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METHOD FOR DETERMINING THE AFFINITY OF MONOCLONAL ANTIBODY USING NON-COMPETITIVE ELISA : A COMPUTER PROGRAM

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ABSTRACT

A simple and reliable method based upon law of mass action for calculating affinity of a monoclonal antibody using non-competitive ELISA, is described. In this method, the binding of an antibody (Ab) with an antigen (Ag) is measured by ELISA using serial dilutions of both antigen (coated on the plate) as well as antibody. When the OD measured after the antigen antibody interaction was plotted against the concentration of Ab, added to the wells, a hyperbolic curve was obtained. The OD, at any point of the curve, was considered as a direct reflection of the amount of antibody bound to the antigen. The OD-100 denotes the occupancy of maximum no. of epitopes available on the antigen molecules, accessible to the antigen. The concentration of antibody (Ab, Ab′) at corresponding levels of antigen concentration (Ag, Ag′), presents the value obtained at OD-50. The [Ag] and [Ag′] are not the true antigen concentrations but are the measurement of antigen density on the plate. The affinity constant K_{aff} was calculated by using the formula K_{aff} = (n - 1)/2(n[Ab′] - [Ab]), derived from law of mass action, where n = [Ag]/[Ag′]. A computer program to calculate the affinity of antibody to the antigen using method described in this manuscript has been developed and discussed.

Key words: Affinity constant; Monoclonal antibody; Non-competitive; ELISA; Computer program

INTRODUCTION

Affinity constant is one of the most important parameter for determining the strength of an antibody-antigen interaction. Several competition methods are available

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in the literature for calculating affinity of the antibodies and prominent amongst them are radioimmunoassay (RIA) [1,2] and enzyme-linked immunosorbent assay (ELISA) [3,4]. ELISA, is preferred for measuring antibody concentrations as it is relatively easier and does not employ the use of radioactive isotopes. Numerous reports in the past have shown that affinity plays a significant role in the quantification of antibodies [5,6]. However, the main problem in determining the affinity constant of antibody by competitive ELISA, is fluid phase equilibrium disruption by solid phase [7].

A method for measuring affinity of a monoclonal antibody by using non-competitive ELISA has been previously described by Beatty, *et al.* [8]. In this method a new equation was derived based upon the law of mass action for calculating affinity of antibody from ELISA data. Beatty *et al.* [8,9] estimated the maximum OD (OD-100), and antibody concentration at OD-50 (half of OD-100) by fitting ELISA data in sigmoid curve (logit-log), for calculating the affinity. Subsequently, it was shown that the ELISA data can be fitted better by hyperbolic curve as compared to the sigmoid curve [10,11]. Recently, we have demonstrated that accuracy of antibody concentration measured from ELISA data may be further increased by using a graphical method [12]. In the graphical method ELISA data is fitted by linear regression in semilogarithmic linear range (Sl-range), and to fit the data beyond Sl-range the hyperbolic curve fitting method is used. Thus, in the light of observations made in ref. 10, 11, 12, their is need to modify the method described by Beatty *et al.* [8].

In the present paper, we describe a method for calculating the affinity of antibody by non-competitive ELISA. Our method is similar to that of Beatty *et al.* [8], except that we have calculated OD-100 and derived antibody concentrations at OD-50 for various concentrations of the antigen, by using graphical method[12, 13], which is more accurate for determining the affinity. A computer program has also been developed which calculates the affinity of antibody to antigen by using non-competitive ELISA, described in this contribution.

MATERIALS AND METHODS

ELISA Reagents

The MAb GORK was kindly provided by Erik Wiersma, University of Uppsala Biomedical Center. MAb GORK was prepared against 2,4,6-trinitrophenyl at an initial concentration of 70 μg/ml. The ovalbumin (OVA), bovine serum albumin (BSA) and substrate orthophenylene diamine (OPD) were obtained from Sigma, St. Louis, USA. Trinitrophenyl (TNP) and peroxidase conjugated antibody were obtained from Pierce and Sera Lab (Sussex, England), respectively.

