Guidelines and Recommendation

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International consensus on antinuclear antibody patterns: definition of the AC-29 pattern associated with antibodies to DNA topoisomerase I

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Abstract: The indirect immunofluorescence assay (IFA) on HEp-2 cells is the reference method for autoantibody screening. The HEp-2 IFA pattern provides useful information on the possible autoantibodies in the sample. The International Consensus on Antinuclear Antibody Patterns (ICAP) initiative seeks to define and harmonize the nomenclature of HEp-2 IFA patterns. The most relevant and usual patterns have been assigned an alphanumeric code from anti-cell (AC)-1 to AC-28 and were organized into a classification algorithm (www.ANApatterns.org). The systemic sclerosis-associated autoantibodies to DNA topoisomerase I (Topo I) produce a peculiar composite 5-element HEp-2 IFA pattern (Topo I-like pattern) comprising the staining of the nucleus, metaphase chromatin plate, nucleolar organizing region, cytoplasm and nucleolus. In a recent assessment of the European Consensus Finding Study Group on autoantibodies, a well-defined anti-Topo I sample was blindly analyzed and classified according to ICAP AC patterns by 43 participant laboratories across Europe. There were wide variations among these laboratories in reporting nuclear, nucleolar and cytoplasmic patterns, indicating the inadequacy of the existing AC patterns to report the Topo I-like pattern. Several ICAP member laboratories independently demonstrated the overall consistency of the HEp-2 IFA Topo I-like pattern using HEp-2 slides from different manufacturers. The ICAP committee reviewed 24 candidate images and selected the four most representative images to be available on the ICAP website. The proper recognition of the AC-29 pattern should trigger suspicion of the presence of anti-Topo I antibodies, which may engender appropriate analyte-specific reflex tests to confirm the autoantibody specificity.

Keywords: antinuclear antibodies; autoantibodies; HEp-2 cell; indirect immunofluorescence.

Autoantibodies are key elements in the diagnosis of systemic autoimmune diseases [1]. The indirect immunofluorescence assay (IFA) on HEp-2 cells (HEp-2 IFA) is a well-established method for the screening of autoantibodies against a wide array of cellular autoantigens [2]. Traditionally known as the antinuclear antibody (ANA) test, HEp-2 IFA has been increasingly recognized as an anti-cell (AC) antibody screening test because in addition to detecting antibodies directed against nuclear targets, it also detects autoantibodies reactive with

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components in the cytoplasm and mitotic apparatus [3]. The semi-quantitative information on the serum level of autoantibodies (represented by the titer in regular microscopy or fluorescence intensity assessed by computer-assisted image analysis) provided by the HEp-2 IFA is augmented by very useful information on the possible molecular targets and the cognate autoantibody specificities, given that the HEp-2 IFA pattern reflects the putative topographic distribution of the target autoantigens across the various cellular domains. Because several autoantigens display peculiar cellular distribution patterns, supervised inferential analysis of HEp-2 IFA patterns allows approximation on the possible autoantibody specificities present in a given serum sample. This property has been recognized in the characterization of a series of AC autoantibodies, including those reacting with the centromere [4], proliferating cell nuclear antigen [5, 6], U3-ribonucleoprotein/fibrillarin [7, 8], CENP-F [9], Cajal bodies [10], NuMA-2/HsEg5 [11, 12], rods and rings [13] and others. Information provided by the HEp-2 IFA pattern is also regularly used in the interpretation of HEp-2 IFA results in the clinical laboratory.

