**EFLM Paper**

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# Methodology and quality assurance practices in performing anti-extractable nuclear antigen (ENA) analysis: an EFLM report on an international survey

https://doi.org/10.1515/cclm-2026-0273
Received February 18, 2026; accepted May 6, 2026;
published online June 8, 2026

**Abstract**

**Objectives:** Autoantibodies to extractable nuclear antigens (anti-ENA) play a crucial role in the diagnosis of systemic autoimmune rheumatic diseases (SARD). Analytical methods differ in antigen composition and diagnostic performance. We aimed to document the extent of variability amongst laboratories in how anti-ENA testing is conducted, controlled and verified.

**Methods:** A survey was developed to collect information on anti-ENA methodology and quality assurance practices. The questionnaire was distributed amongst laboratories by Sciensano (Belgium), national EASI groups (Italy, Croatia, Portugal, Estonia, Greece) and ICAP (worldwide).

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**Results:** A total of 427 laboratories participated (61 % European, 21 % American, 14 % Asian). More than half (52 %) use two or more anti-ENA methods. Turnaround time varied, but 71 % perform the analysis within 72 h. Urgent testing was offered in 42 % of laboratories. Internal quality control (IQC) was performed in 91 % of laboratories, predominantly with commercial materials. External quality assessment (EQA) participation was also high (87 %), with nearly half participating in more than three distributions annually. Reagent lot evaluation was less widely implemented (36 %). Forty-three percent tried to limit variability by limiting lot changes. Method verification was performed by 81 % of the laboratories, though sample type and numbers, as well as reproducibility replicates varied considerably.

**Conclusions:** This survey highlights substantial heterogeneity in anti-ENA laboratory practice. While most laboratories apply IQC and EQA, significant variation remains in test method(s), turnaround times, lot variability evaluation and verification strategies. Development of more comprehensive recommendations on these topics are essential to improve reliability and comparability of results across laboratories.

**Keywords:** anti-extractable nuclear antigen; antinuclear antibodies; quality assurance; harmonization; methodology

## Introduction

Detection of autoantibodies to extractable nuclear antigens (anti-ENAs) is considered essential in the (differential) diagnosis of systemic autoimmune rheumatic diseases (SARD), including systemic lupus erythematosus, Sjögren's disease, immune-mediated inflammatory myopathy and systemic sclerosis [1]. In line with international recommendations, this test usually follows a positive HEp-2 indirect immunofluorescence antinuclear antibody (ANA) analysis, which serves as a screening test for the presence of anti-ENA [2, 3].

