Letter to the Editor

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Analytical performance of the single well titer function of NOVA View®: good enough to omit ANA IIF titer analysis?

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To the Editor,

Antinuclear antibodies (ANAs) are a diagnostic marker for ANA-associated rheumatic diseases [1]. The gold standard method for ANA detection is considered indirect immunofluorescence (IIF) on human epithelial (HEp-2) cells because of its high sensitivity [2]. Most recent ANA IIF guidelines recommend not only to report the presence of ANA as positive or negative but also to give a quantitative result [3, 4].

Nowadays, automated digital reading systems for ANAs by IIF testing have been integrated in routine immunodiagnostic laboratory practice [5, 6]. Several studies already showed a good correlation between fluorescence intensity by automated reading and end point titer (ET) obtained by manual reading [7, 8]. Furthermore, recent studies objectified that the likelihood for a systemic rheumatic disease increases with increasing ANA IIF fluorescence intensity [8, 9]. This illustrates that estimation of fluorescence intensity by automated IIF systems (without serial dilution) has clinical utility.

NOVA View® (Inova, San Diego, CA, USA) is a digital IIF microscope that can be used for automated ANA detection. The system is able to assign five basic fluorescent ANA patterns (homogeneous, speckled, centromere, nucleolar and nuclear dots) and reports the measured average nuclear fluorescence intensity in nominal units, called Light-intensity units (LIUs). For positive ANA IIF samples, the instrument allows estimation of a "single well titer" (SWT) based on the LIUs measured at the 1/80 screening dilution and pattern-specific dilution curves [5, 7]. We evaluated the analytical performance (total imprecision and accuracy) of the SWT function on the NOVA View® for different ANA IIF nuclear patterns.

Total imprecision of LIU measurement and of SWT function was evaluated by analyzing samples with a high and low level of a homogeneous, speckled, centromere and nucleolar pattern in 10 different ANA IIF runs. The target ET of the samples was determined by manual serial dilution. The total imprecision values of LIU measurement for the high-level and low-level samples were, respectively, 8% and 34% for a homogeneous pattern, 26% and 45% for a speckled pattern, 35% and 47% for a centromere pattern, and 38% and 39% for a nucleolar pattern (Supplemental Data, Table 1). When results were expressed as SWT, the estimated SWT deviated from the target ET by maximum 1 SWT for all patterns except for the centromere pattern where the deviation was 2 titers in 30% of the sample with a low antibody level and in 10% of the sample with a high antibody level. Of note, for the sample with a high level of nucleolar antibodies, 60% of the determinations deviated by 1 titer from the target value.

To evaluate the accuracy of the SWT function, samples with an isolated homogeneous (n = 389), speckled (n = 458), centromere (n = 101) and nucleolar (n = 69) ANA IIF pattern were analyzed in four hospitals: University Hospital Leuven, Gasthuiszusters Hospital Antwerp, OLV

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Hospital Aalst and AZ Sint-Jan Hospital Brugge. All serum samples were obtained as part of routine screening for ANA. SWT results obtained at the 1/80 screening dilution were compared to manual ET determination. When the results from the different laboratories were pooled, SWT and ET give identical results in 51.4%, 54.1%, 44.6% and 36.2% of the samples with, respectively, a homogeneous, speckled, centromere and nucleolar pattern (Figure 1). An overestimation of 1 titer by SWT compared to ET was found in 40.1%, 31.0%, 36.6% and 44.9% of samples with, respectively, a homogeneous, speckled, centromere and nucleolar pattern. In 15.9% and 17.4% of samples with, respectively, a centromere or nucleolar pattern, the difference between ET and SWT was ≥2 titer steps (with higher values for SWT compared to ET). The breakdown of the results per laboratory is given in Figure 1 and shows that the results are comparable between the laboratories, and thus that the overestimation of SWT compared to ET was site independent.

Figure 2A shows the pattern-specific association between LIU and SWT. The figure illustrates how the company applied pattern-specific conversion factors to assign SWT. For example, an SWT of 1:1280 corresponded to LIUs between 486 and 756 for a centromere pattern, to LIUs between 801 and 924 for a nucleolar pattern, to LIUs between 1529 and 2647 for a speckled pattern and to LIUs between 1421 and 1809 for a homogeneous pattern. Thus, a same SWT corresponded to lower LIUs for a centromere and nucleolar pattern than for a speckled or homogeneous pattern. The rationale behind this relates to the fact that centromere and nucleolar antibodies react to only a part of the cell and thus generate less fluorescent signal than antibodies that react to larger parts of the cell (speckled or homogeneous pattern).