In recognition of the important role of HEp-2 IFA pattern in the interpretation of the test, an international group of specialists has recently promoted an initiative dedicated to define and harmonize the nomenclature of HEp-2 IFA patterns. The International Consensus on ANA Patterns (ICAP) was launched as a workshop in conjunction with the 12th International Workshop on Autoantibodies and Autoimmunity (IWAA) in 2014, São Paulo, Brazil [14]. Three subsequent ICAP workshops were held, respectively, at the 12th Dresden Symposium on Autoantibodies (DSA) in 2015, Dresden, Germany [15, 16]; at the 13th IWAA in 2016, Kyoto, Japan; and at the 13th DSA, in 2017, Dresden, Germany. Through these meetings and related efforts, ICAP established a comprehensive classification of the most relevant and prevalent HEp-2 IFA patterns and harmonized their nomenclature (Figure 1). Each HEp-2 IFA pattern was assigned an alpha-numeric code (AC-#, for Anti-Cell). For example, the nuclear homogeneous pattern is designated AC-1 pattern and the cytoplasmic mitochondrial-like pattern is designated AC-21. ICAP originally classified the HEp-2 IFA patterns into three main groups: the nuclear group of 14 AC patterns, the cytoplasmic group of nine AC patterns and the mitotic apparatus group of five patterns. Each group is further subclassified into subgroups according to the texture of IFA staining or other common features (Figure 1) [14]. The AC pattern algorithm, representative images, detailed descriptions, historically used terminology and autoantigen associations are available in nine languages at www.ANApatterns.org.

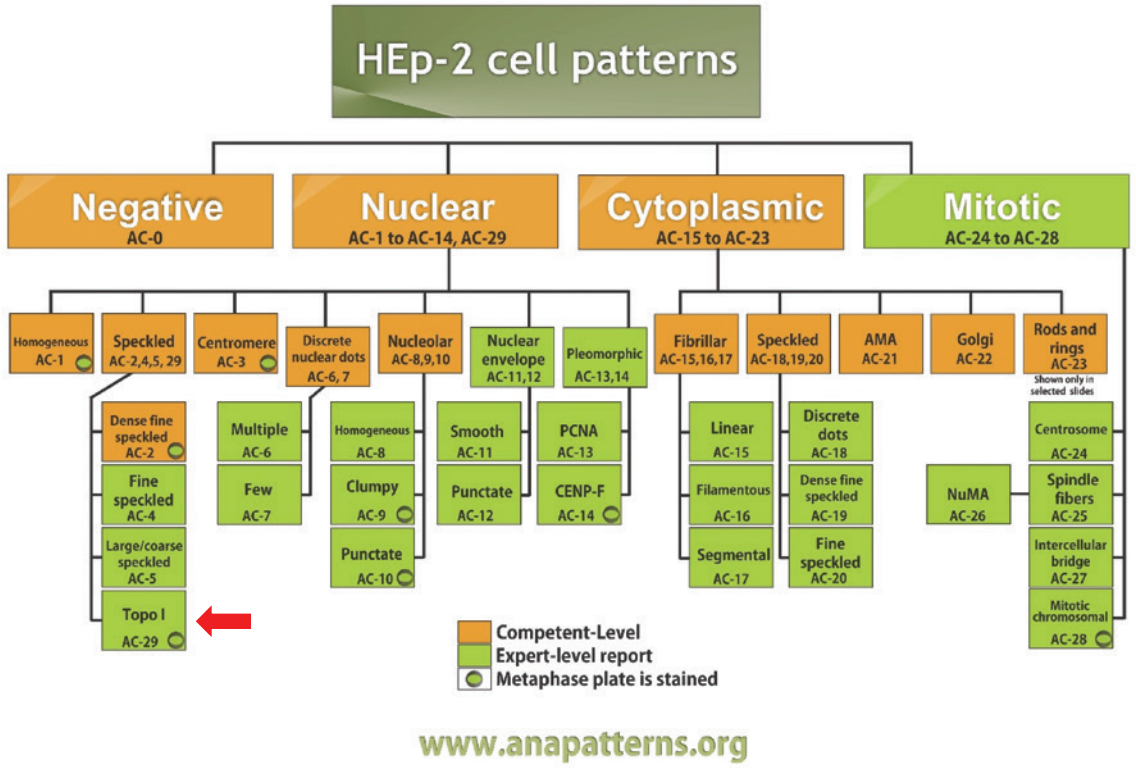

Figure 1: New ICAP decision tree for HEp-2 IFA AC patterns.
The expert-level AC-29 pattern (red arrow) belongs to the speckled pattern group in the nuclear division of the classification tree.

