Comparison of Affinity-Isolated and Non-Isolated Antibodies Used as Capture Antibodies in ELISA

Application Note

In Thermo Scientific Nunc Bulletin No. 11[1] it was discussed that the higher ELISA sensitivity found with a non-saturated capture antibody (CAb) surface compared to a saturated CAb surface, using affinity isolated, polyclonal CAb, might be due to a density dependent difference in the detection molecules' access to the analyte (target) molecules.

Recent findings from the comparison of non-isolated CAb and affinity isolated CAb suggest that this phenomenon is due to steric hindrance of the CAb at high surface densities.

In addition, this work proposes that the affinity isolated CAb is more sensitive to density dependent steric hindrance than the non-isolated CAb.

Materials & Methods

Thermo Scientific Nunc Immuno Plates F96 MaxiSorp (Cat. No. 439454) were coated overnight with a 1:2 dilution series of affinity isolated, polyclonal swine anti-rabbit (SaR) capture IgG antibodies = iCAb (Dako Z 400) in PBS, pH 7.2. Coating was followed by overnight incubation with rabbit target IgG antibodies = TAb (Dako A 008) in PBS with 0.05% Tween 20, in concentrations of either 20 ng/mL (low TAb concentration, LTC), or 10 µg/mL (excess TAb concentration, ETC). The captured target antibodies were detected by a 1 hour incubation with an excess of either SaR peroxidase conjugate (Dako P 217), or 1 part conjugate attenuated with 2 parts SaR (Dako Z 196), in PBS with 0.05% Tween 20, and subsequent substrate reaction with H_2O_2/OPD in phosphatecitrate buffer, pH 5.0, stopped with 2N H_2SO_4, 150 µL/well. All other reaction volumes were 200 µL/well. The Dako reagents used here and in the previous investigation[1] were from the same supplied ampoules. Between the reaction steps, the wells were washed three times with PBS containing an extra 0.2 M NaCl and 0.05% Triton X-100. All reactions were performed at room temperature. Unspecific adsorption of target or detection molecules was diminished by the overall use of detergent[2]. The results are presented in Fig. 1.

For proper comparison of affinity isolated (iCAb) and non-isolated (nCAb) capture antibodies, the above experiment was repeated (with unattenuated conjugate only) using, in parallel, iCAb and nCAb from the same SaR serum preparation (that is corresponding lots of Dako Z 400 and Z 196, respectively, obtained by courtesy of Dr. P. Kaastrup, Dako). The results are presented in Fig. 2.

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Results

The results in Fig. 1 show that for the low TAb concentration (LTC), an "internal" maximum signal is obtained at an iCAb coating concentration close to 0.5 µg/mL, corresponding to 1/10 surface saturation in the Nunc™ MaxiSorp™ F-well using 200 µL coating liquid [1]. This is not the case for the excess TAb concentration (ETC), where a "terminal" maximum signal plateau is obtained at saturating iCAb coating concentrations, i.e. 5 µg/mL and above. These findings are seen to be independent of conjugate attenuation.

The results in Fig. 2 show that for the LTC, the nCAb curve does not share the peculiar signal peak with the iCAb curve. Rather it climbs to a terminal maximum signal plateau, like both curves do for the ETC.

Discussion

In Fig. 1, the qualitatively identical pictures with both conjugate reagents (see Materials & Methods) suggest that the results are free from possible substrate reaction inconsistencies. The common leveling-off of the ETC curves at an iCAb coating concentration of 5 µg/mL suggests that the MaxiSorp surface area utilized is just saturated at this concentration, consistent with the estimated MaxiSorp IgG binding capacity of 650 ng/cm^2 [1,3].

In Fig. 2, similar nCAb and iCAb performance pictures would have been expected, not only for the ETC, but also for the LTC, according to the previous explanation of the internal LTC maximum signal ref. 1. This explanation states that the detection (conjugate) molecules have a better access to the target molecules on a non-saturated CAb surface than on a saturated surface due to the spaced CAb molecules on the non-saturated surface. In that case, the lack of a corresponding internal ETC maximum signal could be explained by assuming that the poorer conjugate access with increasing CAb density is compensated by capture of more TAb.