Next, we looked at the relationship between the LIU and the ET. Here again we found an association between the LIU and the ET: the higher the LIU, the higher the ET. The LIU values between different ET categories revealed more overlap than the overlap between the LIU and the SWT categories (which understandably do not overlap). The overlap between LIU and ET categories was limited to 1 ± titer for all patterns, except for the centromere ANA IIF pattern where significant LIU overlap between ET categories of more than 2 titers was observed. This is probably

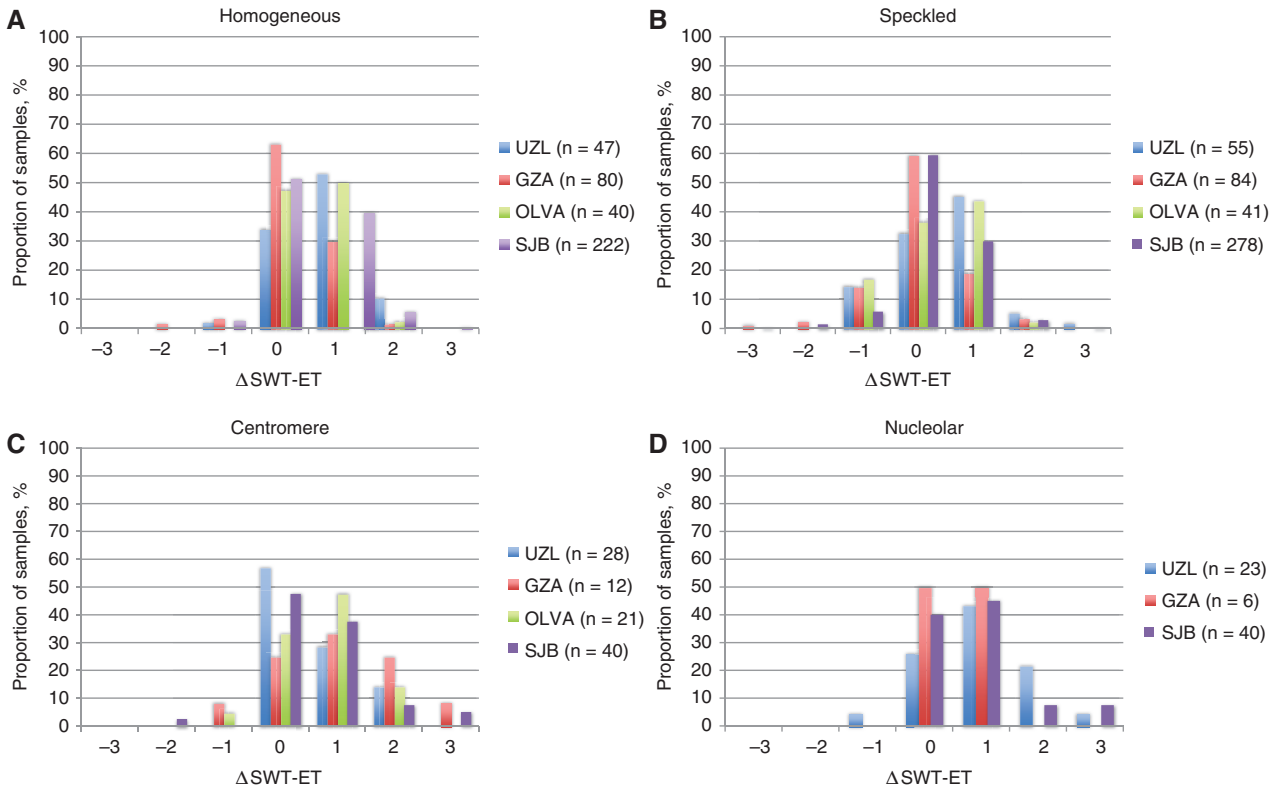

Figure 1: Differences in single well titer (SWT) and end-point titer (ET).
The frequency histograms show the difference in SWT and ANA IIF ET (serial dilution) for isolated homogeneous (A), speckled (B), centromere (C) and nucleolar (D) ANA IIF patterns. Both titer results were obtained from the routine screening for ANA by NOVA View® in four different laboratories: University Hospital Leuven (UZL), Gasthuiszusters Hospital Antwerp (GZA), OLV Hospital Aalst (OLVA) and AZ Sint-Jan Brugge (SJB).

