2 IFA test results.

ANA titers in pediatric systemic rheumatic diseases

Malleson et al. [62] reported that ANA at a screening dilution of 1:40 had a high sensitivity, but a very low positive predictive value (0.10) for pediatric SLE, MCTD or overlap syndromes. Thus, a negative test result at 1:40 cutoff has a high negative predictive value, but low titers occurred commonly in children without a systemic rheumatic disease. The positive predictive value increased with increasing antibody levels and the authors suggested that a screening dilution of 1:160 or 1:320 would increase the utility. Based on the data provided for girls in this study, we calculated the titer-specific LR to be 0.2, 0.93 and 2.7 for titer 1:80, 1:160 and 1:320, respectively.

In accord with Malleson et al. [62], McGhee et al. [63] reported that children with SLE have high ANA titers and that low ANA titers are common in children without chronic inflammatory disease.

Hilario et al. [38] reported that 12.7% of Brazilian healthy children and adolescents had positive ANAs (screening dilution 1:80). The titers found in healthy children were lower than the ANA titers found in children with an AARD (28 SLE, nine JDM and eight SSc). Based on their data, we calculated the titer-specific LR for a systemic rheumatic disease to be 0.94, 2.7, 3.9, 9.4 and 52 for a titer of 1:80, 1:160, 1:320, 1:640 and ≥1:1,280, respectively (with healthy children as controls). This illustrates that the LR for an AARD in children increases with increasing antibody titer. Moreover, the 1:80 and 1:160 titer-specific LR for an AARD were comparable to those observed in adults (0.5 for 1:80, six for 1:160) [15]. Watanukul et al. [40] and Arroyava et al. [41] also reported that the ANA titers found in children with SLE were higher than the ANA titers in healthy children.

> There is no evidence that a lower screening dilution for the HEp-2 IFA test should be applied in children to screen for AARD.

HEp-2 IFA patterns

Recent insights (advancements) and recommendations

The most recent and comprehensive effort to define and describe HEp-2 IFA patterns has been done by ICAP [45, 64, 65]. This consensus is a good basis for pattern description and includes pattern names with a corresponding alphanumeric AC code (anti-cell), a detailed description of the main features for pattern designation, possible associated autoantibody specificities and clinical relevance of each HEp-2 pattern. ICAP differentiated between expert and competent level patterns [45, 64, 65]. National guidelines might adapt the ICAP consensus to their local situation, as has been done, for example in Brazil and the Netherlands [66, 67].

For an overview of the HEp-2 IFA patterns and the associated antigens, we refer to ICAP publications [45, 64, 65] and see www.anapatterns.org [1]. Except for the centromere pattern (AC-3), a HEp-2 pattern cannot be used to reliably deduct the antigen-specificity of the antibody as the same pattern can be due to different antigen-specific antibodies. On the other hand, the pattern can suggest follow-up testing for specific antibodies [1, 65]. Also, it is helpful to verify whether the patterns observed on HEp-2 IFA correspond to (fit with) the specific antibody (antibodies) identified in antigen specific SPA. Certain cytoplasmic patterns are often clinically relevant and should be reported [1, 46, 47, 65, 67].

In a recent large international survey on reporting and interpreting HEp-2 IFA patterns, the centromere (AC-3) and homogeneous (AC-1) pattern obtained the highest scores (followed by the speckled and nucleolar pattern) for clinical relevance of the nuclear competent patterns and the DFS pattern (AC-2) the lowest. Of the cytoplasmic patterns, the reticular/mitochondria-like (AC-21) pattern obtained the highest scores for clinical relevance and the polar/Golgi-like (AC-22) and rods and rings (AC-23) patterns the lowest [68, 69]. The low score for anti-Golgi antibodies corresponds to the limited clinical association of anti-Golgi antibodies with AARD [70, 71]. Anti-rods and rings are not revealed by HEp-2 substrates from all IVD manufacturers and are predominantly reported in the clinical setting of Hepatitis C virus infection treated with α-interferon and/or ribavirin [65]. Of the rare ANAs, anti-multiple nuclear dots (at higher titers) (AC-6) and antinuclear envelope (AC-11, 12) autoantibodies were found to have the highest clinical association with autoimmune liver disease in a large retrospective study [71]. Of note, a recent revision of ICAP reconsidered the nuclear envelope (AC-11, 12) and pleomorphic pattern (AC-13, 14) as 'competent' [45]. For the nuclear envelope (AC-11, 12) this change is in accord with the recent studies [68, 71]. In contrast, for the pleomorphic pattern the change was not supported in recent studies [68, 72]. A study in the US showed good accuracy in identifying nuclear patterns, but less so for cytoplasmic and mitotic patterns [69]. A recent multicenter observational study from Spain found that detection of infrequent ANA patterns with no specific antibodies might lead to the suspicion of an autoimmune disorder [73]. PCNA antibodies were initially described to be highly specific for

SLE, but these antibodies are rare and have also been found in other diseases [74]. The pattern is not revealed by all HEp-2 substrates [75].

Many serum samples yield more than one HEp-2 IFA pattern and some combinations of multiple patterns have clinical relevance. The co-occurrence of two or more of the following patterns: centromere (AC-3), punctate nuclear envelope (AC-12), multiple nuclear dots (AC-6), and AMA-like cytoplasmic reticular (AC-21), should raise the possibility of PBC [76].

Important HEp-2 IFA patterns with the highest potential clinical relevance for AARD, AIH and PBC include:

- AARD:
  - Nuclear: centromere (AC-3), homogeneous (AC-1), (fine, large/coarse) speckled (AC-4, 5), nucleolar (AC-8, 9, 10), multiple nuclear dots (AC-6).
  - Cytoplasmic: (dense) fine speckled (AC-19, 20).
- PBC:
  - Nuclear: multiple nuclear dots (AC-6), nuclear envelope (AC-11, 12).
  - Cytoplasmic reticular (mitochondrial like) (AC-21).
- AIH:
  - Nuclear: homogeneous (AC-1), (fine, large/coarse) speckled (AC-4, 5).
  - Cytoplasmic: linear fibrillar (AC-15)

Recognizing the DFS pattern (AC-2) on HEp-2 cells requires specific training [77]. An international internet-based survey corroborated that recognition of the DFS pattern is challenging [78] and concluded that the presence of anti-DFS70 antibodies should be confirmed by a specific immunoassay before definitive results are reported to the clinician [78], as is also the case for all HEp-2 IFA patterns with the exception of the centromere pattern (AC-3) [65]. However, in a scenario with highly trained analysts, samples with bona fide nuclear DFS pattern (AC-2) is strongly associated with monospecific anti-DFS70 antibodies [79]. It should be noted that differences in the performance of commercial assays for anti-DFS70 exist [80]. The prevalence of monospecific DFS70 antibodies in healthy subjects ranges from 1 to 8% (mainly in females) but is rare in AARD [81, 82]. It should be noted that isolated anti-DFS70 has been reported in SLE [80, 83].

ICAP recently defined the Topo-I-like pattern associated with anti-DNA topoisomerase I antibodies [83]. The pattern description is new for many laboratories and additional studies are needed to document the value of this pattern for guiding follow-up testing for anti-topoisomerase I antibodies.

Taken together, ANA patterns should be interpreted in combination with the antibody titer and the results of the antigen-specific SPA. Some patterns have a higher clinical relevance than others.

- HEp-2 IFA pattern may provide useful information in terms of clinical relevance and guidance for follow-up tests.
- The clinical relevance of the HEp-2 IFA test result is pattern-dependent.
- Correlating the HEp-2 IFA pattern with the result of antigen-specific assays adds value to the interpretation of both.
- The main ANA patterns (with the highest clinical relevance) comprise:
  - nuclear centromere (AC-3), homogeneous (AC-1), dense fine speckled (AC-2), speckled (AC-4, 5), nucleolar (AC-8, 9, 10), multiple nuclear dots (AC-6), nuclear envelope (AC-11, 12).
  - cytoplasmic reticular (AC-21), (dense) fine speckled (AC-19, 20), linear fibrillary (AC-15).

ANA by HEp2 IFA: methodology

Substrate: HEp-2 vs. HEp-2000

It is important to maximize the chances of identifying anti-SSA/Ro60, as this autoantibody is associated with SjS, cutaneous lupus erythematosus, congenital heart block, neonatal lupus syndrome, SLE and other AARD [85–87]. However, the use of HEp-2 IFA to detect SSA/Ro60 antibodies was limited by a lack of sensitivity due to the low abundance of the SSA/Ro60 antigen and reported diffusion of the antigen from the nucleus during fixation and subsequent sample preparation [88]. In this context, the HEp-2000 substrate, a modified HEp-2 substrate transfected with SSA/Ro60 cDNA was developed [49]. The overexpression of SSA/Ro60 antigen results in a characteristic bright speckled pattern with nucleolar staining in 10–20% of interphase cells (sometimes referred to as 'atypical speckled' or SSA/Ro60-pattern) [86, 89]. This distinctive pattern in HEp-2000 cells was shown to be highly specific and, hence, allows for direct identification of SSA/Ro60 antibodies [88, 90–92]. Associations between the distinctive 'SSA/Ro60 pattern' in the presence or absence of other staining patterns on HEp-2000 and some clinical conditions have been proposed [93]. High sensitivity (88%) of the HEp-2000 substrate for detecting SSA/Ro60 positivity as defined by conventional reference techniques (such as double immunodiffusion and counter immune electrophoresis) was observed in two studies [90, 91]. However, it should be mentioned that the HEp-2000 substrate is not considered an ideal screen for anti-SSA/Ro60 as it may fail to detect these antibodies in some sera [90, 91, 94, 95]. Next to HEp-2000, other manipulated cellular substrates are available. In the HEp-20-10 substrate, cell cycle progression is manipulated to increase the proportion of mitotic cells.