An alternative explanation of the observed internal LTC maximum signal by density dependent steric hindrance of the CAb did not seem to fit with the lack of an internal ETC maximum signal. Likewise, even though a higher active percentage of CAb molecules was estimated to be present on the non-saturated surface, the real number of active molecules remained higher on the saturated surface [1].

The present results, demonstrating different iCAb and nCAb performances for the LTC, suggest that the phenomenon may in fact be related to density dependent first layer CAb activity, dependent on the CAb character, rather than to density dependent conjugate abundance. Therefore, the explanation by steric hindrance was reconsidered - under the following assumptions (Figs. 3 and 4):

1. The nCAb preparation consists of three types of IgG antibodies, one having high target affinity (Y), another having medium affinity (I), and a third having low or no affinity (X).

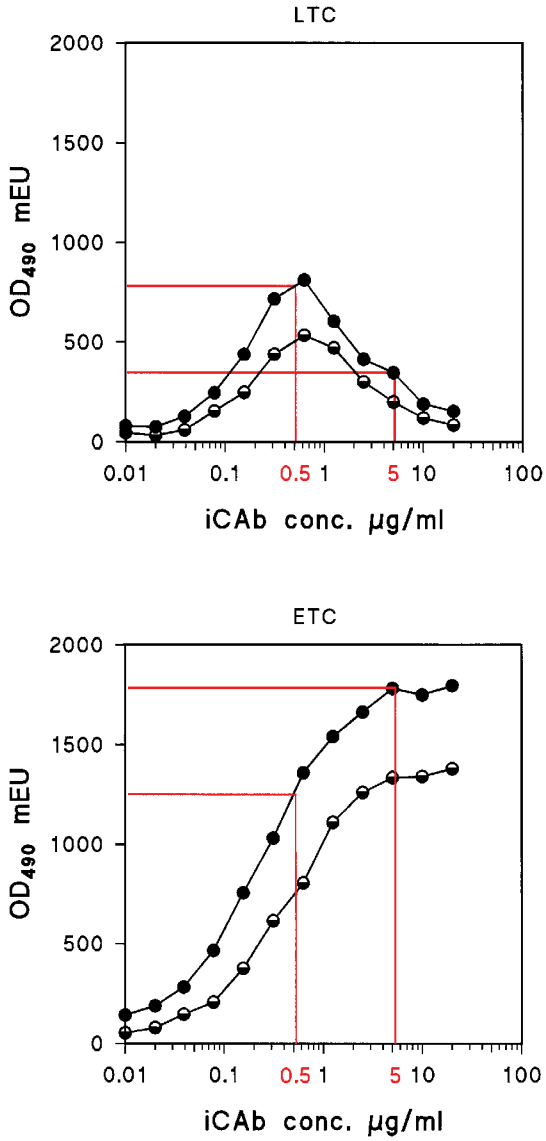

Fig. 1

Signals of captured target antibodies (TAb) in MaxiSorp F-wells coated with increasing concentrations of affinity isolated capture antibodies (iCAb), at low TAb concentration, LTC (above), or at excess TAb concentration, ETC (below), using unattenuated (●), or attenuated

(◒) conjugate. The OD values represent the total readings minus the respective background readings from wells without target molecules.

The red line interpolations correspond to the conditions in the previous work [1], using 1/10 saturating (0.5 µg/mL) and 1/1 saturating (5 µg/mL) iCAb concentrations, and unattenuated conjugate. Note that the LTC signals are higher in an interval of non-saturating iCAb concentrations than at saturating concentrations, and that the results are, qualitatively, independent of conjugate attenuation. See text for further explanation.

2. The iCAb preparation consists primarily of I-antibodies from the nCAb preparation. This seems plausible since most of the X-antibodies may have passed unhindered through the affinity isolation column during application of the nCAb and been

discarded with the primary eluate, whereas most of the Y-antibodies may not have been eluted under the necessarily gentle, non-denaturating conditions.

3. I- and X-antibodies are more sensitive to density dependent steric hindrance than Y-antibodies. The idea is that steric hindrance is a graduated phenomenon: the higher the antibody's affinity is, the less impaired is its target binding strength by neighboring molecules.