At the first ICAP workshop, it was acknowledged that not all known HEp-2 IFA patterns could be included and that subsequent editions of the ICAP initiative might add new patterns to the consensus classification tree [14]. At the fourth ICAP workshop, three novel AC patterns were approved after extensive exchange of images and discussion among the panel of experts. The AC-0 pattern was defined as a negative HEp-2 IFA test, and the AC-XX pattern was coined as an alternative to facilitate reporting unusual HEp-2 IFA patterns not covered by the current ICAP classification [17]. In this paper, we describe the features of the AC-29 pattern, which was recently defined and achieved consensus at the fourth ICAP as strongly associated with antibodies against the enzyme DNA topoisomerase I (Topo I).

Autoantibodies from systemic sclerosis (SSc) sera that recognized DNA Topo I were first reported as reactive to a 70 kDa "Scl-70" antigen by Dr. Eng M. Tan's laboratory in 1979 [18]. Subsequently, the Scl-70 antigen was demonstrated to be a fragment of the 100-kDa Topo I [19, 20], the enzyme responsible for cleaving, relaxing and re-annealing one of the two strands of double-stranded DNA during transcription and duplication of DNA [21]. Anti-Topo I antibody is a very specific biomarker for SSc, especially for the diffuse cutaneous form with more severe disease [22].

In 2009, Dellavance et al. [23] reported on a composite HEp-2 IFA pattern specifically associated with anti-Topo I antibodies. The so-called anti-Scl-70 (hereafter called Topo I-like) pattern was defined by the presence of five elements (Figure 2): (1) prominent nuclear compact fine speckled pattern in interphase cells; (2) consistent strong fine speckled staining of condensed chromatin in mitotic cells (depending on the serum dilution used, the mitotic chromatin staining may appear homogeneous); (3) strong staining of nucleolar organizing region (NOR) associated on condensed chromosomes in mitotic cells (this NOR staining may be obscured by the bright chromosomal staining as NOR are not always in the same focal plane); (4) delicate and weak cytoplasmic weblike staining radiating from the perinuclear area to the vicinity of plasma membrane (in general, the cytoplasmic staining becomes more prominent during titering the sample to higher dilutions); and (5) inconsistent staining of the nucleoli. One additional and subtle feature of the Topo I-like pattern is the hazy interface between the nuclear fine speckled staining and the cytoplasmic staining. In contrast to most nuclear patterns that show a sharp nuclear border, the Topo I nuclear staining has a rather blurry border in which the nuclear fine speckling overreaches the neighbor cytoplasmic region (Figure 3). The strong association of the HEp-2 IFA Topo I-like pattern with anti-Topo I antibodies was demonstrated by showing that 81 serum samples consecutively selected by presenting the Topo I-like pattern yielded a positive reactivity in specific assays to Topo I [23]. In addition, 16 samples consecutively selected by providing a positive reactivity to Topo I yielded the characteristic Topo I-like pattern [23]. The complete Topo I-like pattern was also obtained with the international anti-Topo I standard provided by the Autoantibody Standardization Committee, affiliated with the International Union of Immunology Societies [24]. Finally, two anti-Topo I affinity-purified antibody preparations reproduced the complete Topo I pattern [23].