Historically, anti-ENA testing was mostly limited to SS-A/Ro60, SS-B/La, RNP-A, RNP-C, RNP-70, SmD, SmB, Scl-70, centromere protein B, Jo-1 and Ribo-P [4], and performed with a set of conventional immunological methods, such as western blotting (WB), double immunodiffusion (DID), protein immunoprecipitation (P-IP) and RNA immunoprecipitation (RNA-IP). These historical techniques are often referred to as the golden standard as these techniques were initially used to characterize the various specific reactivities in relation to their clinical presentation. However, today these techniques have been mostly abandoned due to their time-consuming nature, safety issues (radioactivity) and difficulty to standardize [5, 6]. Indeed, considerable progress has been made in the detection of anti-ENA antibodies with a shift towards less time-consuming, automated high-throughput methods [7]. Also, the number of reactivities that are today classified as anti-ENA has increased significantly and the target antigens of the anti-ENA tests are no longer confined to 'nuclear extrable antigens' but also include cytoplasmic reactivities [3, 8]. Commonly used modern platforms for anti-ENA testing consist of, amongst others, addressable laser bead immunoassay (ALBIA), chemiluminescent immunoassay (CLIA), fluorescent enzyme immunoassay (FEIA), enzyme-linked immunosorbent assay (ELISA), dot blots and line immunoassay (LIA). Most of these systems allow for the detection of the different anti-ENA either by a multiplexed analysis of antibodies to different individual antigens or by a single test that detects antibodies to an antigen pool (by some companies referred to as 'CTD' screen). Currently, the spectrum of reactivities assessed by these modern platforms collectively covers more than 35 antigens and are often organized in panels linked to specific disease subsets, such as myositis and systemic sclerosis. Moreover, emerging technologies such as immunoprecipitation mass spectrometry are identifying novel autoantibody reactivities and can be used as a reference technique for antibodies to conformational antigens [9]. Such technologies will likely become available for reference centra [10]. Many studies have compared commercial platforms with conventional techniques and ANA HEp-2 IFA, and have also provided direct comparisons between different commercial platforms; a selection of such studies is cited here [11–22]. These studies indicate variability between methods. This may be linked to diverse factors such as difference in sensitivity between technologies and variation in composition, source and purity of the antigens. Of note, most of these newer techniques use recombinant/synthetic antigens rather than native antigens, and it has been suggested that the use of such antigens might decrease sensitivity towards conformational-dependent epitopes and epitopes originating from post-translational modification [23, 24]. On the other hand, one of the biggest advantages of these newer methods is that they often generate (semi-)quantitative results, while the conventional techniques only deliver qualitative results. Yet, standardization of quantitative methods is lacking and there is a lack of 'good' standard materials for each of the individual reactivities [3]. Thus, standardisation of the anti-ENA tests has not yet been achieved and is probably a utopia. Harmonisation may offer an alternative approach, as it aims to minimize differences between methodologies, test results and procedures of the different laboratories by developing guidelines and recommendations [25]. Indeed, specifically in the field of auto-immune

testing different international and national guidelines/recommendations have been published that offer laboratories tools to implement good clinical practice and to align results [26–30]. Only limited recommendations focused on anti-ENA testing are available and to our knowledge no published data are available on the variability in quality assurance practices [31, 32], but some studies already documented method variability [33, 34]. In line with our previous work [35], where we addressed the methodological aspects of the ANA test, we here aimed to document the extent of international variability on how the anti-ENA test is conducted, controlled and verified, in order to identify potential aspects that could benefit from better harmonization.

# Methods

Questions on anti-ENA testing methodology, internal and external quality control practices, and strategies for controlling lot variability and method verification were designed by the Expert Committee Non-infectious Serology of Sciensano and EASI Belgium as part of the previously published survey on ANA testing [35]. The survey was first distributed to Belgian laboratories by Sciensano in 2018 and subsequently internationally distributed by national European Autoimmunity Standardization Initiative (EASI) groups (Italy, Croatia, Portugal, Estonia, Greece) and ICAP (International Consensus on Antinuclear Antibody patterns) in 2019. Except for 7 questions out of 33, in which Belgian participants could select only one answer in contrast to international participants who could select a combination of answers, the questionnaire was identical. For these 7 questions Belgian answers were omitted. Finally, data analysis was performed by the European Federation of Laboratory Medicine (EFLM) Working Group: “Autoimmunity Testing”.

# Results

A total of 427 laboratories performing ANA testing responded to the questionnaire. Participants were mainly European (61 %), American (21 %) and Asian (14 %) (Table 1). The majority of these laboratories perform anti-ENA testing (392/427, 92 %). See Table 1 for more details.

**Table 1:** Anti-ENA: geographical distribution of the participants.

| Continent | Number of responses, n (%) | Number of labs performing anti-ENA, n (%) |
|---|---|---|
| Europe | 262 (61.3) | 238 (90.8) |
| Asia | 58 (13.6) | 50 (86.2) |
| South-America | 54 (12.6) | 53 (98.1) |
| North-America | 34 (8.0) | 30 (88.2) |
| Africa | 11 (2.6) | 11 (100) |
| Oceania | 8 (1.9) | 8 (100) |
| **Total** | **427** | **392** |

## Methodological and organizational aspects

Different analytical methods can be used to detect anti-ENA antibodies. Fifty-two percent (204/392) of the laboratories used a combination of methods (two or more), while 48 % (188/392) sticked to solely one method (Table 2). Dot/line blot and enzyme-linked immunosorbent assay (ELISA) were the most frequently used assays, while addressable laser bead immunoassay (ALBIA) was the least frequently used assay by the participants at the time of the survey.