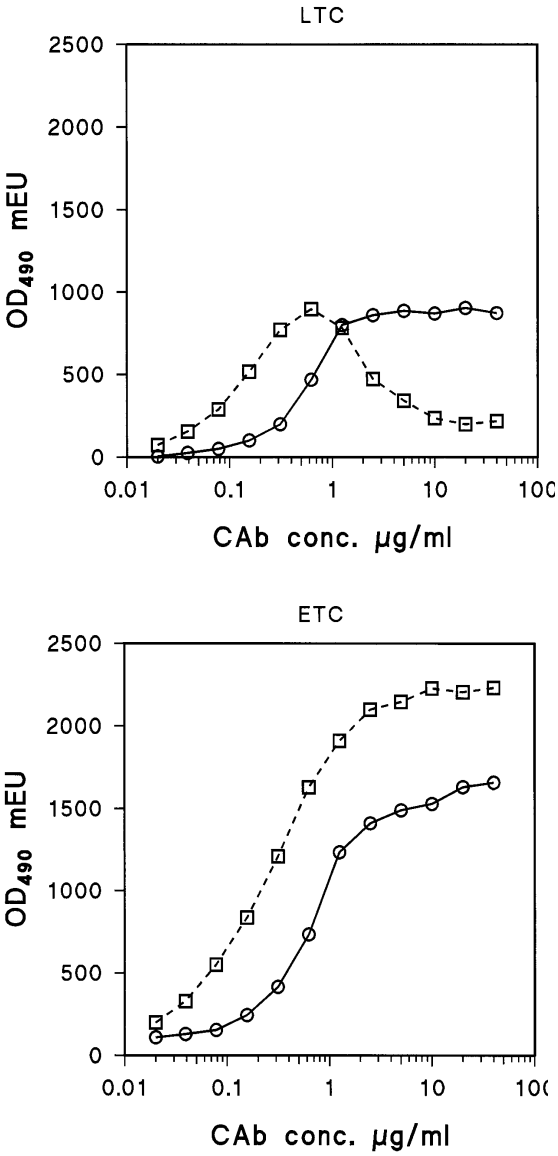

Fig. 2

Signals of captured target antibodies (TAb) in MaxiSorp F-wells coated with increasing concentrations of affinity isolated capture antibodies, iCAb (□ - - □), or non-isolated capture antibodies, nCAb (○ –– ○), at low TAb concentration, LTC (above), or at excess TAb concentration, ETC (below), using unattenuated conjugate. The OD values are the total minus background means from three independent experiments. Note that the LTC maximum signal occurrence at a non-saturating CAb concentration is a phenomenon connected with iCAb only. See Fig. 1 and text for further explanation.

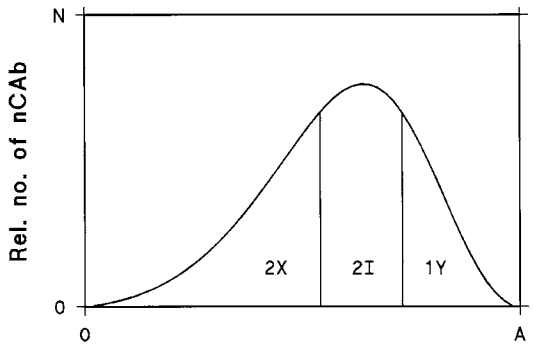

Fig. 3

Empirical model of the target affinity profile of the nCAb preparation used, assuming a 1Y:2I:2X relationship between the numbers of IgG antibodies having high affinity (Y), medium affinity (I), and low or no affinity (X). The iCAb preparation is assumed to consist of I-antibodies only.