Results on the direct comparison between type of substrates are conflicting, and probably also obfuscated by differences in conjugate specificity (IgG-specific or not) between kits. In one study, the HEp-2000 substrate was able to detect SSA antibodies in 4% of HEp-2 IFA negative samples [96]. In a study comparing HEp-2000 with four commercially available conventional HEp-2 assays, the HEp-2000 substrate was significantly more sensitive compared to all four HEp-2 assays for detecting samples with isolated SSA (blot-confirmed) (HEp-2000 sensitivity ranged from 24 to 91%) [93]. Comparable sensitivity between HEp-2000 and HEp-2 for SSA antibody detection (72% [CI 68–76%] for HEp-2 vs. 75% [CI 70–78%] for HEp-2000) was observed by Hoffman and colleagues on a set of 68 anti-SSA/Ro60 line blot positive samples [94]. In contrast, a recent study performed on diagnosed (and potentially treated) SLE patients in the context of the ongoing discussion on the applicability of ANA serology as entry criterion for clinical trials, showed significantly lower sensitivity (72.4%) for the HEp-2000 substrate compared to three HEp-2 substrates (sensitivity ranged 86.2–96.7%) and one HEp-2010 substrate (a substrate enriched with mitotic cells) (sensitivity 99.4%) [97]. Of note, this study did not focus on anti-SSA/Ro60 detection and did not compare the specificity of the HEp-2 assays tested [95]. Interestingly, however, the HEp-2000 substrate (and one HEp-2 substrate) correlated better with disease activity [97].

Despite the high sensitivity of HEp-2000 for SSA/Ro60 antibodies, there has been a shift in certain parts of Europe from HEp-2000 towards HEp-2 over the last years (decrease of HEp-2000 users by 47% over a 6 years period [2013–2019] [source Sciensano, Belgium] and by 40% over a 5 years period [2014–2019] [source UKNEQAS, UK]). It is unknown whether this shift applies also for other parts of Europe or other continents. Factors that attribute to this shift are probably diverse (e.g., the limited number of CAD systems using HEp-2000, the availability of alternative automated SPA for SSA/Ro60 antibodies, …).

The HEp-2000 specific SSA/Ro pattern was initially not included in the ICAP nomenclature as they focused on unmanipulated cellular substrates [65, 86]. This decision was recently questioned by Lee and colleagues, mainly in the context of the high sensitivity of the HEp-2000 substrate for anti-SSA/Ro60 detection and risk assessment for neonatal lupus [85, 87]. In reply to the Comment of Lee et al. [87], ICAP acknowledged that the decision on not including patterns of manipulated cellular substrates may be disputable [86]. On their website, ICAP has added information on HEp-2000 cells stating that anti-SSA/Ro60 staining on HEp-2000 slides is different from other HEp-2 slides (see www.anapatterns.org, pattern AC-4). Recently, variants of the fine speckled nuclear pattern (AC-4) were proposed to increase correlation with presence of anti-SSA/Ro60 [98].

> HEp-2, HEp-2000 and HEp-20-10 cells can be used as substrate for ANA IFA screening.

Conjugates

Published comparative data on the use of different ANA HEp2 IFA conjugates are very limited. One study on ANA detection by HEp-2 IFA in healthy donors found IgG isotype antibodies in 23/62 (37.1%) of sera that were ANA positive using a polyvalent anti-Ig conjugate (mostly titer lower than or equal to 1:80) [99]. A second study on ANA detection in 100 individuals with a connective tissue disease found that all sera had IgG class ANA, either exclusively or combined with IgM and/or IgA ANA [100]. The authors suggested the use of a polyvalent conjugate until the role of IgA ANA in diagnosis, prognosis and follow-up is elucidated [100]. According to some authors and the CLSI guideline on HEp-2 IFA the use of a polyvalent conjugate may produce background fluorescence and may detect clinically irrelevant antibodies [48, 101, 102]. Therefore, the use of an anti-human IgG (Fc)-specific conjugate has been promoted in order to enhance the positive predictive value of the ANA test [102, 103]. IgM-class ANA are also associated with rheumatoid arthritis, therapeutics, older age, and are usually of no diagnostic significance in AARD [48, 104]. In addition, it must be mentioned that treatment with biological therapeutics such as Infliximab, induces ANAs, especially IgM and IgA anti-dsDNA antibodies [105, 106]. In rare cases, these antibodies have been associated with development of drug-induced lupus [105, 106].

> – The use of IgG specific conjugate is sufficient to detect most clinically relevant ANA.
> – The isotype specificity of the used conjugate (polyvalent/IgG specific) contributes to assay variability.

Automated microscopy (CAD)

Alternative for manual microscopy: positive/negative discrimination and pattern recognition

During the last decade, digital systems for HEp-2 IFA allowing automated positive/negative interpretation and pattern recognition have been extensively evaluated. Several studies compared these CAD systems with the

classic manual HEp-2 IFA and reported a good overall concordance (positive/negative) between automated and classic visual interpretation (overall concordance rate: median 93.7%, range 71–99%) [107–122]. Compared to classic manual HEp-2 IFA, CAD systems had a high sensitivity for ANA detection at clinically relevant titers (mean sensitivity: 95.7%; range 87–99%) [107, 110, 116]. Detailed review of the discordant samples revealed that correct positive/negative classification by CAD systems is mostly challenged by low titer ANA, cytoplasmic (e.g., ribosomal, mitochondrial, Jo-1, lysosomal, vimentin) staining and peculiar nuclear (e.g., nuclear dots) and mitotic (e.g., centrosomes, midbody) patterns [109, 110, 116]. The diagnostic accuracy of automated HEp-2 IFA in AARD cohorts has also been documented in several studies [109, 112, 119, 120, 122, 123]. The summary diagnostic sensitivity of CAD compared to classic visual HEp-2 IFA was recently calculated on a pooled dataset of four studies and was 84% (95% CI=81.4–87.7) vs. 78.2% (95% CI=74.5–81.7) for combined AARD, 95.5% (95% CI=90.4–98.3) vs. 93.9% (95% CI=88.4–97.3) for SLE, and 86.5% (95% CI=78.4–92.4) vs. 83.7% (95% CI=75.1–90.2) for SSc, respectively (for systematic review and meta-analysis see [124]). Overall, these findings support integration of these platforms in the first step of ANA screening (positive/negative discrimination).

In addition to positive/negative discrimination, most but not all CAD systems also provide an automated pattern interpretation for a pre-defined set of patterns, mostly including nuclear homogeneous (AC-1), speckled (AC-4, 5), nucleolar (AC-8, 9, 10), centromere (AC-3) and cytoplasmic patterns. Some CAD systems also claim to be able to identify nuclear dots (AC-6, 7), nuclear dense fine speckled (AC-2) and nuclear envelop patterns (AC-11, 12). Nevertheless, even though data must be interpreted in light of the limitations (in terms of the patterns that can be recognized) declared by the manufacturers, published data on the performance of pattern recognition is a concern. A meta-analysis based on 11 studies reported a pooled positive concordance for all patterns (including homogeneous, speckled, nucleolar, and centromere patterns) of 68.5% (95% CI=67.2–69.7) (for systematic review and meta-analysis [124]). The accuracy of the pattern recognition was dependent on the type of pattern and the substrate. CAD systems clearly had difficulties to correctly identify the rare nuclear patterns, cytoplasmic and multiple patterns [109–111, 116, 119], with the lowest summary positive concordance (11.7%) observed for the nuclear dots pattern (95% CI=6.8–18.3) [124]. Therefore, pattern recognition by CADs needs to be further enhanced, necessitating expert review before HEp-2 IFA result validation to date.

End point titer vs. single well titer

An interesting feature of the CAD HEp-2 IFA platforms is that they often provide a system-specific (arbitrary) quantitative measure for fluorescence intensity (FI measure). For several of the available CAD systems, the FI measure showed high analytical reproducibility [109, 110, 125, 126] and significant correlation with the endpoint titers obtained by manual reading [107–110, 116, 119, 122, 127]. In addition, it was shown that the LR for an AARD increased with increasing FI measure [109, 119, 122, 128]. These observations illustrate that estimation of the fluorescence intensity by CAD for HEp-2 IFA has clinical utility.

The above data suggest that the FI measure may provide a useful alternative for endpoint titration. Moreover, some of the current available CAD HEp-2 IFA platforms provide a single dilution titer estimation function, the so called "single well titer" [125]. Yet, there are several limitations in directly applying this correlation between FI measure and end-point titer to eliminate serial dilution. First, the relation between the FI measure and endpoint titer is system-specific and seems to be dependent on substrate [119] and ANA pattern [126, 127]. Moreover, an underestimation of endpoint titer and LR for AARD may be possible in case of ANA patterns that stain only limited parts of the cells (e.g., nuclear dots, speckled cytoplasmic Jo-1 related pattern) [128]. Secondly, overlapping antibody patterns can be masked and overlooked if the samples are analyzed only in a single dilution.

In summary, the added-value of the FI measure generated by CAD systems lies in the quantification of the fluorescence intensity that can be used to estimate the endpoint titer and to generate information regarding likelihood for disease. However, this does not *per se* signify that conventional serial dilutions should be eliminated. Recent ICAP guidelines stated that the test result report should specify whether the end titer is an estimate or achieved by serial dilutions [46].

Added value of CAD in quality assurance

Over the last few years, FI measures of CAD HEp-2 IFA systems have also shown potential in evaluating and controlling several variables impacting the quality of the total HEp-2 IFA testing process. The foundation for this potential is the high analytical reproducibility of the FI measures as has been documented for some of the CAD systems [109, 110, 126, 129]. Both experimental as well as retrospective studies illustrated that monitoring FI measurements on quality control material as well as routine samples are adequate quality indicators if accompanied by well-defined acceptance criteria [129–132]. The acceptance criteria should be based

on identifying clinically important shifts in FI. Indeed, monitoring of FI reproducibility on selected samples (within and between runs within one laboratory or over different laboratories) as well as monitoring of the median FI per run were able to identify technical issues with the equipment in both the pre-analytical (e.g., pipetting issues with the slide processor) as well as the analytical phase (e.g., calibration issues with the CAD). Monitoring the FI was also able to identify shifts in FI related to inter-lot conjugate variability [131, 132]. Importantly, this approach was shown to be more sensitive than the traditional, more subjective, quality control indicators such as quantitative monitoring of IQC samples and monitoring of the monthly percent positives in patient samples [129–132].

