

Review

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The spectrum of nuclear patterns with stained metaphase chromosome plate: morphology nuances, immunological associations, and clinical relevance

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Abstract: The indirect immunofluorescence assay (IFA) on HEp-2 cells is the prevailing method used to screen for autoantibodies in the investigation of systemic autoimmune diseases (SAID). When positive, the titer provides a semi-quantitative assessment of the autoantibody serum concentration whereas the immunofluorescence pattern indicates the possible autoantibody specificities. The Brazilian Consensus on ANA Patterns (BCA) and the International Consensus on ANA Patterns (ICAP) provide recommendations for the harmonization on the pattern nomenclature and test reporting. Nuclear patterns are among the most frequent in the clinical laboratory and some of them are highly relevant in the diagnosis of SAID. Nuclear patterns with stained metaphase plate (MP) indicate autoantibodies against chromatin components or against chromatin-bound antigens. These include the nuclear homogeneous (AC-1), nuclear dense fine speckled (AC-2), Topo 1-like (AC-29), and nuclear fine speckled with stained MP (AC-30) patterns. The Brazilian consensus has also classified the *quasi-homogeneous* nuclear

pattern (QH). The correct identification of these patterns is important because each one is associated with different autoantibody specificities and clinical scenarios. However, the recognition of the nuances in texture of the staining pattern and other specific features that characterize each of them may be challenging for the analyst at the microscope. This review focuses on the morphological characteristics, immunological identities, and clinical relevance of nuclear patterns with stained MP. The aim is to assist laboratory analysts and clinicians in identifying and interpreting these patterns, thus optimizing the use of the HEp-2 IFA test in the investigation of patients under suspicion of SAID.

Keywords: HEp-2 cells; AC-1; AC-2; QH pattern; AC-29; AC-30

Introduction

The indirect immunofluorescence assay on HEp-2 cells (HEp-2 IFA) is a laboratory technique in which specific serum autoantibodies are immobilized on a human cellular substrate (HEp-2 cells) and subsequently detected by fluorochrome-labeled secondary antibodies. Although originally characterized as a human laryngeal carcinoma, HEp-2 cells are now known to be contaminated with HeLa cells [1], but regardless of its tissue origin this cell line is a suitable substrate [2]. The HEp-2 IFA is the most frequently used methodology for the detection of autoantibodies in systemic autoimmune rheumatic diseases (SARD) and other systemic autoimmune diseases (SAID), allowing for the screening of numerous clinically relevant autoantibodies [3]. The test provides three crucial pieces of information: the presence or absence of autoantibodies in the sample, the antibody titer, and the immunofluorescence pattern. The titer and immunofluorescence pattern help differentiate positive results obtained in non-autoimmune individuals from those of

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patients with a probable autoimmune disease. In addition, the HEp-2 IFA pattern provides preliminary indication of the possible targeted autoantigens thereby guiding the investigation of the most likely specific autoantibodies, thus contributing to medical diagnosis and treatment [4–7].

Given the importance of the HEp-2 IFA test in autoimmunity diagnostics, a group of Brazilian experts established the Brazilian Consensus on Antinuclear Antibodies in HEp-2 cells (BCA) in 2000. Since then, successive workshops were conducted aiming at harmonizing the HEp-2 IFA technique and establishing recommendations for standardization of the test and pattern classification [2, 8–11]. The BCA presented the first proposal of classification of the most relevant patterns into a hierarchical tree, harmonizing the nomenclature of HEp-2 cell patterns, which was previously heterogeneous across the country. The hierarchical classification tree comprised four major groups addressing the most prominent cell domains, i.e., nucleus, nucleolus, cytoplasm, and mitotic apparatus [2].

In 2014, the Autoantibody Standardization Committee of the International Union of Immunological Societies (IUIS) launched the International Consensus on ANA Patterns (ICAP) initiative [12] that created a HEp-2 IFA pattern classification system built on the Brazilian Consensus experience. Since then, ICAP's goal has been to promote worldwide harmonization of HEp-2 IFA procedure and interpretation. The guidelines for the interpretation and classification of HEp-2 IFA patterns ultimately aim to help laboratory personnel in the analysis of the test and clinicians in the diagnosis and optimization of medical management of patients under suspicion of SAID [12, 13].

ICAP recognizes 32 different HEp-2 IFA patterns (AC-0 to AC-31) in addition to unusual and non-catalogued patterns, designated as AC-XX [14]. These patterns are categorized into four main groups: negative ($n=1$), nuclear ($n=17$), cytoplasmic ($n=9$), and mitotic ($n=5$) patterns [14]. The consensus nomenclature is organized into a classification tree displayed on the interactive ICAP website (www.ANAPatterns.org). For each pattern, an alpha-numeric AC (anti-cell) code is assigned, for example, the homogeneous nuclear pattern is designated as AC-1. The ICAP website provides representative images for each pattern along with other important information such as the recommended designation, previous nomenclature, a description of the main morphological characteristics, potential antigenic specificities, and clinical relevance [13]. The most relevant patterns that are relatively easy to identify are classified as competent-level patterns, i.e., these are expected to be recognized by any HEp-2 IFA analyst. This is in contrast to the group of expert-level patterns, comprised of less common patterns or those that require higher expertise for recognition [12].

Over the years, experts from several countries have made efforts to absorb and harmonize the ICAP recommendations according to their local specific contexts, for instance, in Argentina [15], Uruguay [16], Italy [17], Ukraine [18], and Brazil [10, 11]. In particular, the Brazilian Consensus (BCA) has implemented several modifications towards harmonization with ICAP, including the adoption of the alpha-numeric AC code. However, certain patterns classified in the BCA algorithm are not present in the ICAP classification tree, and, therefore, these received a provisional “BAC” (Brazilian AC) codification [10, 11].