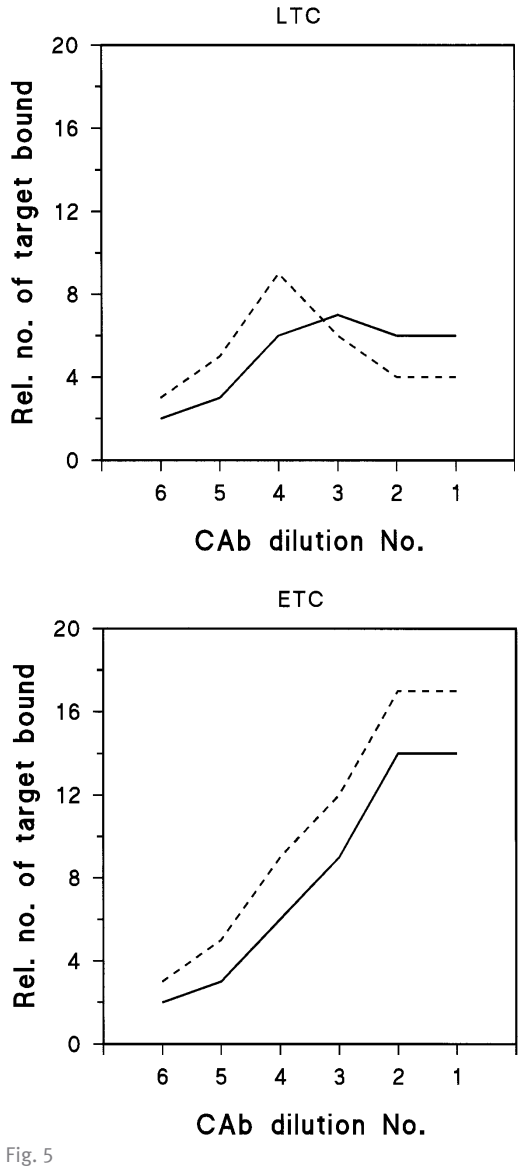

Fig. 5

Graphic presentation of the numbers of firmly bound target molecules from the model surface scenarios in Fig. 4, for increasing densities of iCAb (---), or nCAb (–––), at low target concentration (above), or at excess target concentration (below). Note the qualitative resemblance between these model curves and the data curves in Fig. 2.

4. The more sterically hindered anti-bodies will compete with the less hindered antibodies for capture of target molecules, but the more hindered antibodies will tend to lose the target molecules by subsequent washing.

The validity of these assumptions is demonstrated by the simplified approach outlined in Figs. 3 and 4, since the resulting model curves in Fig. 5 have the essential characteristics in common with the data curves in Fig. 2.