The turn-around time of anti-ENA analysis may depend on the setting. Our results showed that anti-ENA analysis was performed on a daily basis in 35 % (135/382) of the laboratories, while 36 % (137/382) of laboratories performed the analysis 2–3 times a week. The remainder of laboratories had a turn-around-time of a week or more. Forty-two percent (or 101/242) of participating laboratories offered anti-ENA analysis in an urgent setting.

## Quality assurance

### Internal quality control

The vast majority (91 %, 271/299) of the participating laboratories included internal quality control (IQC) samples in each analytical run (Table 3). Variables in the IQC procedure for anti-ENA testing include: the IQC strategy, the number of IQC samples, their origin, antigen specificity and preservation. Two thirds of the laboratories opted to integrate at least one positive sample and one negative sample (67 %, 165/247), while one third of the laboratories omitted the negative control (33 %, 82/247). The level of positivity of the IQC sample was highly variable across the different laboratories: 34 % used a high positive level (71/210), 37 % used a medium positive level (77/210), 13 % used a level around the cut off (27/210), and 17 % combined different levels of positive samples (35/210). Moreover, if more than 1 positive IQC sample was analysed, they were mostly located at the beginning or at the end of the run (76 %, 87/115). Laboratories that alternated specificities over different runs (29 %, 63/216), did it mostly

**Table 2:** Anti-ENA: methodological and organizational aspects.

| Topic | Number of responses | Answers | %[a] |
|---|---|---|---|
| **Methodology** | | | |
| Method used (total) | 392 | | |
| – Only 1 method | 188 | Dot/line blot | 36 |
| | | ELISA | 34 |
| | | FEIA | 15 |
| | | CLIA | 10 |
| | | ALBIA | 4 |
| | | Other | 1 |
| – Combination of 2 methods (either in parallel or consecutively) | 191[b] | Dot/line blot + ELISA | 28 |
| | | Dot/line blot + FEIA | 30 |
| | | Dot/line blot + CLIA | 14 |
| | | Other combinations | 28 |
| – Combination of at least 3 methods (either in parallel or consecutively) | 13[b] | ELISA + CLIA + other | 31 |
| | | FEIA + dot/line blot + other | 54 |
| | | ELISA + dot/line blot + other | 61 |
| **Test frequency & urgency** | | | |
| Test frequency | 382 | Daily | 35 |
| | | 2–3 times a week | 36 |
| | | Weekly | 21 |
| | | Every 2 weeks | 8 |
| Test urgency | 242 | Urgency | 42 |
| | | No urgency | 58 |

[a]Expressed on the total responders, unless stated otherwise; [b]Combinations of different answers are possible. ALBIA, addressable laser bead immunoassays; CLIA, chemiluminescent immunoassay; ELISA, Enzyme-linked immunosorbent assays; FEIA, fluoroenzymatic immunoassay.

on a daily (20 %) or weekly basis (25 %) or at variable time intervals (25 %).

IQC samples can be provided by the manufacturer (mostly as kit controls) or in-house samples from patient origin (either single patients or patient pools). In our survey, 14 % (or 36/250) of laboratories combined in-house controls with commercial controls, while the majority (81 %, 203/250) only used commercial material.

Seventy-one percent of the laboratories that used commercial controls used them undiluted (in contrast to the patient samples) (163/231). As an IQC target, the vast majority of laboratories (83 %, 196/236) opted to use company targets. When patient samples were applied as control material, 62 % (28/45) used single patient samples, 11 % (5/45) used pooled samples and 27 % (12/45) used a combination of single patient and pooled samples.

Fifty-seven percent (54/95) of the participants froze patient IQC samples at −20 °C, while 14 % (or 13/95) stored samples at −80 °C.