ELISA Method

2.4 ml of TNP-OVA was prepared in carbonate-bicarbonate buffer (0.05M,pH 9.6) at a concentration of 625 ng/ml. A series of 4 doubling dilutions were made so that the final concentration was 1/8 of the starting one. In a 96-well microtiter plate (costar), rows A, B, C and D were coated with 100 μl antigen of concentrations 625 ng/ml, 312.5 ng/ml, 156.75 ng/ml and 78.37 ng/ml respectively. 100 μl of OVA (625 ng/ml) was added in row E, this row worked as a control. After overnight incubation at 4°C, the plates were washed three times with PBS(0.01M,pH 7.2)-Tween-20 buffer. The remaining protein binding sites of the wells were saturated with 150 μl/well of 2% (w/v) bovine serum albumin (Sigma) for 2 h at 37 °C. The plates were again washed with PBS-Tween-20 buffer.

MAb GORK against 2,4,6-trinitrophenyl was prepared at an initial concentration of 70 μg/ml and added in each well of column 1. It was then serially double diluted and transferred into the remaining wells of column 2, 3, 4 etc., respectively. The plates were incubated at 37 °C for 2 h and then washed as described earlier. 100 μl/well of peroxidase labeled antibody conjugate (1:1000) diluted in 0.5% BSA was added to the plate and incubated for 2 h at 37 °C. Assay wells were then washed 5 times and 100 μl of freshly prepared substrate orthophenylene diamine was added. The color was allowed to develop for 20 minutes and the reaction was stopped using 100 μl 7% H_2SO_4.

The optical density (OD) of each well was read at a wavelength of 492 nm using a MPR-A4 Eurogenetics EIA reader. This reader can be interfaced to the serial communication port of any computer via RS-232 interface.

MATHEMATICAL DERIVATIONS

The assumptions and detailed derivation based on law of mass action is described earlier by Beatty *et al.* [8]. The Law of Mass Action for two identical antibody binding sites that have no cooperativity can be expressed as:

$K_{aff} = K_1 = K_2$, Thus

$$K = \frac{[AgAb]}{2[Ag]\,[Ab]} = \frac{2[Ag_2Ab]}{[Ag]\,[AgAb]} \tag{1}$$

where K is the Ag-Ab affinity constant and [Ag], [Ab], [Ag Ab], [Ag_2 Ab] are the concentrations of free antigen, free antibody having 2[Ab] antigen binding sites, antibody bound to one antigen and antibody bound to two antigens respectively. $[Ab]_t, [Ag]_t$ are the total antibody and antigen concentration in a well and can be defined by the equation:

$$[Ab]_t = [Ab] + [AgAb] + [Ag_2Ab] \tag{2}$$

$$[Ag]_t = [Ag] + [AgAb] + 2\,[Ag_2Ab] \tag{3}$$

Equ. 1 can be expressed as

$$K\,[Ag] = \frac{2[Ag_2Ab]}{[AgAb]} \tag{4}$$

As the quantity of [Ab] in the well increases, the OD approaches a maximum value OD-100, and free antigen conc. [Ag] approaches to near zero. Thus from equ. (4), [Ag_2 Ab] also approaches to zero at OD-100 . From equ. (3) we thus derive that $[Ag]_t$ = [Ag Ab] at OD-100. The OD directly reflects the amount of antibody bound to the antigen in the well ([Ag Ab] + [Ag_2 Ab]). At 50% of OD-100, OD-50, the amount of antibody bound to the antigen in the well is one half the amount of antibody bound ([Ag Ab] or $[Ag]_t$) at OD-100. Thus, at OD-50:

$$\frac{[Ag]_t}{2} = [AgAb] + [Ag_2Ab] \tag{5}$$

By solving equ. (3) and equ. (5)

$$[Ag] = [AgAb] \tag{6}$$

By solving equ. (1) and equ. (6)

$$[K_{aff}] = \frac{1}{2\,[Ab]} \tag{7}$$

By solving equ. (2), equ. (3), equ. (5) and equ. (7)