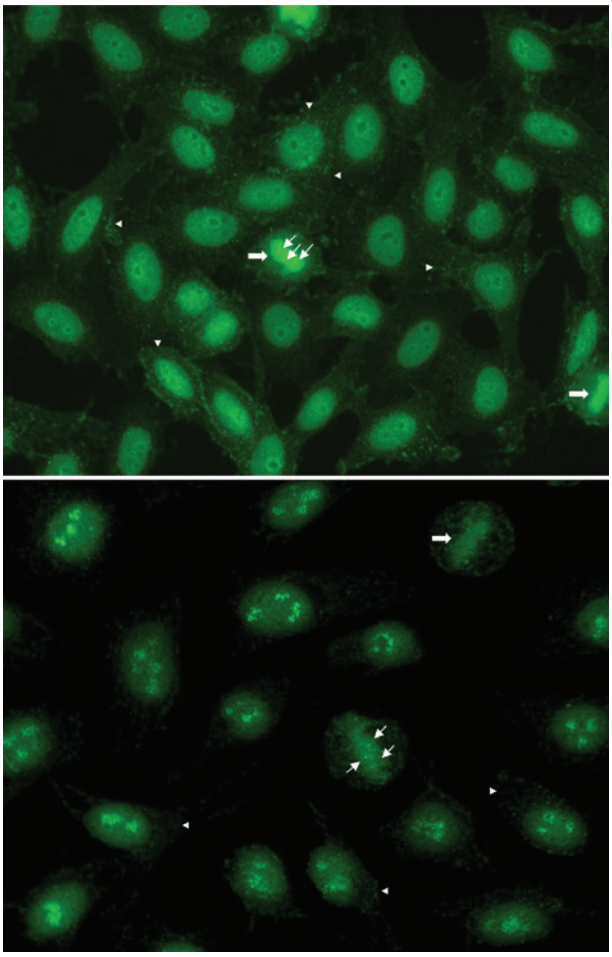

Figure 2: General characteristic features of the Topo I-like HEp-2 IFA pattern (AC-29).
Indirect immunofluorescence on HEp-2 cells with human serum with reactivity to DNA topoisomerase I diluted 1:100. Magnification ×400. Slides from Inova Diagnostics (upper panel) and MBL (lower panel). Interphase nuclei are stained with fine speckled pattern. Metaphase chromatin plate is stained with a compact fine specked pattern (large arrows) as well as with three to eight tiny discrete dots representing the nucleolar organizing regions (small arrows). Upper panel: the cytoplasm depicts a faint weblike staining pattern spanning from the nuclear periphery to the plasma membrane region (arrowheads). Lower panel: the staining of the nucleoli is variable according to the HEp-2 cell preparation.