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

In our survey, 42 % (98/234) of the laboratories indicated not to verify new lots from different batches and not to perform lot reservation to limit the number of lot changes (Table 4). Fifteen percent (35/234) of the laboratories did not do lot reservations but had a lot-to-lot variability evaluation procedure in place, 22 % (51/234) of the laboratories did lot reservations but had no lot-to-lot evaluation procedure and 21 % (50/234) did lot reservations and had a procedure for lot-to-lot evaluation.

### External quality assessment

Of all the participating laboratories in our survey, the vast majority (87 %, 240/277) performs EQA (Table 5). The number of distributions of EQA in which laboratories participated is variable. About 48 % (102/214) of the laboratories indicated to participate in more than 3 external quality control distributions per year, while 10 % (22/214) only participated in one distribution. Usually, laboratories run at least five external quality control samples (63 %, 136/216) per year.

### Method verification

We collected data on verification practices amongst laboratories with a focus on method comparison and reproducibility analysis. According to our data, 81 % (147/181) of the laboratories verified a new method before implementation in routine. The other laboratories did not perform in-house

**Table 3:** Anti-ENA: Internal quality control practices.

| Topic | Number of responses | Answers | %[a] |
|---|---|---|---|
| Performance of run IQC | 299 | Yes | 91 |
| **Details on the IQC procedure** | | | |
| Number of IQC samples | 247[b] | 1 positive and 1 negative IQC | 55 |
| | | >1 positive and 1 negative IQC | 12 |
| | | 1 positive and no negative IQC | 29 |
| | | >1 positive and no negative IQC | 4 |
| Position of positive IQC samples (if ≥2/run) | 115[b] | All at the start or the end of the run | 76 |
| | | Some at the start and some at the end of the run | 24 |
| Level of positive IQC samples | 210[b,e,g] | High level | 34 |
| | | Medium level | 37 |
| | | Around the cut-off | 13 |
| | | Combination of different levels | 17 |
| Alternating specificities over different runs | 216[b] | No, always the same specificities | 71 |
| | | Yes, alternating specificities | 29 |
| Frequency of alternating specificities | 59[d] | Daily | 20 |
| | | Weekly | 25 |
| | | >1/week and <1/month | 10 |
| | | >1/month | 7 |
| | | At the end of same supply | 12 |
| | | Variable | 25 |
| Origin of IQC samples | 250[b] | Commercial origin only | 81 |
| | | Patient origin only | 4 |
| | | Both commercial and patient samples | 14 |
| Preservation conditions of patient IQC samples[f] | 86[c,e] | Fridge with/without preservatives (e.g. sodium azide) | 36 |
| | | −20 °C | 57 |
| | | −80 °C | 14 |
| | | Other | 3 |
| Pooled or single patient IQC samples | 45[c] | Pooled patient sample | 11 |
| | | Single patient samples | 62 |
| | | Pooled and single patient samples | 27 |
| Starting/screening dilution of commercial IQC samples | 231[f] | Undiluted (different from patient samples) | 71 |
| | | Diluted (same dilution as patient samples) | 25 |
| | | Diluted (different dilution as patient samples) | 4 |
| Origin of the target of commercial IQC samples (quantitative method) | 236[b] | Commercial | 83 |
| | | Other | 17 |
| Usage of monthly percentages of (positive patient samples) for IQC purposes | 233[b] | Yes | 13 |
| | | No | 87 |

[a]Expressed on the total responders, unless stated otherwise; [b]Expressed on the total of responses for participants that perform IQC; [c]Expressed on the total responders that perform IQC and use patient samples for IQC purposes; [d]Expressed on the total of responses for participants that use patient material for IQC purposes and perform alternating patterns/antigen specificities in their positive QC over different runs; [e]Combinations of different answers are possible; [f]Expressed on the total of responses for participants that use commercial material for IQC purposes; [g]ICAP only, exclusion Belgian data as for Belgian laboratories combined answers were not possible.