ICAP classifies the HEp-2 IFA patterns into segregated groups according to cell compartments, i.e., nuclear, cytoplasmic, and mitotic apparatus. The nuclear pattern group has the highest level of clinical relevance and has undergone the most updates in recent years. In 2014, the IV Brazilian Consensus incorporated the *quasi*-homogeneous (QH) nuclear pattern into the nuclear pattern group [9]. The QH pattern has a very fine speckled texture tending to homogeneous and is classified as AC-1 in ICAP. However, it has been shown that the frequency of anti-dsDNA and anti-nucleosome antibodies is far lower in samples with the QH pattern in comparison with samples with the bona fide homogeneous pattern [19]. As the QH pattern that has not yet been classified by ICAP, it has received the provisional code BAC-3 [9]. In 2018, ICAP incorporated a compound pattern, previously reported as strongly associated with antibodies against DNA topoisomerase I (Topo I) [20], as the AC-29 pattern [21]. In 2021, there was an update to the ICAP tree, grouping the AC-1, AC-2, and AC-29 patterns to highlight their morphological similarities (www.anapatterns.org). In 2024, additional nuclear patterns were included in the ICAP algorithm, including the nuclear fine speckled with mitotic plate (AC-30) and the nuclear speckled of the myriad discrete type (AC-31). These two patterns were integrated into the group of nuclear patterns with negative plates (AC-4) fine speckled and AC-5, coarse/large speckled [14].

The nuclear patterns are predominant in clinical laboratories and were found to represent more than 70 % of positive samples in one large clinical laboratory [22]. Within this group, there are two main categories, those with no staining of the metaphase chromosome plate and those with distinctive staining of the metaphase plate. These include the nuclear homogeneous (AC-1), nuclear dense fine speckled (AC-2), topo-1-like (AC-29), and nuclear fine speckled with stained metaphase plate (AC-30) patterns. The major segregating factor among these patterns is the staining texture in the interphase nucleus and in the metaphase plate. This difference is subtle and subjective, therefore they are classified at the expert level [14]. Notwithstanding their morphological complexity and potential for

misidentification, the distinction of these patterns is important as each one is associated with a specific clinical context. This review aims to examine the spectrum of nuclear patterns with stained metaphase plate, focusing on the morphology and texture of the patterns, autoantibody associations, and clinical relevance.

Nuclear homogeneous pattern (AC-1)

The homogeneous nuclear pattern (AC-1) is characterized by a diffuse, regular, and homogeneous fluorescence throughout the nucleoplasm of the HEp-2 cell. The nucleolar regions are usually stained, but they may also remain unstained depending on the cellular substrate [12]. In interphase cells, higher titer sera may show more intense staining at the nuclear periphery. In mitotic cells, the metaphase chromatin mass is intensely stained in a homogeneous manner, with a distinctly hyaline appearance. The cytoplasm typically appears negative (unstained) in both interphase and mitotic cells [12]. The morphological features of the AC-1 pattern are illustrated in the upper panel of Figure 1A and detailed in Table 1. This pattern is frequently associated with autoantibodies targeting chromatin components such as double-stranded DNA (dsDNA), histones, and nucleosomes [12]. These antigen associations harmonize with the fact that the chromatin consists of the native complex of histones (~40 %), dsDNA (~40 %), and non-histone proteins (~20 %) [34].

Antibodies that bind to double-stranded DNA (dsDNA) or anti-native DNA (nDNA) are the most clinically relevant antibodies against deoxyribonucleic acid antibodies [23]. These include antibodies against a broad spectrum of fine molecular DNA specificities, including native dsDNA and chemically modified dsDNA containing thymidine dimers. These are frequently high affinity autoantibodies that form stable immune complexes that are relevant in the context of systemic lupus erythematosus (SLE) [23, 35–37]. Anti-dsDNA antibodies can be of different isotypes but IgG-class autoantibodies are predominantly observed in patients with SLE, contributing to the pathogenesis of glomerulonephritis, particularly the high-avidity IgG antibodies [38]. From the pathophysiological point of view, these antibodies are involved in the formation of immune complexes with dsDNA, leading to subsequent deposition in glomeruli. Alternatively, they may cross-react and bind to other autoantigens embedded in the glomerular basement membrane. The immune complexes at the glomerular basement membrane induce an inflammatory process and a variety of

lesions that underlie the distinct histopathological classifications and varying degrees of nephritis [39].

A positive HEp-2 IFA test with the AC-1 pattern suggests the presence of anti-dsDNA antibodies, which must be confirmed in antigen-specific immunoassays, such as the Farr immunoprecipitation radioimmunoassay, the indirect immunofluorescence (IIF) with the protozoan *Crithidia luciliae* (CLIFT), enzyme-linked immunosorbent assays (ELISA), or chemiluminescence immunoassays (CLIA) [40]. Generally, good clinical specificity for SLE is achieved using radioimmunoassay and CLIFT methodologies [41–44], whereas ELISA and CLIA techniques may detect low avidity and cross-reacting antibodies, thereby providing lower clinical specificity. However, CLIFT shows lower sensitivity compared to immunometric methods, such as fluorescence enzyme immunoassay, CLIA, and multiplex technologies, which offer sensitivities ranging from 67 % to 92 % and specificities between 84 % and 98 % in the analyzed cohorts. The high sensitivity and semiquantitative readout of ELISA and CLIA may render these methods appropriate for monitoring the serum levels of anti-dsDNA antibodies [45]. Considering the limited commutability across platforms, the heterogeneous diagnostic performance of CLIFT and immunometric methods in the evaluation of anti-dsDNA antibodies reinforces the need for harmonization among different immunoassays [46].

Regarding the clinical relevance, anti-dsDNA represents a useful parameter for monitoring SLE disease activity, with elevated levels during disease flares, especially in lupus nephritis, and decreasing levels in response to treatment and quiescence [23]. Therefore, in the presence of an AC-1 pattern in patients suspected of SLE, a confirmatory test for anti-dsDNA antibodies is recommended, as it serves as a powerful parameter for classifying and diagnosing SLE, particularly in patients suspected of having renal involvement [23].

The AC-1 pattern may also be associated with the presence of anti-nucleosome and anti-histone antibodies, the latter representing structural protein subunits that provide a core around which chromatin is wrapped, allowing for its spatial organization within the nucleus. The histone core consists of two H2A-H2B dimers and one H3-H4 tetramer, forming a histone octamer with an external H1 histone, the latter being a conserved subunit that assists in stabilizing the chromatin structure [47]. The histone octamer, coupled with approximately 150 DNA base pairs, is known as the nucleosome and forms the fundamental unit of chromatin [48]. Histones not only provide support for chromatin but also play a significant and complex role in regulating gene expression, being encoded by multiple genes spanning several chromosomes [49].