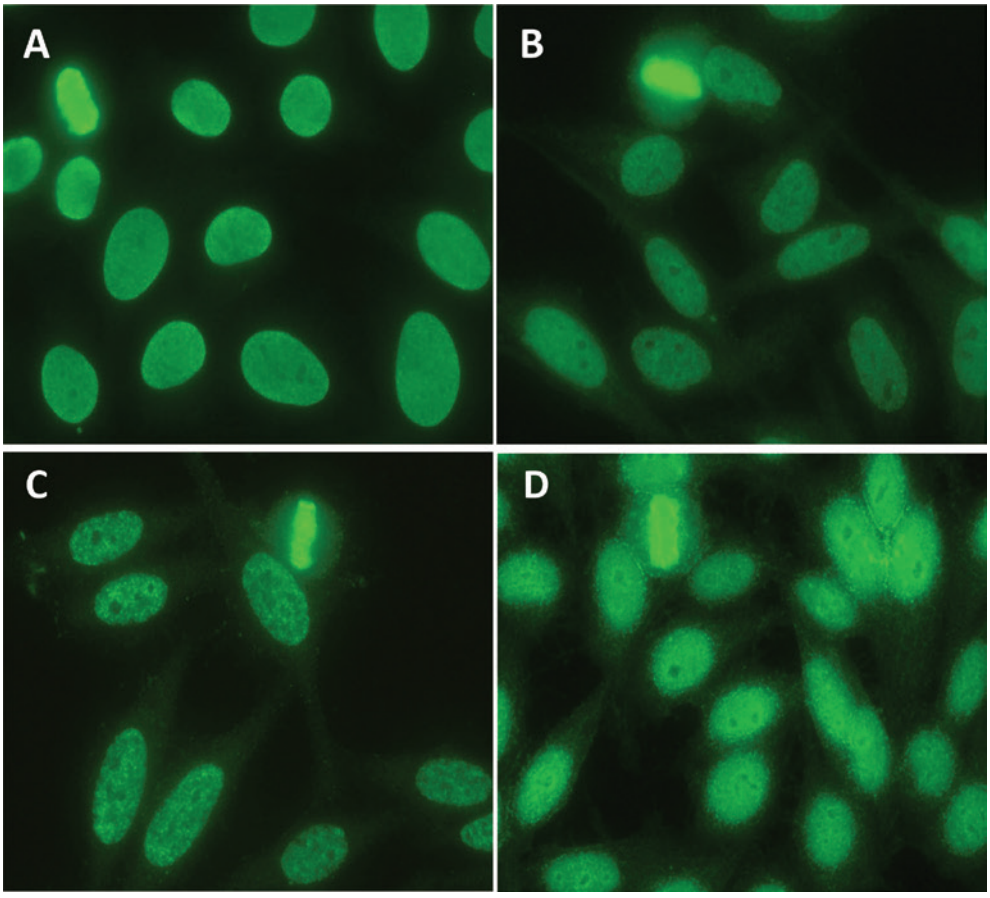

Figure 3: Peculiar aspect of the Topo I-like HEp-2 IFA pattern (AC-29): the fuzzy nuclear border.
Indirect immunofluorescence on HEp-2 cells (Inova Diagnostics) with human serum with reactivity to Topo I diluted 1:100. Magnification ×400. Nuclear borders are sharply defined in (A) nuclear homogeneous pattern (AC-1); (B) mixed nuclear pattern (AC-1+AC-4); and (C) nuclear dense fine speckled pattern (AC-2). By contrast, the AC-29 composite pattern shows ill-defined nuclear borders (D).

The incorporation of the Topo I-like pattern into the ICAP algorithm was initially proposed at the 2nd ICAP edition at the 12th DSA in 2015, Dresden, Germany [15]. The consensus at the time was that more experience with the Topo I-like pattern should be acquired, and in the meantime, experts sought to analyze the reproducibility of this pattern using various HEp-2 slide brands and particular laboratory conditions. Soon it became evident that the main features of the Topo I-like pattern could be observed independently by at least seven ICAP member laboratories who used a variety of HEp-2 slides from different manufacturers (i.e. Bion, Biorad, Bioscientifica, Inova, Euroimmun, Menarini, Immco [including DFS70 knock-out cells], MBL). The need to incorporate an AC pattern specific for the Topo I-like pattern was evidenced by the inadequacy of reports by clinical laboratories across Europe in a recent assessment of the European Consensus Finding Study Group on autoantibodies (ECFSG) (Figure 4). In this ECFSG exercise, a well-defined anti-Topo I serum was blindly analyzed by 43 participant laboratories that were asked to classify the HEp-2 IFA reactivity of the samples according to the existent ICAP classification algorithm. As shown in Figure 4, there was wide variation among the several laboratories, ranging from nuclear (AC-1, AC-2, AC-4), nucleolar (AC-8, AC-9, AC-10) and cytoplasmic (AC-15, AC-17, AC-18, AC-19, AC-23) patterns. Similar findings were obtained in a survey by the Austrian Association for Quality Assurance and Standardization of Medical Diagnostic Tests (ÖQUASTA) (Figure 4). This wide variation clearly demonstrated the inadequacy of the existing AC patterns for classification of the HEp-2 IFA pattern associated with anti-Topo I antibodies.

At the fourth ICAP meeting in 2017, there was a consensus that the Topo I-like pattern be designated as AC-29. The peculiar features that compose the Topo I-like pattern were depicted in great detail and acknowledged by most experts. The ICAP committee reviewed 24 candidate images provided by four ICAP laboratories and achieved consensus on those considered as representative of the proposed AC-29 pattern. Seven images were approved by consensus