verification, but relied on published literature (4 %, 8/181), the kit insert (14 %, 25/181) or a combination of both (0.5 %, 1/181) to get insights into the performance of the applied method (Table 6). From the laboratories that performed method verification, 48 % (70/147) of them performed verification experiments exclusively on patient samples, 11 % (16/147) exclusively on EQA samples and 41 % (61/147) on both. From the laboratories that included patient samples for anti-ENA method verification, 58 % (76/131) characterized the patient samples minimally based on clinical information and 28 % (37/131) combined it with laboratory test results of other methods. Forty-two percent (55/131) used only other laboratory test results as reference and did not take the clinical diagnosis into account.

**Table 4:** Anti-ENA: strategies to control lot variability.

| Topic | Number of responses | Answers | %[a] |
|---|---|---|---|
| Strategies to control lot variability | 234 | Lot variability procedure only | 15 |
| | | Lot reservation only | 22 |
| | | Combination of both strategies | 21 |
| | | No lot reservation nor procedure for evaluating lot variability | 42 |

[a]Expressed on the total responders, unless stated otherwise.

**Table 5:** Anti-ENA: EQC practices.

| Topic | Number of responses | Answers | %[a] |
|---|---|---|---|
| Participation in EQC schemes | 277 | Yes | 87 |
| | | No | 13 |
| Number of distributions | 214 | 1 | 10 |
| | | 2 | 25 |
| | | 3 | 17 |
| | | >3 | 48 |
| Number of samples distributed | 216 | 1 | 6 |
| | | 2 | 13 |
| | | 3 | 8 |
| | | 4 | 10 |
| | | >5 | 63 |

[a]Expressed on the total responders, unless stated otherwise.

To obtain representative data, a substantial number of samples should be included in the method comparison. Our data documented that laboratories that used lab-characterized (non-clinically defined) samples for verification used mostly more than 20 samples (69 %, 52/76); 33 % of them used 40 or more samples. In terms of sample positivity, the vast majority preferred to use a combination of highly and weakly positive samples (86 %, 106/123) to verify the method. Our survey also showed that 84 % (59/70) of the laboratories that tested clinically defined samples used at least 2 samples per specificity/antigen.

Based on our survey results, precision was verified by 73 % of the laboratories (150/205), from which 79 % verified both within-and between-run (119/150), 17 % verified only between-run (25/150) and 4 % only verified within-run precision (6/150). A relatively small fraction of the participating laboratories conducted at least 10 replicates in within-and between-run precision experiments, respectively, 24 % (29/123) and 30 % (41/137). The other laboratories perform less replicates.

## Discussion

In this paper, the results of an international survey performed by the Expert Committee Non-infectious Serology of Sciensano and EASI Belgium in cooperation with national (EASI groups of Italy, Croatia, Portugal, Estonia, Greece) and international (ICAP) expert organizations on the methodological aspects of performing anti-ENA are presented. The questionnaire addressed various topics, mainly on methodological and organizational aspects, but also on internal/external quality control, lot-to-lot variability monitoring, and method verification.

Anti-ENA tests are widely commercially available, and according to our study 92 % of autoimmunity laboratories that perform ANA testing also test anti-ENA. The lowest frequencies in anti-ENA testing were found in Asia (86 %) as well as North-America (88 %), while higher frequencies were observed in Europe (91 %) and South-America (98 %). For the other continents lower participant numbers did not allow for a representable image on this matter.

EASI advices that preliminary results of anti-ENA antibodies are available within 48–72 h [36]. Our survey showed that anti-ENA analysis was performed within 72 h in 71 % of laboratories. The remainder laboratories had higher turnaround-times. Urgent ANA/anti-ENA testing can be of added value in suspected severe autoimmune disease where the result will influence clinical decision-making and/or treatment, such as rapidly progressing inflammatory myopathy/interstitial lung disease or systemic sclerosis [37, 38]. However, in many laboratories, anti-MDA5 (rapidly progressing interstitial lung disease) and anti-RNA polymerase III (scleroderma renal crisis) testing are not included in the basic anti-ENA panel. The latter might have created confusion amongst the participants, and it is unclear who of the participants have taken these reactivities into account upon answering this question. Yet, 42 % of participating laboratories offered anti-ENA analysis in urgent setting.