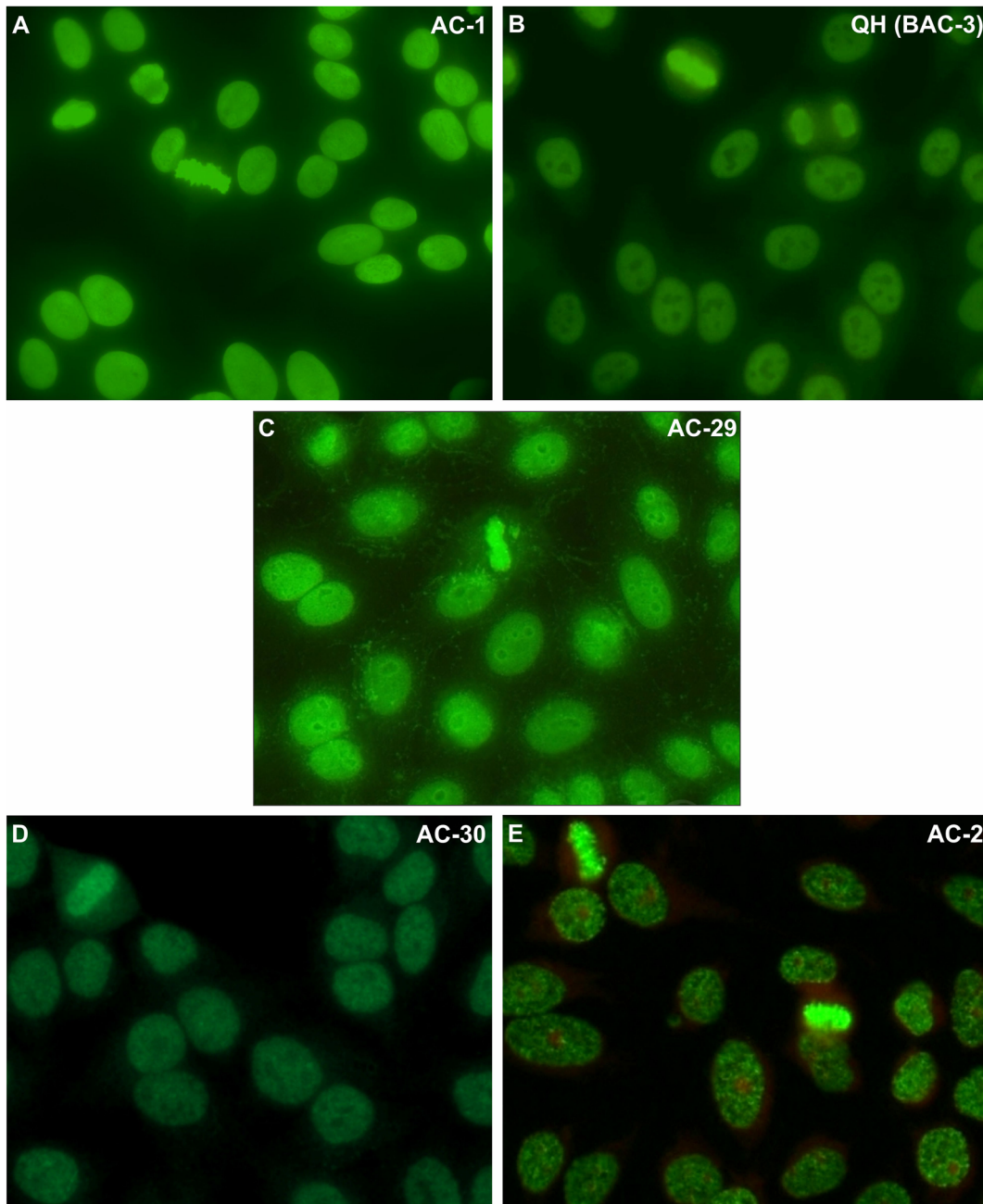


Figure 1: Morphological details of the nuclear patterns that show homogeneous or speckled staining of the metaphase plate: nuclear homogeneous – AC1 (A), nuclear quasi-homogeneous - BAC-3 (B), DNA topoisomerase I/topo I-like – AC-29 (C), nuclear fine speckled with mitotic plate - AC-30 (D), and nuclear dense fine speckled – AC-2 (E). Images from the International Consensus on ANA Patterns, photographs (C) and (E): Werner Klotz & Manfred Herold (Sample dilution: 1:80; microscope magnification: 400×).

Anti-nucleosome antibodies consist of a large family of autoantibodies directed against epitopes of histones exposed in chromatin and against conformational epitopes generated by the interaction between dsDNA and core histones [24]. Free nucleosomes are generated during cellular apoptosis by endonuclease-mediated chromatin cleavage [50]. These components may become immunogenic during apoptosis when

cellular debris containing chromatin is not properly cleared [51]. There is evidence that, as a result of impaired phagocytic clearance of apoptotic nuclear debris, nucleosomes bound to the cell surface and circulating polynucleosomes stimulate and promote the production of autoantibodies [52].

Various technologies have been used to measure anti-nucleosome antibodies, starting with the now obsolete LE

Table 1: Nuclear patterns with stained metaphase chromosome plate: morphological characteristics, antigenic associations, clinical associations, and recommended follow-up testing.