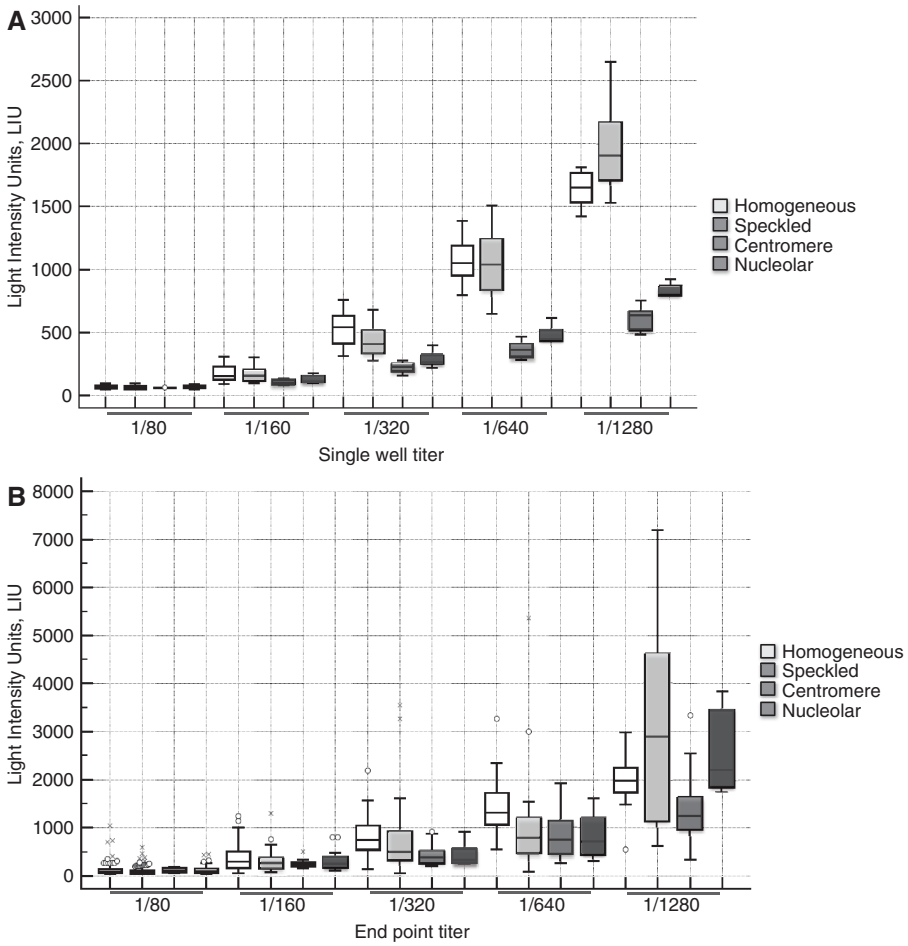

Figure 2: Pattern-specific association between Light Intensity Units (LIU), single well titer (SWT) and end-point titer (ET).
The Box whiskers plots represent the fluorescence intensity results reported by NOVA View® in LIU in function of ANA IIF SWT (A) and ET (serial dilution) (B) for isolated homogeneous (n = 389), speckled (n = 458), centromere (n = 101) and nucleolar (n = 69) ANA IIF patterns.

related to our finding that the imprecision of SWT for the centromere pattern was high (see Table 1 in Supplemental Material). We also found that there were pattern-dependent differences between LIU and ET; however, these differences were less pronounced than the differences applied by INOVA to assign pattern-dependent SWT. For example, the median LIU values that corresponded to an ET of 1:640 were 756, 718, 788 and 1321 for, respectively, a centromere, nucleolar, speckled and homogenous pattern, whereas the median LIU values that corresponded to a SWT of 1:640 were, respectively, 360, 449, 1040 and 1054. These pattern-dependent discrepancies between LIU, SWT and ET might explain why the overestimation of the SWT was more pronounced for the centromere and nucleolar pattern.

Single well testing of high titer sera bears the risk of antibody masking due to the presence of a dominant antibody or hook/prozone effects from antibody excess. In our study, antibody excess was observed, but only in a minority (0.7%) of the samples. Furthermore, SWT on NOVA View® cannot be used for mixed nuclear and cytoplasmic patterns. Therefore, we only focused on the SWT performance on isolated nuclear patterns. When mixed ANA IIF patterns are observed, end point titration by serial dilution remains the recommendation [3, 4].

In conclusion, when compared to ET, SWT on isolated nuclear patterns tended to overestimate the titer in a substantial fraction of samples. The overestimation was 1 titer step difference in 36.0% of all samples, but ≥2 titer steps in 16.5% of samples with a centromere or nucleolar pattern. The company-defined pattern-specific estimation of SWT may result in a biased estimation of the ET, as observed in four different Belgian laboratories.

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