Based on these observations, it can be concluded that the implementation of CAD systems with a documented reproducible FI quantification enables the introduction of objective and more sensitive quality control procedures to monitor the total HEp-2 IFA testing process from dilution up to result interpretation within one laboratory (internal quality control procedures) as well as between laboratories (external quality control programs) [131]. Of note, not all CAD systems provide a (reproducible) FI measure and monitoring of the end-point titer estimate (based on single well reading) as provided by the CAD may be an alternative option, if available.

- A computer-aided diagnosis system (CAD) can support HEp-2 IFA, but expert review remains recommended for positive/negative discrimination.
- A CAD can support HEp-2 IFA, but expert review remains mandatory for pattern recognitions.
- Several CAD systems for HEp-2 IFA provide a fluorescence intensity score (FI measure) which contains:
  - information on titer estimation
  - information on the likelihood for AARD
- When a CAD system for HEp-2 IFA is used that provides a reproducible FI measure, follow-up of this FI measure can contribute to monitoring of the quality of the analysis (e.g., as part of the IQC program) (not applicable for all systems on the market).

Quality assurance approaches for HEp-2 IFA testing

Quality management is an important task for medical laboratories involved in patient care and translational research. In accord with (inter)national guidelines and EN/ISO 15189:2012 accreditation requirements [133], it is the responsibility of the laboratory to monitor and to control the quality of the total testing process by the development and implementation of a thorough quality assurance (QA) program. Nevertheless, in the context of autoimmune testing, and more specifically ANA analysis, the translation and interpretation of these guidelines/requirements in test-specific and detailed recommendations on how to set up this QA program is extremely challenging (for review see [134]).

Internal quality control

One of the important cornerstones in the QA program of clinical laboratories is the internal quality control (IQC) procedure monitoring the results obtained on quality control (QC) samples with known pattern/specificity and fluorescence intensity in each run. Variables in the IQC procedure for the HEp-2 IFA include: the minimal number of samples needed, the titer, the origin, the patterns of the selected samples and the methodology of monitoring, registration, and evaluation of the QC results.

Minimal number of IQC samples and titer

Classically, quality controls of HEp-2 IFA assay kits include a positive and negative IQC sample. The use of (at least) two IQC samples, one negative and one positive IQC sample, in each run is in keeping with the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [48] as well as other more recent methodology recommendations (including the Brazilian consensus guidelines) [66, 135]. There is also consensus on the importance of analyzing at least one low positive sample near the cut-off [48, 134, 135]. In contrast, an exact target titer for this low positive QC sample (1/80–1/160) is only suggested in one of these guidelines [135]. An older version of the CLSI (former NCCLS) guidelines on HEp-2 IFA analysis defined the preferred titer of the QC material as one dilution beyond the cut-off point in relation to the applied cut-off value [47]. The more recent 2006 version of the CLSI recommendation (CLSI LA02-A2), including guidelines for both IFA and ELISA, states that the positive control should be chosen at a level that is important for clinical decision making [48]. Translating this latter into the concept of applying/reporting titer-specific LR for AARD, we recommend matching the target titer of the low positive control sample to a LR of 2–5 for AARD (as this LR suggests small but potentially relevant clinically important differences). This would correspond to a titer of 1:160 for HEp-2000 substrate [15] or a LIU (light intensity unit) of 522–910 (LR of

5.2) with Werfen HEp-2 substrate on NOVA View CAD [16]. Appropriate studies should be conducted to establish analogous thresholds for other commercial products.

Origin of the IQC samples

The selection of IQC samples with known patterns/specificity and fluorescence intensity is advised [134]. In practice, most kits provide a positive and negative kit IQC fulfilling this criterion. However, these kit controls may not be ideal for several reasons. First, as they are often strongly positive and completely negative, they characterize the far extremes that are not representative of most routine samples and do not detect (minor) analytical problems [135]. Secondly, they are often 'ready to use' and do not require pre-dilution and, thus, they do not cover the whole analytical process [130]. Thirdly, in-kit controls might miss longevity, as it cannot be ruled out that companies may change their in-kit controls (e.g., over different reagent lots) [134]. Therefore, undiluted patient-derived IQC samples (either pooled or single patient samples selected from the routine lab samples, which have been aliquoted [to limit the number of freeze–thaw cycles] and frozen until analysis [allowing to last for a longer period]) are the preferred choice. Moreover, in a recent study monitoring the CAD specific FI as a QA indicator, the latter were shown to be more sensitive compared to kit controls. Comparing both type of materials, in-kit controls proved of less significance as they were not able to highlight (artificially introduced) errors in contrast to the patient-derived IQC [130]. CLSI suggests prolonged storage of the samples at −70 °C with a preservative [47, 48]. Although, it is a common practice to preserve serum/plasma samples with immunoglobulins in the freezer at −20 °C, as it represents a feasible option in many labs. Nevertheless, a recent study suggests that not all autoantibodies (including anti-ENA screen) as detected by SPAs are stable at −30 °C over 12 months [136].

Patterns/specificity of the IQC samples

To our knowledge, only one guideline mentions further details on the 'known' specificity and/or patterns that should be selected for the positive IQC samples. The 2009 German EASI guidelines suggested the use of three positive serum samples with different fluorescence patterns resulting from defined antibody reactivity (e.g., centromeres, dsDNA, SSA/Ro60) in alternation over the runs [137]. This strategy is also in line with the recent EASI best practice guidelines suggesting the IQC samples should include as many antigens as possible [134]. Another strategy may be to select a high prevalent potentially precarious/fragile specificity such as SSA/Ro60. An alternative option, especially in view of the recent CAD developments and possibilities, would be the selection of a pattern that has shown high inter-assay reproducibility of the FI measure such as isolated speckled or homogeneous patterns [125, 130]. Of note, alternation of the IQC specificity/pattern over the consecutive runs encumbers optimal usage of quantitative FI measures for QA provided by CAD systems.

Methodology of monitoring, registration and evaluation of the IQC results

Traditionally, IQC measurements in HEp-2 IFA are evaluated for their expected pattern and endpoint titer against predefined target values and criteria. Some authors suggest not to allow pattern deviations or titer changes >1 titer [129, 134]. CLSI states that detectable differences of the HEp-2 IFA method are typical ±2 serial, twofold dilutions [48]. Regulatory requirements in some countries accept titer changes of maximal ±2 dilutions [102]. The disadvantage of this approach is that it only allows for qualitative monitoring/judging. Recent advances with CAD systems have enabled semi-quantitative monitoring options using Levey-Jennings plots accompanied by Westgard rules [138], as shown in several studies using the reproducible system-specific FI measures as a basis [129, 130, 132], as already delineated in the paragraph on the added value of CAD systems in QA. Of note, these studies also suggest acceptance criteria [129, 130, 132]. Moreover, this approach allows for the analysis of the IQC samples in a single dilution instead of performing an end-point titration.

Training and controlling inter-observer variability

To ensure homogeneity in HEp-2 IFA reading, technicians who read the slides should receive regular training and evaluations. Obviously, a laboratory should have a certain threshold of ANA requests in order to maintain sufficient expertise. In addition, training should be organized internally as well as externally. Moreover, in some countries documentation on the inter-observer variability should also be included in the verification report [139]. One approach that has been suggested is the internal organisation of (i) regular sessions in which cases are discussed and (ii) periodic blinded reading (by each technician) of representative routine samples with various patterns and titers [135]. CAD systems generating high quality digital images enable organizing such reviews on a wider level (e.g., national EQC

assessments in Belgium). The frequency of these 'observer' trainings/reviews as well as the requirements of these training sessions should be defined and may be dependent on national regulations (e.g., in Germany four internal trainings/year). Moreover, these trainings should not be limited to analytical competence but should also include training on how to interpret the data in a clinical context [134].

Homogeneity of HEp-2 IFA reading may also be further improved by the application of double (blinded) reading of the slides by independent technicians (on all routine samples) [140]. In one study, homogeneity on fluorescence intensity evaluation between observers improved when reading was performed on the CAD monitor (digital images) instead of under the classic microscope [141]. In contrast, the improvement was less pronounced for pattern classification, probably related to the inability for sharp focussing using a CAD monitor [141]. In this regard, it should also be mentioned that variations in CAD monitor settings may also include an additional factor of variability. Procedures should be in place to keep these settings in line with the settings as were validated. Finally, despite the lack of formal studies, homogeneity in Hep-2 IFA results might be further improved by the use of the ICAP consensus nomenclature (e.g., the introduction of the AC-29 pattern associated with antibodies to DNA topoisomerase I [84]).

External quality assessment

External quality assessment programs (EQA) for HEp-2 IFA (often combined with anti-ENA analysis) should be used to monitor inter-laboratory variability [48, 66]. By providing advice to the participating laboratories, EQAs have shown a positive impact on HEp-2 IFA performance [142]. The frequency and character (e.g., voluntary vs. obligatory) of EQA participations should be defined by national regulations.

QC approaches based on patient results

According to CLSI, monitoring the frequencies of test results that fall in different reference range categories (e.g., negative, borderline, positive) may detect changes related to the introduction of new lots of test reagents [48]. More details on how to approach this practically can be found in reference [132]. Obviously, this approach is only feasible if the laboratory performs a certain threshold of ANA requests in an acceptable timeframe. CAD systems also allow to monitor patient medians of the system-specific FI measures generated in each run, an approach which has proven to be useful for systems that generate a reproducible FI measure [130, 132]. Moreover, this concept supports the idea that a dichotomous interpretation (positive vs. negative) of ANA test results is an oversimplification.

- Performance of HEp-2 IFA should be monitored by internal (IQC per run and periodic blinded reading of representative cases) and external quality assessment programs.
- Factors minimizing HEp-2 IFA inter-observer variation include educational programs, application of double reading, usage of ICAP nomenclature and CAD monitor reading (digital images).
- At least 2 IQC samples (one negative and one low positive [with a target level matching a LR of 2–5 for AARD]) should be included in each run and judged semi-quantitatively (either by end-point titration or automated intensity scoring).
- In addition to kit controls, it is advised to run IQC samples of patient origin, either pooled or unique samples, as they are processed as routine samples (thus allowing monitoring of the whole assay procedure).
- The preferred pattern of the positive control sample preferentially has a high reproducibility (e.g., isolated homogeneous or speckled on CAD systems).
- Monitoring of the % of (low, medium, high) positive results in Levey-Jennings plots allows for the evaluation of assay stability over time (e.g., to document the effect of manufacturer inter-lot changes).
- When a CAD system for HEp-2 IFA is used that provides a reproducible FI measure (not applicable for all commercial systems on the market): monitoring of the IQC sample and median patient FI in Levey-Jennings plots is a sensitive method to detect assay stability over time (e.g., to document the effect of lot changes).