| Code | Morphological characteristics | | Antigenic associations | Clinical associations and follow-up testing | References |
|--|--|---|---|--|---|
| | Interphase nucleus | Metaphase plate | | | |
| Nuclear homogeneous | AC-1 The nucleolar regions are usually stained, but they may also remain unstained depending on the cellular substrate | The metaphase chromatin mass is intensely stained in a homogeneous manner, with a distinctly hyaline appearance | Anti-dsDNA, anti-nucleosome, anti-histone | In patients suspected of SLE with an AC-1 pattern, testing for anti-dsDNA and anti-histone antibodies is recommended. Anti-histone antibodies, though classically associated with SLE and drug-induced lupus, are also found in Sjögren's disease, inflammatory myositis, autoimmune hepatitis, and rheumatoid arthritis. Anti-nucleosome antibodies, the first described in SLE, are valuable diagnostic biomarkers, especially for lupus nephritis | Rekvig OP [23]; Bizzaro et al. [24]; Krippner et al. [25]; Zirwas et al. [26] |
| Nuclear quasi-homogeneous | QH Extremely fine speckled diffuse nuclear fluorescence, resembling but not quite homogeneous in texture | Metaphase chromatin mass is intensely stained in a homogeneous texture | Anti-dsDNA, anti-nucleosome, anti-histone, anti-Sm, anti-U1-RNP, anti-SS-A/Ro, anti-SS-B/La (frequently more than one antinuclear antibody) | Although with lower correlation, it can be observed in patients with systemic autoimmune rheumatic diseases. In cases of clinical suspicion, the relevant autoantibodies should be investigated. | França et al. [19] |
| Topo-1-like | AC-29 Fine, compact, and prominent speckled diffuse nuclear fluorescence in interphase cells with delicate and weak cytoplasmic fluorescence with a web-like appearance radiating from the perinuclear area to the vicinity of the plasma membrane and inconsistent fluorescence of nucleoli with varying aspects. | Consistent and strong fine speckled staining of condensed chromatin in mitotic cells, with the chromatin metaphase plate appearing as homogeneous in high titer samples. Strong staining of the nucleolar organizing regions (NOR) at the metaphase plate | Anti-Scl-70 | In suspected SSC, follow-up testing for anti-topoisomerase I (Scl-70) antibodies is recommended, as they are present in 20–30 % of SSC patients and associated with diffuse cutaneous involvement, interstitial lung fibrosis, and a more aggressive disease course, including higher risks of renal, gastrointestinal, and cardiac complications. | Shero et al. [27]; Ho and Reveille [28]; Gabrielli et al. [29] |
| Nuclear fine speckled with stained metaphase plate | AC-30 Nuclear speckled fluorescence with uniform distribution of speckles and the nuclear border is regular, | The metaphase plate displays a uniformly distributed fine speckled pattern | Most samples have none of the clinically relevant autoantibodies, but some may have a combination of anti-dsDNA, anti-nucleosome, anti-histone, anti-Sm, anti-U1-RNP, anti-SS-A/Ro, anti-SS-B/La, and anti-Scl-70 | The AC-30 pattern is not associated with anti-DFS70 antibodies and up to 25 % of sera may recognize one or more clinically relevant antinuclear antibodies. So, in cases of clinical suspicion, the disease-related autoantibodies should be investigated. | Landoni et al. [30]; Andrade et al. [14] |
| Nuclear dense fine speckled | AC-2 Non-uniform speckled fluorescence distributed throughout the interphase nucleus, with characteristic heterogeneity in the size, brightness, and distribution of the speckles. Across the interphase nucleus, some areas exhibit denser or sparser staining. The border of the nucleus is irregular (moth-eaten pattern). | The metaphase plate displays a strong speckled pattern, with some coarse speckles standing out | Anti-DFS70/LEDGF, most frequently as the unique antinuclear antibody in the sample. | If anti-DFS70 autoantibody is confirmed in the absence of relevant autoantibodies against extractable nuclear antigens (including anti-dsDNA), it is rarely associated with systemic autoimmune rheumatic diseases (SARD) [31–33]. | Muro et al. [33]; Fitch-Rogalsky et al. [31]; Mariz et al. [7] |

cell test and eventually evolving to immunoprecipitation methodologies, ELISA, and CLIA [47]. Clinically, anti-nucleosome antibodies have proven to be a good diagnostic biomarker for SLE, representing the first autoantibody described in association with the disease, particularly in the development of lupus nephritis [24].

Anti-histone antibodies can recognize total histones or restricted sets of histone subunits, and can bind free histones or histones bound to DNA [25, 53]. In the HEp-2 IFA test, anti-histone antibodies may yield the AC-1 (homogeneous nuclear) pattern, as previously described [54] and can be determined using ELISA or CLIA [55]. The Western blotting technique has been used in the original characterization of autoantibodies against histone subunits. In this case, polyacrylamide gel electrophoresis is used to separate histones into their constituents based on molecular weight [56].

In terms of clinical relevance, anti-histone antibodies have been classically associated with SLE and drug-induced lupus but they are also found in other conditions, including Sjögren's disease, inflammatory myositis, and rheumatoid arthritis [25, 26].

The AC-1 pattern is one of the common patterns in autoimmune hepatitis but other HEp-2 IFA nuclear patterns may also occur, although the corresponding autoantigens have not yet been fully defined [13, 57].

Nuclear *quasi*-homogeneous pattern

An interesting and subtle pattern within the group of nuclear patterns with a positive metaphase plate is the *quasi*-homogeneous speckled nuclear pattern (QH). The QH pattern is characterized by extremely fine speckled diffuse nuclear fluorescence, resembling but not quite homogeneous in texture, and it also includes a similar staining of the metaphase plate. The morphological similarity to the AC-1 pattern may lead to confusion between these two patterns and one needs to exercise the ability to differentiate those two patterns [9].

The immunological characterization of the QH pattern was performed by analyzing 60 samples with this morphological profile on four different HEp-2 cell brands, compared with an equal number of samples with the nuclear homogeneous (AC-1) and nuclear dense fine speckled (AC-2) patterns. Reactivity to double-stranded DNA (dsDNA), nucleosome, and histones was tested in all samples. Autoantibodies against nucleosome, histones, and dsDNA were present in 96.7 %, 60 %, and 36.7 % in AC-1 samples, respectively, compared to 35 %, 16.7 %, and 7 % in the QH samples.

None of the AC-2 samples presented any of these autoantibodies [58]. Thus, the QH pattern exhibited an intermediate autoantibody profile between the AC-1 and AC-2 patterns and was seldom observed in patients with systemic autoimmune rheumatic diseases [58].

Considering that the QH pattern does not rule out the possibility of an association with SARD, although with a lower post-test probability than the AC-1 pattern [58]. In a retrospective analysis of 11,478 HEp-2 IFA positive samples in a large Brazilian clinical laboratory, the QH pattern presented a 10 % total prevalence among positive samples [22]. The morphological details of the QH pattern can be observed in Figure 1B and detailed in Table 1.