$$[K_{aff}] = \frac{1}{(2\,[Ab]_t - [Ag]_t)} \tag{8}$$

By using two different concentrations of Ag in coating solutions, one [Ag'] being half the other [Ag], we find at OD-50 that:

$$[Ag]_t = 4\,([Ab]_t - [Ab']_t) \tag{9}$$

Thus, from equ. (8) and equ. (9)

$$[K_{aff}] = \frac{1}{2\,(2\,[Ab']_t - [Ab]_t)} \tag{10}$$

The general formula is thus

$$[K_{aff}] = \frac{(n-1)}{2\,(n\,[Ab']_t - [Ab]_t)} \tag{11}$$

Equ. (11) represents the general formula for calculating affinity of monoclonal antibody to antigen, where n = $\frac{[Ag]}{[Ag']}$ and K_{aff} affinity constant.

READING AND CALCULATING ELISA DATA

The OD data were collected using a 96-well microplate reader (Eurogenetics) and fed to the microcomputer from the keyboard. The computer can also be directly linked to microplate reader using RS-232 interface, for inputting the data. The program uses the OD data information to complete 8 × 12 table of the data points which is then printed on the output sheet. The OD data was fitted by graphical method for given concentrations of Ag and maximum OD (OD-100), and concentrations at OD-50 for different Ag concentrations is described as below.

In graphical method ELISA data is fitted by linear regression in semilogarithimic linear range (Sl-range) and by hyperbolic curve fitting method beyond the Sl-range. The

detail derivation of graphical method is described previously by Raghava *et al.* [12].

a) The semilogarithmic curve of OD *vs* logarithm of concentration for given Ag concentration was used for calculating linear interpolation formula. In this curve, the Sl-range was depicted, denoting the linear portion of the curve with maximum slope and data in this range fitted by linear regression [12,13]. Sl-range of the curve was fitted by using following linear equation

$$\mathrm{OD} = A_0 + A_1 \times \log_{10}(C) \tag{12}$$

Where C, A_0 and A_1 represent the concentration, constant and slope of the curve respectively. The value of A_0 and A_1 was then calculated by fitting the data in the range using least square curve fitting method.

$$C = 10^{(M \times \mathrm{OD} + K)} \tag{13}$$

Where $K = -\frac{A_0}{A_1}$ and $M = \frac{1}{A_1}$ are constants.

Equ. (13) represents the linear interpolation formula obtained for given concentration of [Ag] coated on the plate.

b) The Hyperbolic interpolation formula was calculated as below. The detailed derivation has been described previously [10,11,12]. The hyperbolic equation can be written as

$$(C - X_0)(\mathrm{OD} - Y_0) = C_0 \tag{14}$$

$$C = \frac{C_0}{(\mathrm{OD} - Y_0)} + X_0 \tag{15}$$

where variables C and OD represent antibody concentration and optical density respectively. The regression constants which define the curve are X_0, Y_0 and C_0 represent capacity, (signal+background) and flatness. Constants could be obtained for N set of standard data points as described below.

$$A = N \sum X_i Y_i - \sum X_i \sum Y_i \tag{16}$$

$$B = N \sum X_i X_i - \sum X_i \sum X_i \tag{17}$$

$$C = N \sum Y_i Y_i - \sum Y_i \sum Y_i \tag{18}$$

$$D = N \sum X_i X_i Y_i - \sum X_i Y_i \sum X_i \tag{19}$$

$$E = N \sum X_i Y_i Y_i - \sum X_i Y_i \sum Y_i \tag{20}$$

These three equations (for X_0, Y_0 and C_0), along with the definitions of five constants ($A - E$) above are sufficient to perform hyperbolic regression on any experimental data set

$$X_0 = \frac{DA - EB}{A^2 - CB} \tag{21}$$

$$Y_0 = \frac{AE - CD}{A^2 - CB} \tag{22}$$

$$C_0 = \frac{\sum X_i Y_i - X_0 \sum Y_i - Y_0 \sum X_i}{N} + X_0 Y_0 \tag{23}$$

Equ. (13) represents the hyperbolic interpolation formula, where C is the concentration of the Ab the values of constants X_0, Y_0 and C_0 were calculated by using equ. (16– 20). The signal value Y_0 (OD-100) was calculated from equ. 23.