Reagent lot acceptance and monitoring of lot-to-lot variability

Review of existing recommendations and guidelines

Variation in HEp-2 IFA has been shown between brands [75, 143, 144], but it has also been suggested that variations in HEp-2 IFA results could be related to lot changes of the same brand [66, 75, 125, 132], presumable inherent to the manufacturing process [5, 66].

In line with (inter)national guidelines and EN/ISO 15189:2012 accreditation requirements, each new reagent lot and shipment should be validated before use [133]. CLSI recommends testing patient samples with both the current and the candidate new lot [145]. Patient materials are preferred to commercial QC materials as the latter may not reflect the performance of patient samples (reviewed in [146]), but they can be used in parallel with the patient samples and/or to verify a new shipment of the same reagent

lot that had previously been validated [145, 146]. The lot-to-lot comparison approach as outlined in CLSI EP26-A is however found impractical and several alternative approaches have been proposed (reviewed in [146]). Though these proposals are generally intended for pure quantitative methods and therefore not simply transferable to the HEp-2 analysis.

For HEp-2 IFA, several more specific recommendations advise on the best approach for reagent lot acceptance. According to CLSI LA02-A2, each lot number of HEp-2 cells should be tested with *a low titer, known positive anti-SSA* sample and a set of other positive and negative known sera which are collected/preserved for the purpose of lot-to-lot comparisons [48]. The Brazilian guideline suggests to test a panel of samples representative of a broad array of patterns, with emphasis for those antigens susceptible to damage (i.e., Jo1, SSA, PCNA, RNA polymerase) and which are immunologically and morphologically well characterized [5, 48, 66, 135].

For conjugate lot changes, the CLSI LA02-A2 as well as the Brazilian guidelines recommend chessboard titration and/or comparison with standard conjugates for determining the optimal conjugate dilution [5, 48, 66, 135]. However, in the context of the current IVDR regulations in some jurisdictions this may not be universally recommended. In fact, kits with a validated combination of reagents are extensively being used, and any deviation from the manufacturer's instructions requires extensive validation (see validation).

Current recommendation

We recommend verifying lot changes with patient-derived IQC samples supplemented with samples selected for this purpose minimally covering different cell compartments (nucleus and cytoplasm) and different titer levels including negatives. Lot changes should be minimized as much as possible.

Acceptance criteria

When performing lot-to-lot comparisons according to CLSI EP26-A, the estimated difference between two lots should not exceed a critical difference [145]. This critical difference (medically allowable error) should be based on e.g., clinical outcome studies, biological variation, analytical performance, professional practice guidelines and published professional recommendations [145–147]. For HEp-2 IFA, it is very challenging to define this critical difference. In case HEp-2 IFA is considered qualitative/semi-quantitative, the ANA IFA CLSI guideline suggests that in lot-to-lot comparability studies, at least 85% of the samples should give equivalent results [48]. Conventionally, the accepted maximum clinical variability is no deviation from the initial pattern and a one or two titer step difference [48, 102, 129, 132, 134]. It may be disputed whether this generally accepted variability really mirrors the total allowable error that can be tolerated without invalidating the medical usefulness of the analytical result in terms of the % of ANA positives and the % anti-ENA positives detected after HEp-2 IFA screening [132]. It should be noted that this criterion can also be translated in CAD system-specific criteria that can be applied by using the FI measures of the HEp-2 IFA results in lot-to-lot comparisons [129]. Using Levey-Jennings charts (and Westgard rules) may help to evaluate/monitor the impact of lot changes (see above) [129, 130, 132, 137].

> Lot-to-lot variability of conjugate and/or substrate should be evaluated before implementing a new lot. This can be done by patient-derived IQC samples supplemented with samples selected for this purpose minimally covering different cell compartments (nucleus and cytoplasm) and different titer levels.

ANA by SPA

SPAs for screening of ANA specificities and for (multiplexed) identification of these specific antibodies are increasingly being introduced in clinical laboratories (reviewed in [1, 148]). Even though some assay formats apply cell extracts as the analyte for the solid phase, the most recently developed automated assays screen for the presence of antibodies to a selected set of relevant antigens that are associated with AARD. It is beyond the scope of this work to summarize the performance characteristics of the different assay platforms and methodologies. For a recent comprehensive review, see Bossuyt et al. [1]. In short, the performance of these assays is assay- and disease-dependent. One should be aware of the strengths and weaknesses of the SPAs as a screening tool when applied in a clinical setting. The best clinical performance for AARD is obtained when SPA and HEp-2 IFA are combined (see above under ANA in AARD). Defining test result specific LR adds clinical value to SPA test results and allows to harmonize interpretation [57].

- The performance of SPA assays for AARD screening/detection is disease- and assay-dependent.
- There is no single SPA threshold that has both optimal sensitivity and optimal specificity for AARD screening.
- Reporting test result (interval)-specific LR for AARD may improve clinical interpretation of SPA test results.
- Combining HEp-2 IFA with SPA for AARD screening increases clinical value.

Validation/verification

Validation is defined by the WHO (World Health Organization) as '*the action or process of proving that a procedure, process, system, equipment or method works as expected and achieves the intended result*' [149]. Method validation establishes objective evidence (by documenting performance characteristics) that a method/application is adequate for the intended use [133]. CE-IVD – (Conformité Européenne *In vitro* diagnostics)/US FDA-approved tests are validated by the manufacturer [150, 151]. When the laboratory uses non-standard methods, modifications of standard methods, standard methods outside the intended scope, or laboratory-designed or developed methods, validation is the responsibility of the laboratory [133]. According the 2017 EU IVD Regulation, validation is the responsibility of the manufacturer or of the laboratory in case no commercial assay is available [152]. The 2017 CE IVD Regulation will require laboratories that run laboratory developed tests to meet certain standards and to be compliant with the IVDR's Annex 1 "General Safety and Performance Requirements" and quality management system framework [152].

Medical laboratories often use CE IVD/FDA-labelled tests (manufacturer/developer-validated methods) in an unmodified way (according to manufacturers' instructions). In this case, method verification instead of validation applies. Verification is an abbreviated process that confirms via objective evidence that an already validated examination procedure is appropriate for a specific intended use in one's own laboratory [151, 153–157]. Prerequisite for verification is the availability of information provided by the manufacturer/developer [133]. Guidance on how to verify/validate in a state-of-the-art way can be found in general guidelines issued by professional organizations such as the Clinical Laboratory Standards Institute [158, 159], books or publications [160, 161].

ISO standards have been translated and interpreted by national authorities, some of them specifying how to meet the ISO requirements, possibly resulting in small differences between countries both in the definition of validation/verification as well as in the minimal requirements.

An overview of the definitions of validation/verification according to CAP, ISO15189 and national guidelines is given in Supplementary Table 2 [133, 139, 150, 156, 157, 161]. In general, verification is applicable for CE/FDA labelled tests or published methods. Unique for the Dutch NVKC and CMI is that verification can rely on a combination of locally produced data (for performance characteristics that can be influenced by the local environment e.g., instrument pipetting precision) and referral to documentation (for performance characteristics [e.g., clinical sensitivity and analytical specificity] that have been objectively validated with available documentation elsewhere, e.g., another accredited lab or in multicentre validation) (summarized in Supplementary Table 2) [156, 162]. Some characteristics can be considered irrelevant or impossible to evaluate (e.g., due to low disease prevalence, unavailability of a reference method) [156, 162]. In this case, documentation/argumentation is required. The verification report has to mention for every performance characteristic whether or not experimental data were produced to meet local acceptance criteria [156, 162].

Analytical validation and verification

EN-ISO 15189 does not specify which analytical performance characteristics have to be verified [133]. Supplementary Table 3A lists the minimal requirements for validation/verification as included in CAP accreditation guidelines, ISO15189 and national ISO15189 translations/interpretations. Validation/verification of precision (repeatability/intermediate precision), accuracy/trueness, measurement interval, interferences, stability, reference interval and medical decision limits are commonly encountered requirements. According to COFRAC and the NVKC, method comparison with the method already in use is a verification requirement [139, 156], while in other guidelines method comparison is not obligatory for validation/verification purposes but it is demanded to assure comparability of results when using different methods, apparatus or procedures. In some guidelines, method comparison is also suggested to verify trueness/accuracy [156, 162] or to establish/verify reference values [151]. The ISO-independent CAP guidelines indicate that in addition to the commonly encountered requirements, a limited verification of linearity (verification of the reportable range for three points [low/midpoint/high]) [151]. In the next paragraphs, we discuss the challenges related to verification of HEp-2 assays and give an overview of possible approaches to verify the commonly encountered minimal requirements.

Verification of a new HEp-2 IFA method

Detection of ANA by HEp-2 IFA is complex and prone to analytical variability [135, 143]. There are various factors that contribute to the variability. The culture conditions, the fixation method (e.g., methanol, acetone vs. paraformaldehyde) and permeabilization method (e.g., Triton X-100) may affect the expression, preservation (or destruction) and accessibility of cellular antigens [75, 135, 163]. Optimal culture and fixation conditions may be antigen-specific [75, 135, 163]. Moreover, results may be influenced by the fluorochrome-conjugated secondary antibody (isotype, species, nature of immunogen, purification method, fluorescein/protein molar ratio, antifading treatment and concentration), the incubation medium, blocking solution, washing buffer and mounting medium [135, 162]. The equipment, including the type of light sources (stable LED vs. older mercury/argon lamps) and CAD systems used, and the operating procedures applied can further contribute to analytical variability [129].