DNA topoisomerase I/topo-I-like pattern (AC-29)

The AC-29 pattern is highly suggestive of reactivity against the enzyme DNA topoisomerase-I (topo-I), a 765-amino acid (91 kDa) protein localized in the cell nucleus due to the presence of a functional nuclear localization signal in the enzyme N-terminal domain. Both topo-I and topo-II are enzymes involved in relaxing the DNA helix as it unwinds for DNA replication and RNA transcription [59, 60]. Several epitopes are recognized by anti-topo I autoantibodies, with the amino acids 489–573 stretch being identified as an immunodominant epitope [61]. Anti-topo I antibodies of the IgG class are most frequently detected compared to IgA and IgM isotypes [62].

In 1979, Eng M. Tan and colleagues described the presence of autoantibodies recognizing a 70 kDa nuclear antigen, termed Scl-70, in patients with SSc [63]. Later studies by van Venrooij et al. and Guldner et al. (1985 and 1986, respectively) demonstrated that this autoantigen corresponded to the 100 kDa Topo I enzyme [64, 65]. Anti-topo-I autoantibodies are highly specific biomarkers for SSc, being present in 20–30 % of SSc patients and are preferentially associated with diffuse cutaneous involvement and interstitial lung fibrosis, suggesting a more aggressive disease course [27, 29]. Furthermore, anti-topo-I is associated with a higher likelihood of renal vascular damage, renal crisis, severe gastrointestinal involvement, and cardiac fibrosis [28]. In 2009, Dellavance et al. redefined the fluorescence pattern characteristics associated with this autoantibody, determining that the Topo I-like pattern is a composite pattern characterized by reactivity in five distinct cellular regions of the HEp-2 cell [20]. In 2018, ICAP assigned the AC-29 classification code to the HEp-2 IFA pattern specifically associated with anti-Topo I autoantibodies [21].

The AC-29 pattern has been shown to be strongly associated with autoantibodies to DNA topoisomerase I [20, 21] and is composed of up to five morphological elements: (1) fine, compact, and prominent speckled diffuse nuclear fluorescence in interphase cells; (2) consistent and strong staining of condensed chromatin in mitotic cells, with the chromatin metaphase plate appearing homogeneous in high titer samples; (3) strong staining of the nucleolar organizing regions (NOR) at the metaphase plate, the visualization of which may need adjustment in the fine focus to allow distinction from the bright metaphase plate staining as NOR are not always in the same focal plane as the chromatin; (4) delicate and weak cytoplasmic fluorescence with a web-like appearance radiating from the perinuclear area to the vicinity of the plasma membrane. Frequently, the cytoplasmic staining becomes more prominent during sample titration to higher dilutions; and finally, (5) inconsistent fluorescence of nucleoli with varying aspects [20, 21]. Due to

the number of elements and the variation in analysis depending on the brand of the HEp-2 slide, this pattern is considered challenging to identify, and detailed analysis with attention to different focal planes is recommended for proper visualization. For this reason, the pattern is classified at the expert level by ICAP and the Brazilian Consensus on ANA [10, 14]. The morphological details of the AC-29 Topo-1-like pattern can be observed in Figure 1C and revised in Table 1. The five key characteristics of the pattern are highlighted in Figure 2.

Upon identification of the AC-29 pattern by HEp-2 IFA, the presence of anti-topoisomerase I (anti-topo-I) antibodies should be confirmed using an antigen-specific immunoassay, such as ELISA, double immunodiffusion, or immunoblotting. In high-throughput laboratories, ELISA and CLIA are the preferred methods. Notably, CLIA has demonstrated excellent agreement with the gold standard immunoprecipitation technique for detecting anti-Scl-70 (Topo I) antibodies [66, 67].