Currently, various analytical methods (e.g. line/dotblot, CLIA, ALBIA, FEIA, microarray, ELISA) with variable performance characteristics are available for detecting the most clinically relevant anti-ENA [1, 13, 15]. Moreover, the number of autoantibodies covered by the commercial tests may be different. Therefore, laboratories may opt to combine ENA testing methods (either in parallel or in a stepwise (reflex) manner) to optimize diagnostic performance characteristics for SARD [1]. Laboratories sometime also take into account the clinical presentation and ANA pattern(s) to determine

**Table 6:** Anti-ENA: method verification in practice.

| Topic | Number of responses | Answers | %[a] |
|---|---|---|---|
| Performing verification of commercial method | 181[b,c] | Yes | 81 |
| | | No verification, rely on kit insert | 14 |
| | | No verification, rely on publication | 4 |
| | | No verification, rely on kit insert and publication | 0.5 |
| **Details verification procedure** | | | |
| Origin of samples | 147[b,c,d] | EQC samples exclusively | 11 |
| | | Patient and EQC samples | 41 |
| | | Patient samples exclusively | 48 |
| Characterization of patient samples | 131[b,c,e] | Clinically characterized | 30 |
| | | Laboratory characterized | 42 |
| | | Both clinically and laboratory | 28 |
| Number of laboratory- characterized patient samples used for verification | 85[c,f] | <10 | 14 |
| | | ≥10 to <20 | 17 |
| | | ≥20 to <40 | 36 |
| | | ≥40 | 33 |
| Number of clinically characterised samples per antigen/specificity | 70[c,g] | 1 sample | 16 |
| | | 2–3 samples | 54 |
| | | 4–5 samples | 20 |
| | | >5 samples | 10 |
| Level of sample positivity | 123[c,d] | Weakly positive only | 5 |
| | | Highly positive only | 9 |
| | | Combination | 86 |
| **Verification of precision** | | | |
| Evaluation of precision | 205 | Yes | 73 |
| | | – Between-run only | 17[h] |
| | | – Within-run only | 4[h] |
| | | – Between- and within-run | 79[h] |
| Between run procedure: | 136[h] | 0 | 1 |
| –Number of antigen specificities | | 1 | 27 |
| | | 2 | 31 |
| | | ≥3 | 41 |
| –Number of replicates | 137[i] | <5 replicates | 38 |
| | | 5–9 replicates | 32 |
| | | ≥10 replicates | 30 |
| Within run procedure: | 123[j] | <5 replicates | 41 |
| –Number of replicates | | ≥5 to <10 replicates | 35 |
| | | ≥10 replicates | 24 |

[a]Expressed on the total responders, unless stated otherwise; [b]Combinations of different answers are possible; [c]ICAP only, exclusion of Belgian data because for Belgian laboratories combined answers were not possible; [d]Expressed on the number of laboratories that perform a verification of a commercial method; [e]Expressed on the number of laboratories using patient samples for verification; [f]Expressed on the number of laboratories performing verification using non-clinically defined patient samples; [g]Expressed on the number of laboratories performing verification using clinically defined patient samples; [h]Expressed on the number of laboratories verifying precision; [i]Expressed on the number of laboratories performing between run precision verification experiments; [j]Expressed on the number of laboratories performing within run precision verification experiments.