Delavance et al. [75] studied the variability in pattern recognition between different brands of HEp-2 slides and found that different commercial brands can produce different staining patterns for the same serum or can give different results in terms of positivity/negativity. Marked differences were found for the cytoplasmic speckled pattern associated with anti-Jo-1, the PCNA-like pattern and CENP-F [75]. Long-term kit variability was also recently documented in a study reviewing the EQC results conducted by the CAP between 2008 and 2018, with variability strongly associated with the kits, and differences between kits being quite consistent during the 11 years studied [164].

Only limited published guidelines in the context of autoantibody and/or ANA analysis discuss validation/verification aspects (overview in Supplementary Table 3B), some of them only addressing limited topics [5, 47, 66, 134, 135, 137, 162]. In addition, several CLSI guidelines may be useful as guidance for development of the validation/verification processes (e.g., CLSI EP12-A2 [159], CLSI EP15-A3 [158]). However, it should be mentioned that the CLSI EP15-A3 guideline is developed for quantitative assays and therefore not simply applicable for qualitative/semi-quantitative HEp-2 IFA analysis [158].

The verification of a new ANA method starts with controlling the HEp-2 (000) cell density, distribution, morphology and number of mitotic cells (3–5/field at 200×) [137]. The EASI recommendations for autoantibody test verification minimally suggest to verify trueness (by method comparison), repeatability and intermediate imprecision as well as verification of the reference limits [134], in line with requirements defined in ISO and national translations/interpretation (Supplementary Table 3A).

Verification of trueness (by method comparison)

Each laboratory should demonstrate in a method comparison that its ANA method detects the major clinically relevant patterns as well as the major clinically relevant antigen reactivities, both in the nuclear and the cytoplasmic compartment, given the variability in the recognition of immunofluorescence patterns among different HEp-2 brands [5, 66, 75, 137, 146, 163]. In a method comparison this ability is compared between the comparative method and the newly introduced/candidate HEp-2 IFA method, with the comparative method being either the old/former HEp-2 IFA method or ideally, a combination of methods allowing for the detection of patterns/specific antigen reactivities (including also SPAs). It is recommended by the *Brazilian consensus guidelines for detection of anti-cell autoantibodies* that each new brand should be tested with a panel of sera representing the various patterns, preferentially covering the different cell compartments [66]. These guidelines do not list the patterns that should be included in the panel, but do list some patterns/reactivities for which HEp-2 substrates can present problems (e.g., anti-SSA/Ro60, anti-RNP/Sm, anti-PCNA, anti-CENP-F, anti-Jo-1, anti-NuMA-1-like, anti-NuMA-2, rods and rings). The 2009 German EASI guidelines recommend analysis of minimally three positive samples with different fluorescence patterns resulting from defined antibody reactivity (e.g., centromere, dsDNA, SSA/Ro60) [134]. CLSI LA02-A2 recommends evaluation of the detection of clinically significant autoantibodies (dsDNA, U1RNP, Sm, SSA/Ro60, SSB, Scl-70, CENP, Jo-1) [48]. Reference samples (with either defined pattern and/or specific reactivity) were made available by the Autoantibody Standardization Committee and can be ordered on their website (www.AutoAb.org).

The minimum number of samples to be included in a method comparison is a matter of debate. According to the CLSI EP12-A2 user protocol for evaluation of qualitative test performance, at least 50 samples positive with both the new and comparative method (analytical sensitivity) and at least 50 samples negative with the comparative method (analytical specificity) should be tested [159]. In a recent EASI paper on quality and best practice in autoimmune laboratories [134], it is proposed to verify analytical sensitivity and specificity with a method comparison comprising optimally of 50 known positive (analytical sensitivity) and 100 known negative sera (analytical specificity) with a minimum of 30 comparisons including at least 10 positive and 10 negative samples. This minimum was based on the requirements for performing Kappa statistics analysis [165].

We recommend to verify at least the following clinically-relevant patterns and reactivities (i.e., those that are comprised in classification/diagnostic criteria): the nuclear homogeneous (AC-1), nuclear speckled (AC-4, 5), nucleolar (AC-8, 9, 10), centromere (AC-3), multiple nuclear dots (AC-6) and nuclear envelope pattern (AC-11, 12), as well as the cytoplasmic speckled (AC-19, 20) and reticular/anti-mitochondrial pattern (AC-21); and dsDNA, SSA/Ro60, Sm/RNP, CENPB, Scl70, RNA-polymerase III, Jo-1, sp100, gp210, and AMA-M2 reactivities. We propose that the detection of each of the clinically important patterns/reactivities mentioned above should be confirmed in preferentially five samples per pattern/reactivity, if possible. In addition, at least 10 negative samples should be included in the comparison. For rare antibodies, laboratories can refer to publications or participate in inter-laboratory collaborations such as the Dutch initiative for national validation/verification of autoantibody assays [162]. Optimally, the samples included in the method comparison are well-characterized (e.g., documented with results of the SPAs, clinical context [if available], target results [for instance in case of the use of EQC samples or reference samples]).

As there is no golden standard for HEp-2 IFA and differences in method comparisons between the new HEp-2 IFA method and the comparative method are to be expected, correlation with specific reactivities in the SPA and/or clinical information and/or the target (if applicable) can contribute in defining the shortcomings of the new HEp-2 IFA method and/or the former method. Knowledge of the shortcomings of the new assay should then be used to decide on the need to adapt follow-up testing.

> Each laboratory should demonstrate that its HEp-2 IFA method detects the major clinically relevant patterns as well as the major clinically relevant antigen reactivities, both in the nuclear and the cytoplasmic compartment.

Verification of precision

Verification of precision is an essential part of the method verification process [139, 157, 162]. Recent EASI recommendations for autoantibody tests in general propose to test 10 replicates of a negative and 10 replicates of a positive sample (preferentially a low, medium and high titer sample), within the same run and between different runs [134]. The number of proposed replicates by EASI is remarkably lower than the number of replicates suggested in established (inter)national guidelines on calculating intra- and inter-run variability (e.g., 30 replicates over 15 days in the French accreditation guidelines [139], five replicates over 5 days according to CLSI EP15-A3 [158]). The EASI guideline was based on the conclusion of Senant and colleagues that >10 measurements for either intra- or inter-run CVs will not improve the estimation of the assay precision, whatever the type of immunoassay used [134, 166].

Evaluating precision for HEp-2 IFA is challenging, and the preferred strategy will depend on how the data are considered: binomial (positive/negative), ordinal (titers) or continuous (FI results).

If HEp-2 IFA results are considered strict binominal/ordinal data, the focus lies on determining *how often* results differ from the target rather than *how much* the results differ from the target.

For qualitative tests, the recent EASI guideline proposes analysis *of 10 replicates of positive and 10 replicates of negative samples in a couple of consecutive days* in order to establish consistency of results [134]. Of note, the by EASI recommended maximum acceptable variability for HEp-2 IFA is a 1 titer step difference [134].

When using a CAD system, HEp-2 IFA results may be considered qualitative and semi-quantitative results derived from a quantitative value, the system-specific FI measure. In that case, precision can be verified using approaches applied to quantitative assays [161]. However, as reproducibility specifications of most system-specific FI measures are missing (manufacturers do not specify imprecision claims), validation is theoretically required. According to CLSI EP05A3 precision can be established (validation) on double measurements obtained in 2 runs/day over a 20 days period [167]. Alternatively, published data on reproducibility of the FI measures were generated on 6–20 replicates [109, 110, 119, 125, 130] and can be considered as state-of-the-art references for verification. According to CLSI EP15-A3 precision can be verified by five measurements over a period of 5 days [158]. A similar approach has been proposed by Antonelli et al. [168]. Acceptability evaluation is based on F test [158, 168].

> Each laboratory should verify the precision of the method used. The approach will depend on how the data are handled: binomial (positive/negative), ordinal (titers) or continuous (FI results).

Verification/validation of end point titer estimation by CAD

If the laboratory intends to report endpoint titers based on the CAD system specific FI measure (either on a single well or on a dilution series), a correlation between single well titer (estimated titer) and end point titer (as determined by serial dilution) obtained with conventional microscope should be

documented. A correlation between system-specific FI-measures and titers has been documented for several HEp-2 IFA CAD systems [107–110, 116, 119, 122, 127]. Differences between the end point titer and the estimated titer based on the fluorescence intensity measured at a single dilution (single well titer) by CAD systems have been shown. For example, a Belgian multicenter study performed on the NOVAVIEW® CAD system revealed an acceptable over-estimation of one titer difference in 36% of samples, but a difference of ≥2 titer steps in 16.5% of samples with a centromere or nucleolar pattern [125].

Application of single well estimated titer by CAD has limitations and the claims of the manufacturer should be verified by comparison to end point titer for the most prevalent patterns.

Verification of pipetting device

Although the performance of the pipetting apparatus may be considered the responsibility of the manufacturer, it can also be regarded as a local variable that should be verified [162]. Malfunction of automatic pipetting apparatus can contribute to analytical variability, such as fluctuations in fluorescence intensity [131, 132] or carry-over causing a change in ANA patterns in different dilutions of the same serum (personal experience M. Vercammen). This can be done by repeatability/reproducibility tests and by carry-over protocols.

Verification of linearity, measuring range, prozoning, interferences, conjugate titration

According to EASI, evaluating linearity and measuring range, Hook-effect or prozoning, interferences and general handling issues like robustness or carry-over are less relevant when applying CE-IVDs kits for autoantibody measurements such as HEp-2 IFA [134]. Nevertheless, the topics that are not applicable should be documented/argued in the verification report. Of note, prozoning may occur in HEp-2 IFA [169, 170].

CLSI I/A02-A2 and the Brazilian guidelines propose the titration of the conjugate, also when part of a reagent kit [5, 48, 66, 134]. In this case the kit will be used outside the manufacturer's instructions and method validation instead of verification is required. In the context of good microscope systems, we suggest to evaluate the kit using the manufacturer's instructions.

Clinical validation/verification

Acording to the ISO 15189, CE IVDR, CAP directives and the new 2017 IVD regulation the manufacturer is responsible for the clinical validation of a CE/FDA labelled test that is used by the laboratory without modifications [133, 150–152, 155]. As proposed by EASI [134], we emphasize that the information of the clinical validation should be shared with customers.