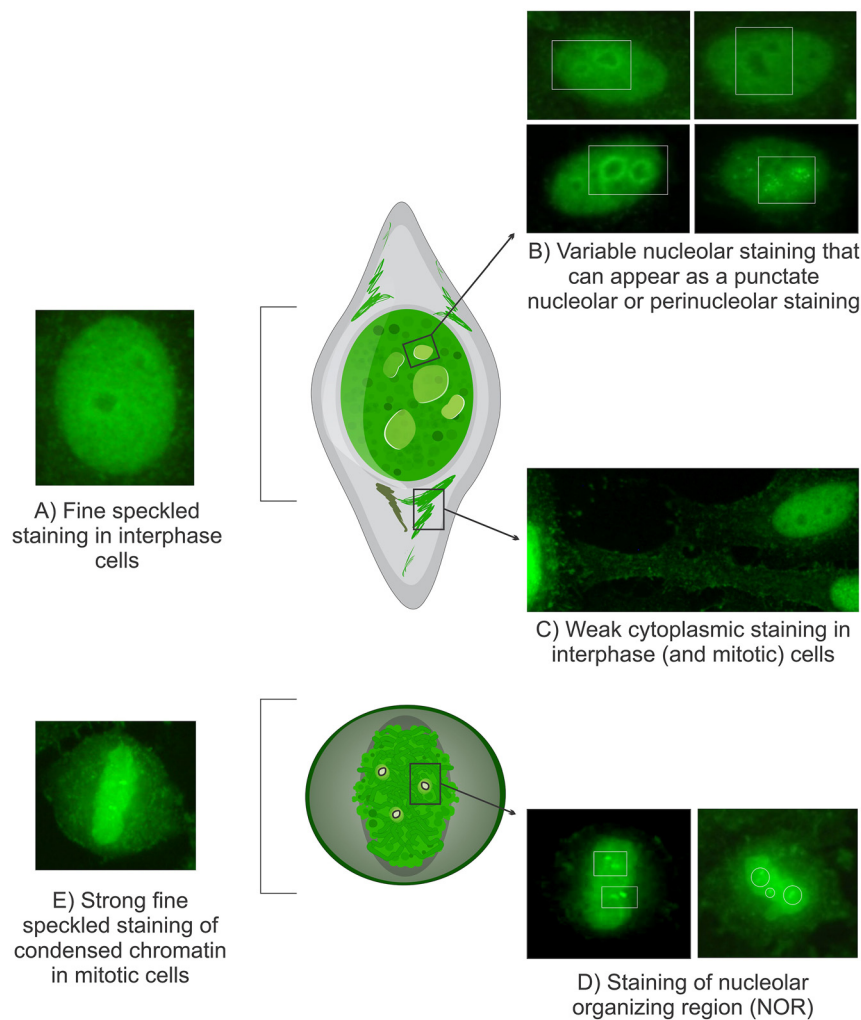


Figure 2: Morphological characteristics of the Topo I-like pattern: The Topo I-like pattern is defined by staining across five distinct sub-cellular regions. (A) Prominent extremely fine and compact speckled staining in interphase nuclei. (B) Variable nucleolar staining, which may present as punctate nucleolar, homogeneous or perinucleolar staining in interphase cells, although this feature is not consistently observed. (C) Faint cytoplasmic staining in interphase and mitotic cells, forming a delicate network radiating from the perinuclear area to the plasma membrane. (D) Intense staining of NOR over condensed chromosomes in mitotic cells, though this can be obscured by bright chromosomal staining. (E) Strong extremely fine compact speckled staining of condensed chromatin in mitotic cells, which may appear homogeneous with high titer samples. Images sourced from the International Consensus on ANA Patterns (www.ANAPatterns.org), photographs by Werner Klotz & Manfred Herold.

Dense fine speckled pattern (AC-2)

The AC-2 pattern is produced by autoantibodies that recognize a 75 kDa chromatin-associated protein designated DFS70, also known as lens epithelium derived growth factor p75 (LEDGF/p75), transcription co-activator p75 (TCP75), and PC4/SRSF1 interacting protein 1 [68–70]. The DFS pattern was first described in 1994 [71] and the target autoantigen was fully characterized as the Dense Fine Speckled 70 (DFS70) protein in the late 1990s [72]. This designation was based on their distinctive nuclear fluorescence pattern and migration near the 70 kDa region in immunoblotting assays [72]. The entire DFS70 sequence corresponds to the p75 transcriptional coactivator positive cofactor 4 (PC4), which is integral to RNA polymerase II complexes and plays a key role in gene transcription [72, 73]. Contemporarily an independent group reported the same protein as the Lens Epithelium-Derived Growth Factor p75 (LEDGF/p75) with properties of protecting cells from oxidative damage [74]. Acting as a hub for protein-protein interactions, DFS70/LEDGFp75 facilitates the recruitment of transcription factors, chromatin remodelers, and regulatory proteins to RNA polymerase II complexes at active chromatin sites [75].

The dense fine speckled (DFS) pattern is characterized by a non-uniform speckled fluorescence distributed throughout the interphase nucleus, with characteristic heterogeneity in the size, brightness, and distribution of the speckles. Across the interphase nucleus, some areas exhibit denser or sparser staining. The border of the nucleus is irregular (moth-eaten pattern). The metaphase plate displays a strong speckled pattern, with some coarse speckles standing out [12]. It should be emphasized that the AC-2 pattern is clearly recognizable only when anti-DFS70 is the only antinuclear antibody in the sample, as the presence of additional antinuclear antibodies tend to mask the distinctive characteristics of this pattern. Details of the AC-2 pattern can be observed in Figure 1E, in the lower panel, detailed in Table 1.

Anti-DFS70/LEDGF antibodies are primarily of the IgG class, although IgE-class antibodies have been detected in some atopic diseases. Anti-DFS70 antibodies target a highly conserved region in the C-terminal domain of DFS70 and can be detected at moderate to high titers with variable frequency in clinical routines [31, 72, 76].

Initial studies of this autoantibody system primarily relied on the HEp-2 IFA pattern elicited by anti-DFS70 autoantibodies, with just a few initial studies confirming the anti-DFS70 reactivity in antigen-specific immunoassays. Progressively, a multitude of solid-phase assays for detecting these autoantibodies has been developed in recent years, providing excellent platforms for confirming the anti-DFS70

reactivity with high specificity and sensitivity. These assays include serum adsorption against recombinant DFS70/LEDGF protein, IFA with DFS70-knock-out HEp-2 cells, CLIA, ELISA, line immunoassays, dot blot assays and fluorescence enzyme immunoassay (FEIA) [70, 77].

The initial reports suggested that the AC-2 pattern was associated with inflammatory diseases, such as interstitial cystitis, asthma, alopecia areata, atopic dermatitis, and ocular diseases [71, 72, 78, 79]. Along the years, it was shown that this pattern and the correspondent anti-DFS70 antibody were frequently found also in apparently healthy individuals [7, 32, 72, 80]. A seminal finding was the demonstration by Mariz et al. that the AC-2 pattern rarely occurs in sera from patients with SARD and, therefore, represent a negative biomarker for systemic autoimmunity [7]. Other studies confirmed that anti-DFS70 autoantibodies in the absence of relevant autoantibodies against extractable nuclear antigens (anti-DNA) are rarely associated with SARD [31, 33, 81]. Therefore, monospecific anti-DFS70 antibodies, strongly speak against the odds of SARD in a given individual [13]. Thus, the accurate identification of the AC-2 pattern produced by autoantibodies to DFS70 is crucial for its clinical use as a potential biomarker to aid in the exclusion of SARD [82].

Nuclear fine speckled with mitotic plate pattern (AC-30)

A further refinement in the classification of nuclear speckled patterns was achieved by the characterization of the nuclear fine speckled pattern with similar staining of the metaphase plate. This was classified by ICAP as the “nuclear fine speckled with mitotic plate” pattern (NFS with plate) and was assigned the AC-30 code [14]. The AC-30 pattern has some similarities with AC-2 as both are fine speckled patterns that stain the interphase nuclei and the metaphase plate. However, in contrast to AC-2, the AC-30 pattern has a uniform distribution of speckles and the nuclear border is regular [81] (Figure 1D, Table 1). The AC-2 pattern, in turn, has a heterogeneous distribution of the nuclear speckles, with areas of dense speckles intermingled with areas of sparse coarse speckles. As a result of such heterogeneity, the border of the nucleus is irregular (moth-eaten pattern). Preliminary evidence indicates that the AC-30 and the AC-2 patterns have distinct immunological associations. While the AC-2 pattern is strongly associated with anti-DFS70 antibodies and the absence of antibodies to most clinically relevant antinuclear antibodies, the AC-30 pattern is not associated with anti-DFS70 antibodies and