which additional anti-ENA specificities should be tested or advised to the requesting clinician. Moreover, difference in testing strategies/algorithms between countries might also be influenced by differences in healthcare organization and reimbursement criteria/limitations, as well as to the adherence to local guidelines and recommendations, which might be shaped by country-specific factors such as resource availability, regulatory frameworks, epidemiological context, and professional consensus, In our survey performed in 2018–2019, 52 % of the laboratories used a combination of methods (two or more), while the others use only one method. At that time the dot/line blot and ELISA were commonly used. Yet, a recent paper that analyzed the UK NEQAS reports between 2012 and 2021, showed a gradual reduction of ELISA over time and an increase in FEIA, CLIA, ALBIA and line/dotblot [33]. A limitation of our report is thus that the survey results are potentially not fully representing the current situation. According to EN/ISO15189:2022, IQC is

of the utmost importance to guarantee the analytical performance of tests in clinical laboratories [39]. Our data showed that in the vast majority of the participating laboratories an IQC sample is analyzed in each analytical run. According to a CLSI guideline and published recommendations, the inclusion of at least two IQC samples (one positive and one negative) in each run is recommended [4, 30]. In line with this guideline, 67 % of laboratories integrated at least one positive sample and one negative sample and 33 % of laboratories integrated at least one positive IQC sample. Also, the majority of laboratories used only IQC material from commercial origin (81 %). However, commercial controls may be less ideal for IQC purpose for several reasons. First, they are often "ready to use" and require no predilution, hereby missing the preanalytical procedure. Second, commercial IQC materials represent mostly 'extreme' values (high positive and extreme negative), not allowing to reveal relevant analytical issues around the cut-off. Third, the use of in-house IQC samples offers the advantage of bridging reagent lot changes, while commercial material is often changed in parallel with lot changes and thus not able to capture response changes caused by lot changes. Fourth, the use of in-house controls allows for the expansion of the antigens covered by IQC, as commercial material is mostly limited to one single antigen. Thus, it may be a better option to combine both commercial and in-house IQC samples to tackle these concerns, as was done by (only) 14 % of laboratories. We do not encourage to omit the use of the commercial IQC materials proposed by the manufacturer. Long-term storage at −70 °C or the addition of preservatives [4, 30] should guarantee the quality of the in-house controls. Of note, only 14 % of the laboratories stored the IQC samples at −80 °C.

External quality assessment (EQA) programs provide insights into the performance of specific tests, allow to identify inter-laboratory discrepancies, and to monitor the success of harmonization efforts [33]. Thus, participation in EQA is considered one of the cornerstones of a good quality assurance policy [39]. Moreover, the frequency of participation, and character of EQA participation (e.g., voluntary vs. obligatory) is often guided by national quality regulations and ISO accreditation policies [39]. For anti-ENA testing, the EQA programs are often combined with EQA programs for HEp-2 IFA. Of all the participating laboratories in our survey, the vast majority (87 %) performed EQA, with about 48 % of the laboratories participating in more than 3 EQA distributions each year.

According to international guidelines and EN/ISO15189:2022, it is good practice to evaluate lot-to-lot variability of different batches before the implementation of a new lot in case lot variability is a known issue [39]. This can be done by testing patient-derived QC samples with both the current and the new candidate lot (reviewed in [30]). Another strategy to reduce lot-to-lot variability is to limit the number of lot changes by requesting lot reservation at the manufacturer. In our survey, lot-to-lot variability was only evaluated by 36 % of the participants. Neither was the limitation of lot changes widely implemented (done by 43 % of participants).

Nowadays, medical laboratories commonly use CE IVD/FDA-labelled tests. If medical laboratories perform the CE IVD/FDA-labelled test according to the manufacturer's instructions, and limit its application to the intended use claimed by the manufacturer, method verification (to assure the procedure is appropriate in one's own laboratory for the given purpose) suffices to document the quality of the assay (reviewed in [30]). Our survey revealed that method verification was done by the vast majority of laboratories (about 81 %) before implementing the test.