However, national legislations can formulate additional requirements. In Belgium, France and The Netherlands, for example, the laboratory medicine specialist is responsible for clinical validation/verification, even when CE-IVD labelled tests are used [139, 157, 162]. He or she decides which aspects need to be investigated and how this should be done. For instance, the Dutch CMI suggests that clinical sensitivity verification may be achieved by documented rationale (e.g., by making reference to multicentre validation data) [162]. According to CAP, laboratories are not obliged to make clinical claims [151, 155]. But if they do, they can refer to the manufacturers' data. 'New' clinical claims should be validated, but for rare conditions or well-accepted uses of a test, referring to peer-reviewed literature is acceptable [151].

Clinical validation of a new HEp-2 IFA method

The CLSI I/LA02-A2 guideline for quality in ANA advises to verify clinical sensitivity and –specificity as well as reference values [48]. Clinical validation relies on analyzing clinically well-defined patients (e.g., fulfilling the classification criteria) and controls. This classically involves determination of diagnostic sensitivity and specificity for the cutoff proposed by the manufacturer. But, as we argued above, we propose to establish test result (titer, fluorescence intensity interval)-specific LR as this adds clinical value. This can be done in (multicenter) studies in which clinical laboratory immunologists, clinicians and manufacturers are involved. In order to establish test result-specific LR a large number of clinically well-characterized patients (ideally taken at the moment of diagnosis) and controls (ideally disease controls) is needed. For example, a recent study that established test result-specific LR for HEp-2 IFA and SPA included >400 patients and >700 controls [57]. Assuming that the technical quality of the assay is well-validated and under control, it is likely that these diagnostic performance characteristics are less prone to local variability. Therefore, we argue that such studies should not/cannot be done by each individual laboratory. National

Table 1: Overview of recommendations/statements on methodological aspects.

R	ANA methodological aspects	Grade	References	Delphi score		
				Median	% high scores	
	ANA by HEp-2 Immunofluorescence analysis (IFA)				**≥8**	**≥7**
1	ANA by HEp-2 IFA should report the antibody titer and the pattern	A	[8, 45, 46]	9	96	97
	Reference range and importance of titer					
2	For many patterns, the likelihood for a ANA-associated rheumatic disease increases with antibody titer	A	[14–17, 48, 55–58]	8	83	92
3	For most patterns, there is no single HEp-2 IFA titer that is associated with both optimal sensitivity and optimal specificity for ANA-associated rheumatic diseases	A/B	[10, 12]	8	89	92
4	Taking into account the antibody level helps to interpret ANA test results	B		8	82	95
5	Reporting titer-specific or test result-specific likelihood ratios (LR) for ANA-associated rheumatic disease improves clinical interpretation of HEp-2 IFA test results	B		8	60	82
	ANA in healthy individuals					
6	ANA can be found in a substantial part of apparently healthy individuals (children, adults, elderly; increasing in prevalence with age) and this should be taken into account when interpreting test results	A/B	[16, 32–41]	9	95	99
	ANA in pediatric systemic rheumatic diseases					
7	There is no evidence that a lower screening dilution for the HEp-2 IFA test should be applied in children to screen for ANA-associated rheumatic diseases	A	[38, 40–42, 62, 63]	8	78	91
	Patterns					
8	HEp-2 IFA pattern may provide useful information in terms of clinical relevance and guidance for follow-up tests	A	[1, 45, 64, 65]	9	93	97
9	The clinical relevance of the HEp-2 IFA test result is pattern-dependent	A	[45, 64, 65, 68–74, 76]	8	82	93
10	Correlating the HEp-2 IFA pattern with the result of antigen-specific assays adds value to the interpretation of both	A	[1, 64, 65, 78, 83]	9	94	99
11	The main ANA patterns (with the highest clinical relevance) comprise: – nuclear centromere (AC-3), homogeneous (AC-1), dense fine speckled (AC-2), speckled (AC-4, 5), nucleolar (AC-8, 9, 10), multiple nuclear dots (AC-6), nuclear envelope (AC-11, 12) – cytoplasmic reticular (AC-21), (dense) fine speckled (AC-19, 20), linear fibrillary (AC-15)	A/B	[45, 46, 64, 65, 67–76, 80, 81, 84]	8	86	96
	Substrate					
12	HEp-2, HEp-2000 and HEp-20-10 cells can be used as substrate for ANA IFA screening	A	[48, 86–97]	8	81	92
	Conjugate					
13	The use of IgG specific conjugate is sufficient to detect most clinically relevant ANA	A/B	[48, 99–106]	9	93	97
14	The isotype specificity of the used conjugate (polyvalent/IgG specific) contributes to assay variability	A/B	[48, 99–106]	9	87	97
	Automated microscopy					
15	A computer-aided diagnosis system (CAD) can support HEp-2 IFA, but expert review remains recommended for positive/negative discrimination	A/B	[107–122], meta-analysis [124]	9	81	93
16	A CAD can support HEp-2 IFA, but expert review remains mandatory for pattern recognitions	A	[109–111, 116, 119], meta-analysis [124]	9	93	99
17	Several CAD systems for HEp-2 IFA provide a fluorescence intensity score (FI measure) which contains: – information on titer estimation – information on the likelihood for ANA-associated rheumatic diseases	A	[107–109, 116, 119, 122, 127, 128]	8	64	87

Table 1: (continued)

R	ANA methodological aspects	Grade	References	Delphi score		
				Median	% high scores	
	ANA by HEp-2 Immunofluorescence analysis (IFA)				≥8	≥7
18	When a CAD system for HEp-2 IFA is used that provides a reproducible FI measure, follow-up of this FI measure can contribute to monitoring of the quality of the analysis (e.g., as part of the IQC program) (not applicable for all systems on the market)	A	[109, 110, 126, 130–132]	8	76	92
	Quality assurance approaches in HEp-2 IFA					
19	Performance of HEp-2 IFA should be monitored by internal (IQC per run and periodic blinded reading of representative cases) and external quality assessment programs	A	[48, 134, 135, 139]	9	98	99
20	Factors minimizing HEp-2 IFA inter-observer variation include educational programs, application of double reading, usage of ICAP nomenclature and CAD monitor reading (digital images)	A/B	[134, 135, 140, 141]	9	94	98
21	At least 2 IQC samples (one negative and one low positive [with a target level matching a LR of 2–5 for ANA-associated rheumatic diseases]) should be included in each run and judged semi-quantitatively (either by end-point titration or automated intensity scoring)	A/B	[8, 48, 134, 135]	9	85	96
22	In addition to kit controls, it is advised to run IQC samples of patient origin, either pooled or unique samples, as they are processed as routine samples (thus allowing monitoring of the whole assay procedure)	A/B	[130, 134, 135]	9	83	93
23	The preferred pattern of the positive control sample preferentially has a high reproducibility (e.g., isolated homogeneous or speckled on CAD systems)	A/B	[109, 110, 125, 126, 130, 135]	8	88	98
24	Monitoring of the % of (low, medium, high) positive results in Levey-Jennings plots allows for the evaluation of assay stability over time (e.g., to document the effect of manufacturer inter-lot changes)	A	[48, 130, 131]	8	71	87
25	When a CAD system for Hep-2 IFA is used that provides a reproducible FI measure (not applicable for all commercial systems on the market): monitoring of the IQC sample and median patient FI in Levey-Jennings plots is a sensitive method to detect assay stability over time (e.g., to document the effect of lot changes)	A	[130, 132]	8	77	91
	Reagent lot acceptance and monitoring of lot-to-lot variability					
26	Lot-to-lot variability of conjugate and/or substrate should be evaluated before implementing a new lot. This can be done by patient-derived IQC samples supplemented with samples selected for this purpose minimally covering different cell compartments (nucleus and cytoplasm) and different titer levels.	A/B	[5, 48, 66, 133, 135, 145, 146]	8	79	91
	SPA for ANA screening					
27	The performance of SPA assays for ANA-associated rheumatic diseases screening/detection is disease- and assay-dependent	A	Reviewed in [1, 148]	9	91	100
28	There is no single SPA threshold that has both optimal sensitivity and optimal specificity for ANA-associated rheumatic diseases screening	A	Reviewed in [1]	9	93	98
29	Reporting test result (interval)-specific LR for ANA-associated rheumatic disease may improve clinical interpretation of SPA test results	A/B	[13–17, 57]	8	66	92
30	Combining HEp-2 IFA with SPA for ANA-associated rheumatic disease screening increases clinical value	A	Reviewed in [1, 11, 12, 17, 18]	9	88	98

Grading: A [experimental data/literature source is available], B [expert opinion]. Delphi scoring on a scale 1–9 [0 – absolutely no agreement with the recommendation; 9 – maximal support for the recommendation; 5 – impartial/undecided], with the option to skip the question based on inexperience; participants with no answer or unclear answer, or indicating that they had no experience on the topic were excluded from the analysis; scores of at least eight or seven on a scale of nine were considered high scores.

initiatives for validation/verification such as the Dutch CMI working group may reduce individual efforts made to introduce new tests in the laboratories [162]. Multicenter validation studies with diagnostic samples is preferable to method comparison by individual laboratories using non–diagnostic samples [162].

Table 2: Overview of recommendations/statements on validation/verification approaches.