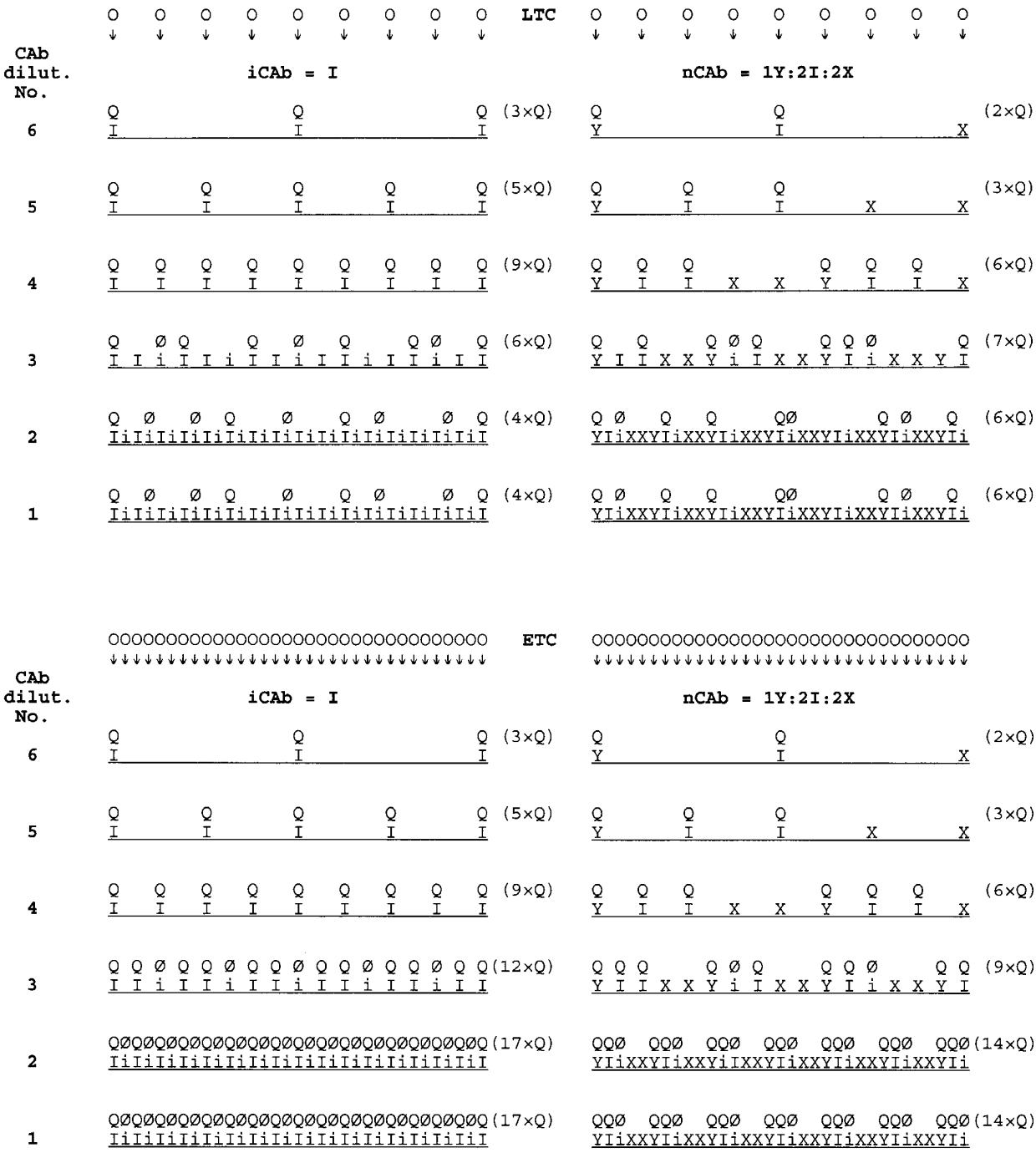

Fig. 4

Simplified surface scenarios of the text assumptions for increasing densities of iCAb (left), or nCAb (right), at low target concentration, LTC (above), or at excess target concentration, ETC (below), using the iCAb and nCAb compositions assumed in Fig. 3. The high affinity (Y) and the low or no affinity (X) antibodies remain unaffected by steric hindrance: the Y antibodies bind target molecules firmly (Q), and the X antibodies do not bind target at all. Only the medium affinity antibodies (I) are affected by steric hindrance, which appears at CAb dilution No. 3 and reaches its maximum at dilution No. 2 (surface saturation), respectively affecting every third and every second I-antibody (i). Bound target molecules are proportionally distributed between the Y-, I- and i-antibodies. However, the i-bound target molecules (Ø) will be detached (and removed) by the subsequent washing due to the sterically impaired binding strength. The numbers of remaining target molecules (Q) are graphically depicted in Fig. 5.

Summary

This investigation demonstrated that, when used as first layer capture antibodies in ELISA, affinity isolated polyclonal antibodies (iCAb) present maximum assay sensitivity at an iCAb density close to 1/10 surface saturation. Non-isolated antibodies (nCAb) present maximum sensitivity (of the same magnitude) at the nCAb density equalling surface saturation. This was explained by density dependent steric hindrance, to which iCAb was assumed to be more sensitive than nCAb.

However, it was necessary to make additional, supporting assumptions. The most essential (and controversial) support assumption states that the more sterically hindered antibodies "i" will compete with the less hindered antibodies for capture of target molecules, and that some, preferably i-bound target molecules will be detached by subsequent washing.

The difference between the iCAb and nCAb performances should be considered in assay construction. Probably, when using nCAb, a surface saturating coating would be optimal, whereas, when using iCAb, a non-saturating coating would be optimal. The particular optimum iCAb density should be determined a priori by adjusting the coating concentration to obtain maximum signal for an appropriately low concentration of target molecules.

In conclusion, the iCAb application presents the advantages of coating antibody uniformity and economy. However, it also has the disadvantages of only moderate antibody affinity and possible adsorptive denaturation in the absence of space-filling support molecules [1].

The interesting question of how monoclonal antibodies would perform as immobilized capture antibodies in comparison with the polyclonal iCAb and nCAb remains to be addressed.

References

1. Esser P. (1993).
   Activity of adsorbed antibodies.
   Thermo Scientific Nunc Bulletin No. 11, 3-6.

2. Esser P. (1990).
   Detergent in polystyrene ELISA
   Thermo Scientific Nunc Bulletin No. 8, 1-6.

3. Esser P. (1988).
   Principles in adsorption to polystyrene.
   Thermo Scientific Nunc Bulletin No. 6, 1-5.

Application Note

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