The EASI recommendations for autoantibody test verification suggest to minimally verify trueness (by method comparison), repeatability and intermediate imprecision as well as verification of the reference limits [36]. However, it must be noticed that a method comparison with the test currently in use in the laboratory does not tell you something about the trueness of the test; it only generates data on agreement between methods. According to CLSI, diagnostic accuracy of a method should be evaluated against the 'best available criterion' for establishing the condition [40]. Conventional techniques for anti-ENA detection may fit this definition, but in practice such studies are difficult to organize by most labs. Alternatively, the study can be set-up with clinically characterized samples, or labs can use samples from EQA with a target response. Of note, in EQA schemes the target response might be defined by the response provided by the majority of the participating laboratories, rather than by the response obtained by reference techniques. Our data show high variability in the type of samples used to verify an assay (EQA vs. patient samples and lab-characterized vs. clinically-characterized patient samples). Eighty-nine percent of laboratories used patient's samples and 41 % combined them with EQA samples, while 11 % used only EQA material. If patient materials were used for verification purposes 58 % of the laboratories took clinical information into account.

According to the CLSI user protocol, a minimum of 50 positive and 50 negative samples (tested with both the new and the comparative method) is needed to establish analytical sensitivity and specificity, respectively [40]. Other numbers for method comparison have been suggested by EASI, stating that an optimal method comparison consists of 50 positive and 100 negative sera, but with a minimum of 30

comparisons including 10 positive and 10 negative samples [36]. Sixty-nine percent of laboratories that used laboratory-characterized (non-clinically defined) samples for verification used more than 20 samples, 33 % used 40 or more samples. As for within- and between-run precision experiments, the majority of laboratories (respectively, 76 and 70 %) performed less than 10 replicates thus not being in line with the EASI guideline [36].

Ideally all anti-ENA reactivities that are included in the commercial test and are reported by the laboratory, are evaluated during the method comparison. However, this is becoming increasingly challenging, both practically and economically, due to the growing number of (often rare) reactivities included in newer anti-ENA tests. In line with the previously published ANA recommendations [30], method verification should at least include the most clinically relevant reactivities specifically those included in classification or diagnostic criteria. These include anti-dsDNA, -SSA/Ro60, -Sm, -RNP, -CENPB, -Scl-70, -RNA polymerase III, -Jo-1 (provided that the corresponding antigens are present in the test and reported by the laboratory). Moreover, also in line with the ANA recommendation, we propose that the detection of each of these clinically important reactivities mentioned above should be confirmed in preferentially at least five samples per reactivity [30]. As a consequence of this approach, laboratories may not verify all antibodies theoretically detectable by the anti-ENA assay and may therefore consider listing reactivities in their report not covered during method validation, or alternatively, choose not to report them at all. In the long term, the organization of anonymized sample exchanges between centers – particularly for rare reactivities – could help to address some of the practical challenges of method verification. It is noteworthy that the survey data generated in 2018–2019 might not be fully representative of the actual situation. However, it can be hypothesized that the challenges related to performing these measures (e.g. sample type, availability of rare samples, economic burden…) might even be greater than in the past due to the expanding repertoire of antigens included in the anti-ENA. To conclude, anti-ENA testing is widely implemented and highly accessible, but there is high variation in test methods and turnaround times. We documented that the majority of laboratories embrace basic measures to control and maintain high-quality anti-ENA analysis. Nevertheless, our results also illustrate that there is high variability amongst the quality assurance and method verification strategies. This may be a consequence of the challenging nature of anti-ENA testing [e.g. wide commercial test arsenal available, multiple reactivities analyzed in one test, rare antibodies, diverse disease spectrum, background autoantibodies in (provisionally) healthy individuals, lack of standardization] and the lack of detailed recommendations. Efforts should be made to improve harmonization of anti-ENA testing.

**Acknowledgments:** The authors would like to thank all the participating labs for their contributions.
**Research ethics:** Not appicable.
**Informed consent:** Not appicable.
**Author contributions:** All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
**Use of Large Language Models, AI and Machine Learning Tools:** Not applicable.
**Conflict of interest:** The authors state no conflict of interest.
**Research funding:** None declared.
**Data availability:** The data that support the findings of this study are available from the corresponding authors, upon reasonable request.

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