R	HEp-2 IFA validation/verfication approaches	Grade	References	Delphi score Median	% high score ≥8	≥7
	Analytical validation/verification					
31	Each laboratory should demonstrate that its HEp-2 IFA method detects the major clinically relevant patterns as well as the major clinically relevant antigen reactivities, both in the nuclear and the cytoplasmic compartment	A/B	[5, 48, 66, 75, 133, 136]	9	91	94
32	Each laboratory should verify the precision of the method used. The approach will depend on how the data are handled: binomial (positive/negative), ordinal (titers) or continuous (fluorescence intensity measure results)	A/B	[134, 139, 157–162, 168]	9	89	95
33	Application of single well estimated titer by CAD has limitations and the claims of the manufacturer should be verified by comparison to end point titer for the most prevalent patterns	B		8	82	96
	Clinical validation/verification					
34	According to ISO 15189, CAP directives and the new 2017 IVD regulation, the manufacturer is responsible for the clinical validation of a CE/FDA labelled test. National legislation can formulate additional requirements	A	[134, 139, 151, 152, 154–156]	9	93	98
35	Validation of a HEp-2 IFA method is preferentially done in large multi-center studies including a sufficient number of diagnostic samples of clinically characterized patients and controls. Such studies should allow to estimate test result specific LR	B		8	85	92

Grading: A [experimental data/literature source is available], B [expert opinion]. Delphi scoring on a scale 1–9 [0 – absolutely no agreement with the recommendation; 9 – maximal support for the recommendation; 5 – impartial/undecided], with the option to skip the question based on inexperience; participants with no answer or unclear answer, or indicating that they had no experience on the topic were excluded from the analysis; scores of at least eight or seven on a scale of nine were considered high scores.

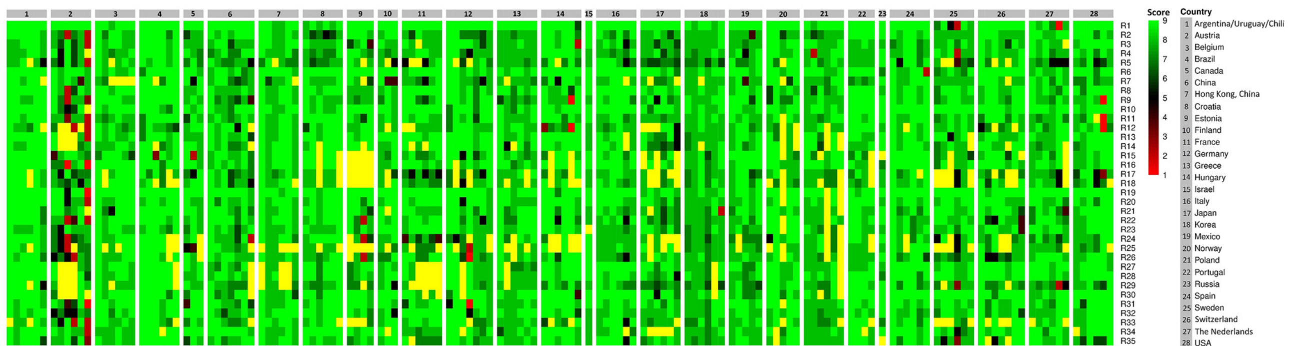

Figure 1: Heatmap of the Delphi scoring results organized by recommendation (R) and country/region. Delphi scoring on a scale 1–9 [0 – absolutely no agreement with the recommendation; 9 – maximal support for the recommendation; 5 – impartial/undecided]. Yellow areas represent either unanswered/unclear scoring or scores 15 (unanswered due to inexperience).

- Acording to ISO 15189, CAP directives and the new 2017 IVD regulation, the manufacturer is responsible for the clinical validation of a CE/FDA labelled test. National legislation can formulate additional requirements.
- Validation of a HEp-2 IFA method is preferentially done in large multi-center studies including a sufficient number of diagnostic samples of clinically characterized patients and controls. Such studies should allow to estimate test result specific LR.

ANA and IVDR

In May 2017, the In-Vitro Diagnostic Medical Devices Regulation (EU) 2017/746 (IVDR) was published and is planned to be enforced in 2022 [152]. HEp-2 IFA testing devices are subject to these regulations and must fulfill their requirements. According to the 2017 IVDR, all devices are divided into classes A,

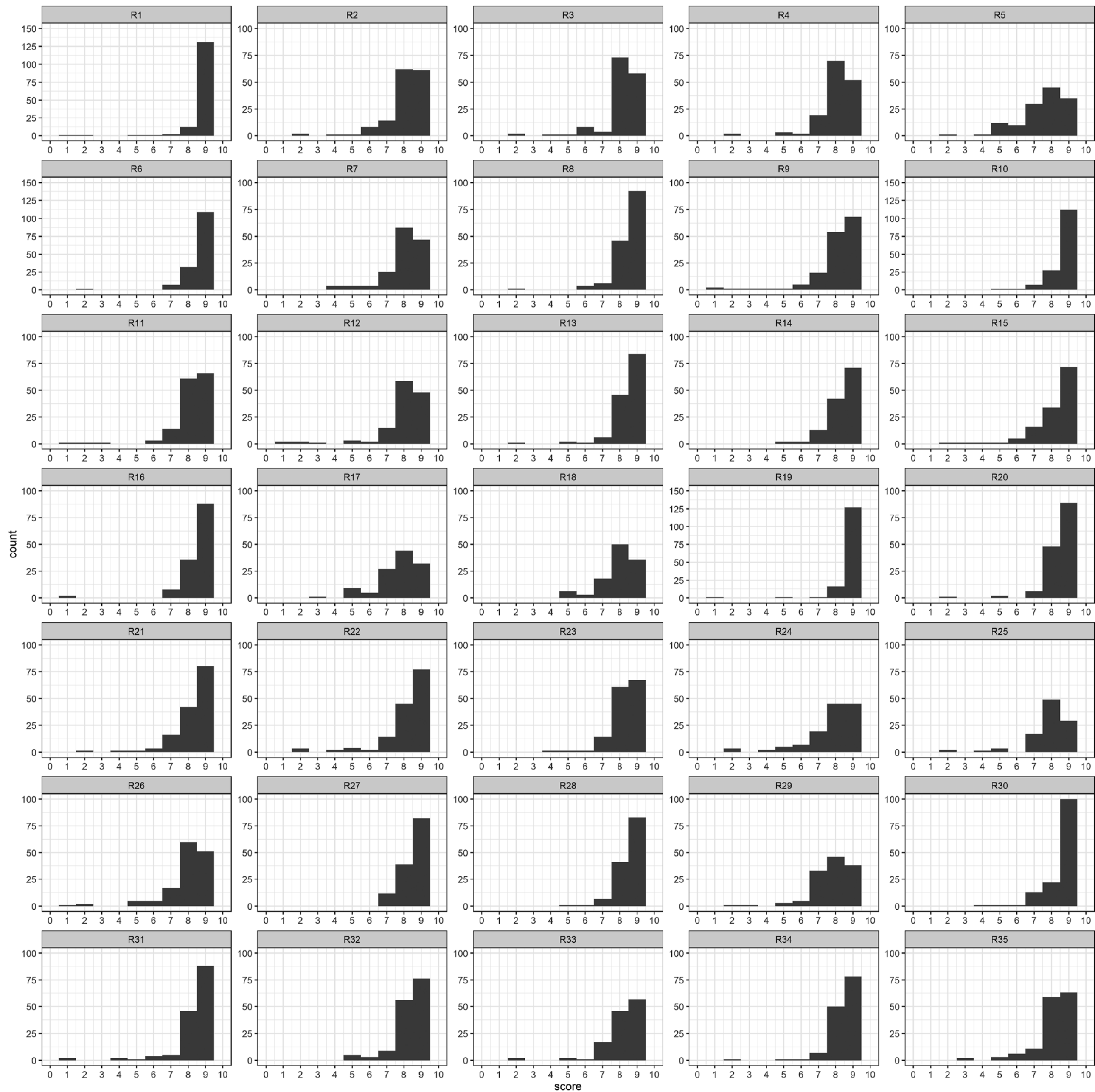

Figure 2: Distributions plots of the Delphi scoring results of the individual recommendations (R). Delphi scoring on a scale 1–9 [0 – absolutely no agreement with the recommendation; 9 – maximal support for the recommendation; 5 – impartial/undecided]. Counts (Y-as) represent number of answers with the particular score (bars) for the particular recommendation.

B, C and D, from uncritical (A) to highly critical (D), taking into account the intended purpose of the devices and their inherent risks. As per the classification rules of the IVDR, ANA test devices fall into group B, which is the default class for all parameters which do not fall within the scope of any of the stated rules. This is mainly because HEp-2 IFA devices do not test for transmissible agents.

Second, clinical evidence of the performance within the intended purpose of the test must be demonstrated and updated throughout the lifecycle of the test device. Such updating entails the planned monitoring by the manufacturer of scientific developments and changes in medical practice. Relevant new information should then trigger a reassessment of the clinical evidence of the device. A guideline for fulfilling these requirements can be found at MedTech Europe (https://www.medtecheurope.org/resource-library/clinical-evidence-requirements-for-ce-certification-under-the-in-vitro-diagnostic-regulation-in-the-european-

union/). An important point is the use of harmonized standards. Devices that are in conformity with the relevant harmonized standards, the references of which have been published in the Official Journal of the European Union, shall be presumed to be in conformity with the requirements. An example is EN 13612:2002 Performance evaluation of *in vitro* diagnostic medical devices (https://standards.iteh.ai/catalog/standards/cen/26676c14-f7c5-4f6c-b054-ffbd4199af54/en-13612-2002).

A critical issue in regard to the demonstration of clinical evidence of HEp-2 IFA tests would be the fact that ANA can occur years before the clinical manifestation of the disease [171]. So the predictive value in the absence of clinical disease would have to be demonstrated.

Recommendations/statements – Delphi scoring

The final 35 recommendations/statements that were formulated in this study are summarized in Tables 1 and 2 and are divided into two subgroups. The first subgroup of statements is dedicated to different methodological aspects of ANA analysis, mostly focused on HEp-2 IFA (Table 1). The second subgroup of statements is focused on IFA validation/verification aspects and covers both analytical as well as clinical validation (Table 2). For each statement, the median Delphi score and the % of high scores are listed (Tables 1 and 2). More details on the results can be found in Supplementary Table 4.

Overall, the global scoring response was 93%, with a minimal of blank/unclear answers (0.3%). In total, mean reported inexperience on the different topics was 9% (range 1–32%) with highest inexperience reported for the CAD-related statements (recommendations 15–18 [11–24% inexperienced], recommendation 25 [32% inexperienced] and recommendation 33 [16% inexperienced]).

Globally, 85% (4,114 out of 4,832) of all submitted scores were above 7 (agree or strongly agree), 95% of scores were above 6 (moderately agree, agree and strongly agree), indicating strong international support for the proposed recommendations. A heat map representation of all scores per geographical region/country is given in Figure 1. Distribution plots for each recommendation are depicted in Figure 2.