over 25 % of sera recognize one or more clinically relevant antinuclear antibodies [14]. This could explain the negative results in anti-DFS70-specific tests in AC-30 samples misclassified as AC-2. In a retrospective analysis from the databank of a large clinical laboratory, we explored 2,560 samples concomitantly tested for HEp-2 IFA and autoantibodies to dsDNA, nucleosome, Sm, U1-RNP, Scl-70, SS-A/Ro60, and SS-B/La. The AC-30 and the AC-2 patterns were reported in 609 (23.8 %) and 85 (3.4 %) samples, respectively. The most frequent autoantibodies observed in the AC-30 samples were directed against nucleosomes (17.8 %), SS-A/Ro60 (7.2 %), and dsDNA (6.9 %). In contrast, these autoantibodies were largely not detected in the 87 AC-2 samples: only three (3.4 %) with anti-SS-A/Ro antibodies. Importantly, among 310 samples with confirmed anti-DFS70 reactivity, the HEp-2 IFA tested showed the AC-2 pattern in 285 (92 %) samples, the AC-30 pattern in 21 (6.8 %) samples, AC-4 in seven (1.9 %) samples, and AC-1 in three (1 %) samples [14, 30]. Thus, while the confirmation of an AC-2 pattern with anti-DFS70 suggests the absence of clinically relevant autoantibodies and systemic autoimmune disease, the presence of the AC-30 pattern may indicate a moderate likelihood of association with clinically relevant autoantibodies and autoimmunity [14, 30].

In contrast, the AC-1 is strongly associated with antibodies to chromatin antigens, such as dsDNA, nucleosome and histones. In a comparative study, 29 of 30 samples with the AC-1 pattern had antibodies to dsDNA and/or nucleosome, whereas none of 30 samples with the AC-2 pattern had these autoantibodies. In comparison, the nuclear QH pattern showed an intermediate behavior, with 21 (35 %) of 60 samples presenting anti-nucleosome antibodies. Reactivity to histones was observed in 18 (60 %) of the 30 AC-1 samples, in 10 (16.7 %) of the 60 QH samples, and in only 1 (3.3 %) of the AC-2 samples [19].

Final considerations

HEp-2 IFA patterns are good indicators of the subjacent autoantibodies in the sample under analysis, however, the suspected autoantibodies should always be confirmed according to the clinical context of the patient. For example, the AC-1 pattern suggests reactivity against multiple chromatin-associated antigens, especially dsDNA and nucleosome. Anti-dsDNA and anti-nucleosome antibodies serve as useful biomarkers for monitoring SLE, with elevated levels observed during disease flares, particularly in lupus nephritis, and a decrease in levels following treatment [23]. Therefore, in suspected SLE cases presenting with

an AC-1 pattern, confirmatory tests for anti-dsDNA and anti-nucleosome antibodies are recommended.

The QH speckled nuclear pattern has weaker association with clinically relevant autoantibodies and with SARD in comparison with the AC-1 pattern [58]. In addition, the QH pattern is associated with a broader range of antinuclear antibodies than the AC-1 pattern. Therefore, if the patient presents clinical manifestations consistently suggestive of SARD, further investigation for autoantibodies to dsDNA, nucleosome, SS-A/Ro, SS-B/La, U1-RNP, and Sm is recommended, according to the most probable autoimmune diseases in consideration [58].

Along the spectrum of nuclear patterns with stained metaphase plate, the AC-30 pattern is characterized by a regular speckled texture. In contrast to the AC-2 pattern, up to 25 % of AC-30 samples may have reactivity to clinically relevant autoantibodies, including those against dsDNA, nucleosomes, Sm, U1-RNP, Scl-70, SS-A/Ro, and SS-B/La, therefore warranting further investigation if the clinical scenario is suggestive of a systemic autoimmune disease. Further down the spectrum of nuclear patterns with stained metaphase plate, when a non-uniform speckled fluorescence is observed throughout the interphase nucleus, characterized by heterogeneity in the size, brightness, and distribution of speckles, the AC-2 pattern is the most likely pattern, which, in most cases, is associated with anti-DFS70 antibodies and is not associated with systemic autoimmune diseases [7, 14, 81].

The AC-29 pattern shows a strong antigenic association with antibodies to DNA topoisomerase I, therefore this pattern is very relevant in cases suspected of SSc, particularly in the diffuse cutaneous SSc and more aggressive forms of the disease. If there is clinical suspicion of systemic sclerosis (SSc), a follow-up test for anti-topoisomerase I antibodies (formerly known as Scl-70) is recommended [13, 21]. Due to the very compact speckled texture of the nuclear staining and also because of staining of the metaphase plate, the AC-29 may be erroneously interpreted as the AC-1 pattern. Therefore, one should carefully look for the presence of the five AC-29 morphological elements in samples provisionally interpreted as AC-1 or QH nuclear pattern. In that sense, the observation of the metaphase under focus fine tuning can be useful in the detection of the NOR, which is not present in the AC-1 and QH patterns but is an integral element of the AC-29 pattern [21]. Figure 3 illustrates the spectrum of morphological characteristics of each of the nuclear patterns with stained metaphase plate, as well as the antigenic identity to be investigated in each context. Figure 4 summarizes the varying likelihoods of autoantibody associations between HEp-2 IFA nuclear patterns and stained metaphase chromosome plates, based on autoantibody specificities. It is important to emphasize that