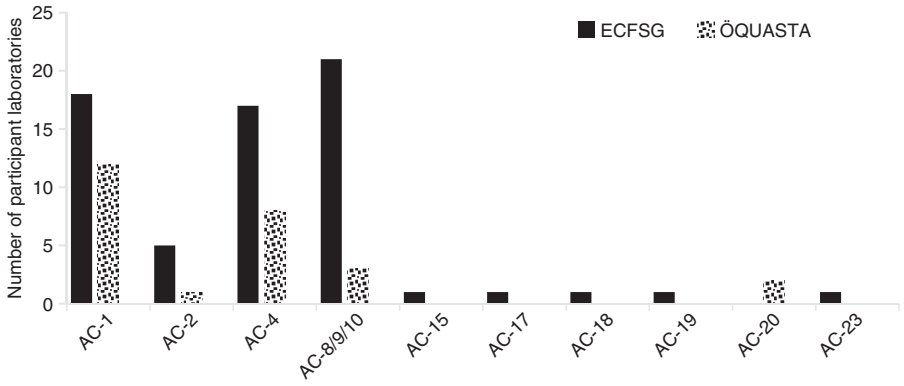

Figure 4: Previous ICAP classification tree was inadequate for reporting on HEp-2 IFA pattern for anti-DNA topoisomerase I antibodies. A blinded reference sample with anti-DNA topoisomerase I antibodies was distributed to 43 participant laboratories in the European Consensus Finding Study Group on autoantibodies (ECFSG) and 25 participant laboratories in the Austrian Association for Quality Assurance and Standardization of Medical Diagnostic Tests (ÖQUASTA). Laboratories should report according to ICAP classification (before establishment of AC-29). Reports from ECFSG laboratories ■ and ÖQUASTA laboratories ▪▪▪ show absence of consensus. AC-1, nuclear homogeneous; AC-2, nuclear dense fine speckled; AC-4, nuclear fine speckled; AC-8/9/10, nucleolar homogenous, clumply and speckled patterns, respectively; AC-15, cytoplasmic fibrillary linear; AC-17, cytoplasmic fibrillary segmental; AC-18, cytoplasmic discrete dots; AC-19, cytoplasmic dense fine speckled; AC-20, cytoplasmic fine speckled; AC-23, cytoplasmic rods and rings.

voting, and the top four are now available at www.ANApatterns.org. Due to the complexity of AC-29 pattern, not all images optimally depict all five of the AC-29 features. Thus, the NOR dots at the metaphase chromatin mass may be in different focal planes and not appear in focus in certain images; the nucleolus is inconsistently stained and may not be apparent on HEp-2 cells subjected to a variety of fixatives and other manufacturing conditions; the delicate weblike cytoplasmic staining may not be readily recognized at lower dilutions (1/80; 1/160), only to become more evident at higher dilutions (≥1/320). In the ICAP classification tree, the AC-29 pattern is arranged together with other nuclear speckled patterns (Figure 1) due to the fact that the nuclear staining is the most prominent of its five features. It is classified as an "expert level" pattern due to its complexity that requires training and expertise in the interpretation of HEp-2 IFA patterns.

Although the five-element compound AC-29 pattern has been observed in most commercial HEp-2 cell slides, there may be some variations in the expression of each element according to the slide brand. The detection of all five elements may be a challenge especially when only a single serum dilution is used (e.g. strong mitotic chromatin staining may obscure NOR) or in semi-automated systems when images are often selected from a single focal plane (e.g. NOR or cytoplasmic staining may not be in same focal plane as interphase nuclei).

There are some practical recommendations on how to routinely screen for the AC-29 pattern. If a compact fine speckled pattern is observed in interphase nuclei and at the mitotic chromatin mass, one should carefully look for positive NOR staining by searching different focal planes at the mitotic chromatin plates. Next, one should look for the presence of the weblike cytoplasmic staining, especially at higher dilutions of the sample. Lastly, the nucleolar staining should be evaluated, although in some HEp-2 slides the nucleolar staining is only visible near the edge of the wells or not visible at all.

In conclusion, the establishment and incorporation of the AC-29 pattern as a reliable representative of anti-Topo I autoantibodies enriches and refines the ICAP classification algorithm and contributes to the nomenclature harmonization and dissemination of this important HEp-2 IFA pattern. The proper recognition of the AC-29 pattern should trigger suspicion on the presence of anti-Topo I antibodies, which should engender appropriate analyte-specific reflex tests to confirm that autoantibody specificity.

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