Acknowledgments: We thank the national EFLM teams (Croatian Society of Medical Biochemistry and Laboratory Medicine, the Georgian Laboratory Medicine Association, the Estonian Society of Laboratory Medicine, the Italian Society of Clinical Biochemistry and Clinical Molecular Biology, the Lithuanian Society of Laboratory Medicine, the Portuguese Society of Clinical Chemistry, Genetics and Laboratory Medicine, the Slovenian Association for Clinical Chemistry and Laboratory Medicine, the Spanish Society of Laboratory Medicine and the Turkish Biochemical Society) for their valuable input and suggestions. We thank all experts for participating in the Delphi questionnaire (round 2) (organized per geographical region/country): Manfred Herold (Rheumatology Laboratory, Medical University of Innsbruck, Innsbruck, Austria); Andrea Griesmacher (Central Institute of Clinical and Chemical Laboratory Diagnostics, University Hospital of Innsbruck, Austria), Georg Endler (labors.at, Vienna, Austria); Jörg Hofmann (Institut für Labormedizin, Sozialmedizinisches Zentrum Ost, KAV Wien, Austria); Nikolaus Wick (Speziallabor Wick, Innsbruck, Austria); Ulrike Demel (Clinical Department for Rheumatology and Immunology, University Hospital of Graz, Austria); Lieve Van Hoovels (Department of Laboratory Medicine, OLV Hospital, Aalst, Belgium); Khadija Guerti (Department of Clinical Chemistry, Antwerp University Hospital, Edegem, Belgium); Laurence Lutteri (Department of Clinical Chemistry, University Hospital Liège, Liège, Belgium); Sylvie Goletti (Cliniques Universitaires Saint-Luc, Brussels, Belgium); Sofie Schouwers (Department of Laboratory Medicine, GZA Hospitals, Antwerp, Belgium); Sara Vijgen (Department of Laboratory Medicine, Jessa Hospital, Hasselt, Belgium); Ana Kozmar (Department of Laboratory Diagnostics, University Hospital Centre Zagreb, Zagreb, Croatia); Katarina Gugo (Department of Medical Laboratory Diagnostics, University Hospital Center Split, Split, Croatia); Jasna Pavela (Institute of Clinical Laboratory Diagnostics, Osijek University Hospital, Osijek, Croatia); Lovorka Đerek (Clinical Department of Laboratory Diagnostics, University Hospital Dubrava, Zagreb, Croatia); Vedrana Drvar (Clinical Department of Laboratory Diagnostics, Clinical Hospital Center Rijeka, Rijeka, Croatia); Andrea Tešija Kuna (Department of Clinical Chemistry, Sestre Milosrdnice University Hospital Center, Zagreb, Croatia); Raivo Uibo (Department of Immunology, Medical Faculty, University of Tartu, Estonia); Kaja Metsküla (United Laboratories, Tartu University Hospital, Tartu, Estonia); Liisa Kuhi (Central Laboratory, Diagnostic Clinic, East Tallinn Central Hospital, Tallinn, Estonia); Meeli Glükmann (Clinical Chemistry Department, Synlab Eesti OÜ, Tallinn, Estonia); Anna-Maija Haapala (Department of Immunology, Fimlab laboratories, Tampere, Finland); Jari Karhukorpi (ISLAB, Finland); Arno Hänninen (Turku University Hospital, Finland); Lucile Musset (Department of Immunology, CHU Pitié Salpêtrière-Ch Foix, APHP, Paris, France); Marie Senant (Cerballiance, Lisses, France); Marie-Agnès Dragon-Durey (Hôpital

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Kucharz (Medical University of Silesia, Katowice, Poland); Bogdan Batko (Department of Rheumatology and Immunology, Faculty of Medicine and Health Sciences, Andrzej Frycz Modrzewski University, Krakow, Poland); Zbigniew Żuber (Department of Pediatrics, Faculty of Medicine and Health Sciences, Andrzej Frycz Modrzewski Kraków University, Kraków, Poland); Piotr Dąbrowski (Department of Rheumatology, Clinical Hospital No. 2 in Rzeszów. University of Rzeszow, Poland); Maria-Jose Rego de Sousa (Immunopathology and Autoimmunity Department, Centro de Medicina Laboratorial Germano de Sousa, Lisbon, Portugal); Catarina Castaldo/Margarida Albuquerque (Immunopathology and Autoimmunity Department, Centro de Medicina Laboratorial Germano de Sousa, Lisbon, Portugal); Esmeralda Neves (Immunology Department, Centro Hospitalar Porto, Porto, Portugal);Ana Paula Cruz (Immunology Department, Centro Hospitalar Vila Nova de

Gaia e Espinho, Gaia, Portugal); Vladimir Malkov (Tafi Diagnostica, Vladivostok, Russia); M Luisa Vargas-Pérez (Immunology, Hospital Universitario de Badajoz, Spain); Aurora Jurado Roger (Immunology, Hospital Universitario Reina Sofia, Biomedical Research Institute of Cordoba (IMIBIC), Córdoba, Spain.); Garbiñe Roy (Immunology, Hospital Universitario Ramón y Cajal, Madrid, Spain); Laura Martínez Martínez (Immunology, Hospital Santa Creu i Sant Pau, Barcelona, Spain); Marco A Montes Cano (Immunology. Hospital Universitario Virgen del Rocío, Sevilla, Spain); Yvelise Barrios Del Pino (Hospital Universitario de Canarias, Tenerife, Spain); Catharina Eriksson (Department of Clinical Microbiology section of Infection and Immunology, Umeå University, Umeå, Sweden); Rille Pullerits (Department of Clinical Immunology, Sahlgrenska University Hospital, Gothenburg, Sweden); Johan Rönnelid (Uppsala University Hospital, Uppsala, Sweden); Rui Da Silva Rodrigues (Department of Clinical Immunology, Karolinska University Hospital, Stockholm, Sweden); Charlotte Dahle (Department of Clinical Immunology, University Hospital, Linköping, Sweden); Maria Lundgren (Clinical immunology, University Hospital, Lund, KITM, Lund, Sweden); Ingmar Heijnen (Immunology, Laboratory Medicine, University Hospital Basel, Basel, Switzerland); Eric Dayer (Synlab Immunologie, Lausanne, Switzerland); Elisabeth Probst-Müller (Immunology, University Hospital Zurich, Zurich, Switzerland); Arlettaz Lionel (Institut Central des Hôpitaux (Hôpital du Valais), Valais, Switzerland); Dehlia Chevalley (Service of Immunology and Allergology, Lausanne University Hospital, Lausanne, Switzerland); Michael P. Horn (Department of Clinical Chemistry, Inselspital Bern, University of Bern, Bern, Switzerland); Luca Bernasconi (Institute of Laboratory Medicine, Kantonsspital Aarau, Aarau, Switzerland). Edward Chan (Department of Oral Biology, University of Florida, Gainesville, FL, United States); Mark Wener (University of Washington, United states); Lisa K. Peterson (ARUP Laboratories, United States); Anne E. Tebo (Mayo Clinic, Rochester, USA); Donald Bloch (Massachusetts General Hospital, Harvard Medical School, United States); Carlos A. von Mühlen (Private Consultant in Rheumatology and Clinical Pathology, United states); Ignacio García-De La Torre (Department of Immunology and Rheumatology, Hospital General de Occidente, Universidad de Guadalajara, Guadalajara, Mexico); Julio Sierra G de Quevedo (Unidad de Patología Clínica, Guadalajara, Mexico); Q.B.P. Andrés Mendiola Jiménez (Servicio de Reumatología H.U. de la U.A.N.L., Monterrey, Mexico); Daniel Perez Covarrubias (Laboratorio de Inmunología Clínica, S.A. de C.V. Guadalajara, México); Carlos A. 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Gutierrez Children Hospital, Buenos Aires, Argentina); María Pino (Hospital de Clínicas “José de San Martín” UBA División Reumatología Laboratorio, Buenos Aires, Argentinia); Carolina Valenzuela (Instituto de Salud Pública de Chile-Laboratorio Nacional y de Referencia de Inmunología, Chili); Minoru Satoh (Department of Clinical Nursing, University of Occupational and Environmental Health and Kitakyushu Yahata-Higashi Hospital, Kitakyushu, Japan); Takao Fujii (Department of Rheumatology and Clinical Immunology, Wakayama Medical University, Wakayama, Japan) Michito Hirakata (Medical Education Center, Keio University School of Medicine, Tokyo, Japan) Tsuneyo Mimori (Ijinkai Takeda General Hospital, and Department of Rheumatology and Clinical Immunology, Kyoto University, Kyoto, Japan) Yoshinao Muro (Department of Dermatology, Nagoya University, Nagoya, Japan); Shinji Sato (Department of Internal Medicine, Tokai University School of Medicine, Isehara, Japan); Kyeong-Hee Kim (Department of Laboratory Medicine, Dong-A University, Republic of Korea) Jimyung Kim (Department of Laboratory Medicine, Chungnam National University Hospital, Republic of Korea) Eun-Jee Oh (Department of Laboratory Medicine, Catholic University of Korea, Republic of Korea) Hee-Won Moon (Department of Laboratory Medicine, Konkuk University Medical Center, South Korea) Sun Min Lee (Department of Laboratory Medicine, Pusan National University Yangsan Hospital, Republic of Korea); Hyun-Woo Choi (Department of Laboratory Medicine, Chonnam National University Hospital, Republic of Korea). 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Research funding: None declared.
Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: JD reports consultancy and/or speakers fees from Werfen/Inova, Thermo Fisher Scientific, and Euroimmun Medizinische Labordiagnostika. MF is a Medical Director of Mitogen Diagnostic Corporation (Calgary, Canada) and a consultant to Werfen (San Diego, CA, USA). XB reports consultancy and/or speakers fees from Werfen, Thermo Fisher Scientific and Menarini.
Ethical approval: Not applicable.
Informed consent: Not applicable.

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Supplementary Material: This article contains supplementary material (https://doi.org/10.1515/cclm-2023-0209).