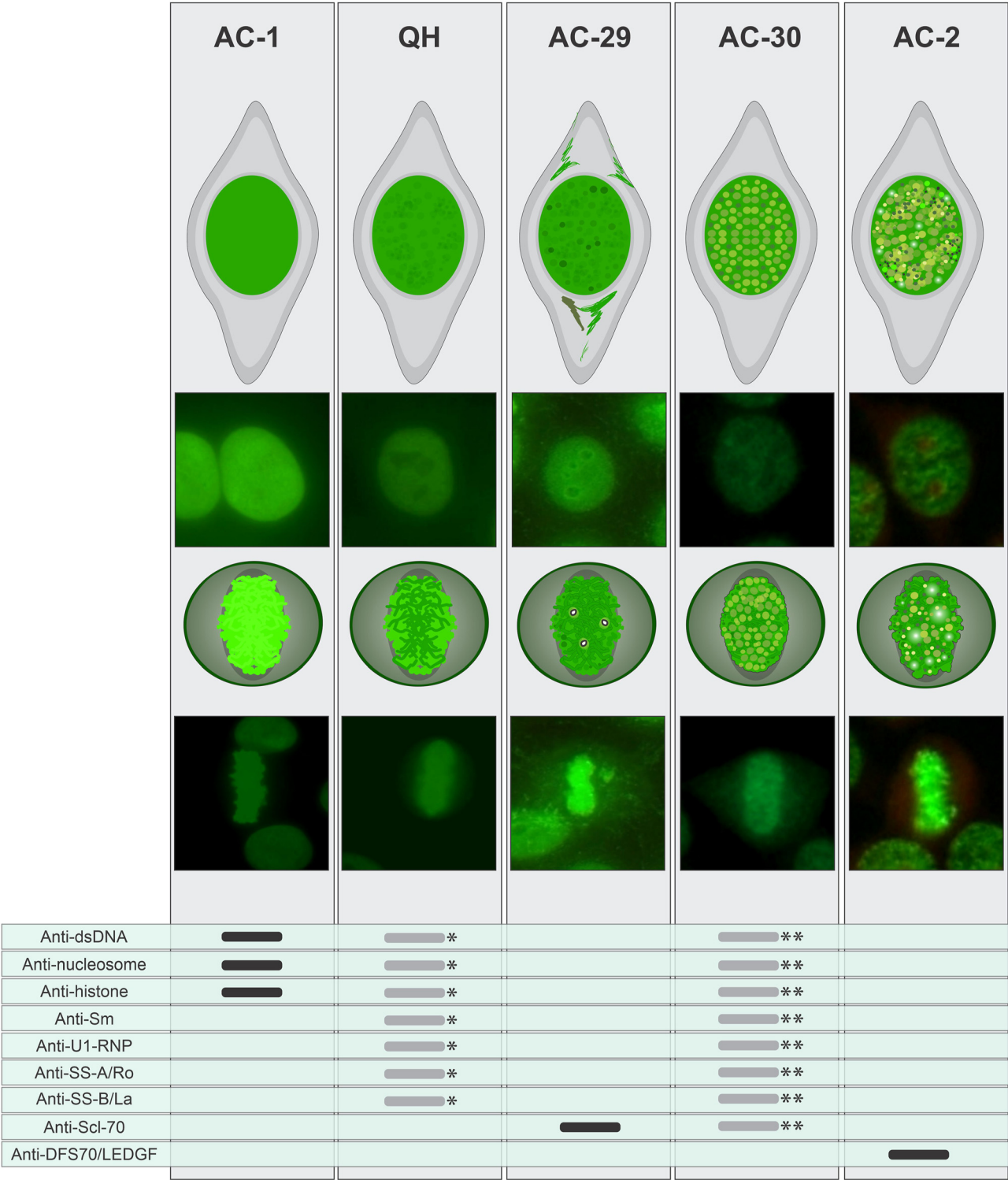


Figure 3: Comparison of morphological characteristics of the AC-1, BAC-3 (QH), AC-29, AC-30, and AC-2 patterns presenting a comparative analysis of the morphological features of the patterns, focusing on the staining in interphase nucleus, metaphase plate, and the reactivity of autoantibodies against immunogenic targets. *If the patient exhibits clinical manifestations suggestive of SARD, further testing for antibodies to dsDNA, nucleosome, SS-A/Ro, SS-B/La, U1-RNP, and Sm is recommended, guided by the most likely autoimmune diseases under consideration. **The AC-30 may exhibit reactivity to dsDNA, nucleosomes, histones, Sm, U1-RNP, Scl-70, SS-A/Ro, and SS-B/La in approximately one-fourth of cases, warranting additional investigation guided by the most likely autoimmune diseases under consideration.

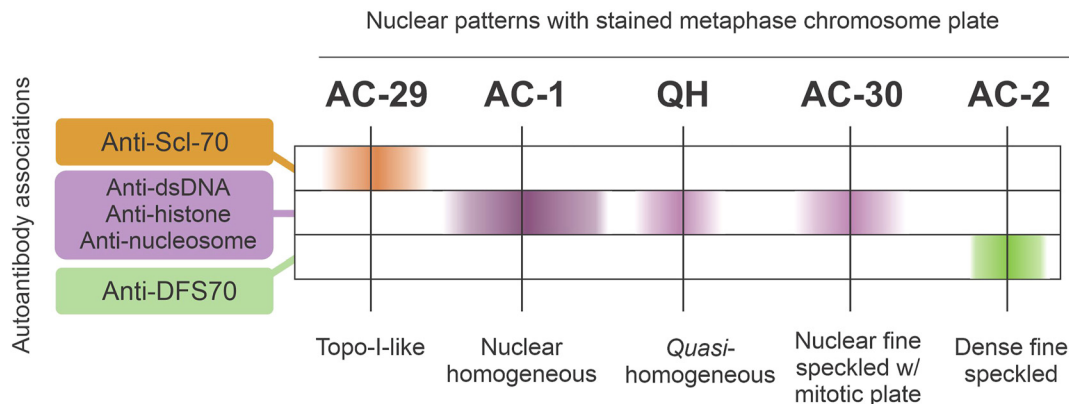


Figure 4: Schematic representation of the likelihood of autoantibody associations (anti-dsDNA, anti-nucleosome, anti-histone, anti-Scl-70, and anti-DFS70) with HEp-2 IFA nuclear patterns that exhibit staining of the metaphase chromosome plate.

not all cases in the laboratory routine will show images with the distinctive characteristics necessary for a conclusive definition of the pattern. In such cases, the analyst can use the umbrella competent-level classification suggested by ICAP, i.e., AC-1/AC-2/AC-30, whereby a broader spectrum of possibilities of autoantibody associations and clinical relevance are addressed.

Finally, it should be noted that samples with multiple autoantibodies to nuclear antigens may cause a mixed nuclear pattern in which none of the standard nuclear patterns can be clearly recognized. For example, a sample with antibodies to dsDNA, SS-A/Ro, and U1-RNP may produce a mixed nuclear pattern that cannot be classified as AC-1, AC-4 or AC-5. The observed staining texture may vary according to the serum concentration of each of these autoantibodies. In some cases, one may see a blurred speckled in the interphase nucleus and a homogeneous metaphase plate. Such mixed nuclear pattern is not unusual in samples from patients with SLE, a disease characterized by the co-occurrence of multiple antinuclear antibodies.

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