The total Ab concentration at OD-50 was calculated by using equ. (13), when OD-50 is in Sl-range, otherwise by using equ. (15). Similarly, the total Ab concentration at OD-50, for different Ag concentrations coated on the plate, were also calculated. Finally, the Ab affinity was calculated by putting the value of Ab concentration at OD-50 in equ. (11).

COMPUTER PROGRAM

The menu-driven computer program for calculating the affinity of monoclonal antibody from non-competitive ELISA data, has been written in GW-BASIC. OD data obtained from the Microplate reader can be fed to computer either directly by interfacing or by using Keyboard and data could be stored for future use. Before starting the calculation, a 'dilution template' should be defined and the information about the serial dilutions of antibody added to the wells and antigen coated on the plate must also be

supplied. The dilution template consists of arrays of dilution factor (DF), corresponding to the given [Ag] coated on the assay plate. It calculates the average OD of samples, which are in duplicate, triplicate, etc.

The computer program, 1) Fits the OD data for given Ag concentration by hyperbolic curve-fitting method and calculates OD-100, OD-50 and also hyperbolic interpolation formula; 2) It depicts the Sl-range and calculates the linear interpolation formula; and 3.) Checks whether OD-50 is in Sl-range and then calculates accordingly the concentration of [Ab] for a given [Ag] concentration coated on the plate using equ. (13) and equ. (15). In a similar way the program calculates the [Ab] concentration at OD-50 for other concentrations of [Ag] coated on the plate. Lastly, it calculates the antibody affinity by using equ. (11). It also calculates the capacity and flatness of hyperbolic curve, which plays a vital role in ELISA optimization. The program also allows the display and printing of the results. Fig. 1 shows an example printout from one of such ELISA plate.

RESULTS

The affinity constant of MAb GORK was calculated by using equ. (11) for different ratios of antigen coated on the plate ($n = \frac{[Ag]}{[Ag']}$). Based on the curve in Fig. 2

(a) 6.82×10^8, 4.46×10^8 and 3.045×10^8 for antigen coated ratio n = 2.

(b) 5.04×10^8 and 3.41×10^8 for antigen coated ratio n = 3.

(c) 3.67×10^8 for antigen coated ratio n = 4.

Similarly, a number of experiments were performed for calculating the affinity constant of MAb GORK for different values of 'n'. The affinity constant calculated by this method was comparable with soluble-phase affinity constants measured by inhibition RIA (competition RIA) for this antibody [2]. The solid phase affinity constants calculated by our method had an excellent correlation coefficient of 0.98. We also calculated the affinity constant of MAb GORK, by Beatty *et al.* [8] method. The overall results, calculated from our method, a previously described non-competitive method, and RIA are summarized in table I.

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                    OPTICAL DENSITY IN 8 x 12 FORMAT

   1     2     3     4     5     6     7     8     9     10    11    12
A 0.946 0.939 0.927 0.903 0.865 0.791 0.606 0.431 0.231 0.135 0.056 0.024
B 0.744 0.738 0.727 0.708 0.669 0.612 0.429 0.250 0.171 0.105 0.053 0.021
C 0.502 0.496 0.482 0.464 0.435 0.341 0.227 0.161 0.107 0.056 0.035 0.019
D 0.271 0.262 0.244 0.219 0.179 0.148 0.113 0.081 0.057 0.041 0.033 0.026
E 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010
F 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010
G 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010
H 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010 0.010

PLATE NO. = 1, DILUTION FACTOR = 2, INITIAL CONC. = 70.000 MICROGRAMS/ML

                          ELISA DATA ANALYSIS


                          [Ag] CONC. =   625

SL-RANGE(OD) = 0.23 - 0.79, SLOPE (SL) = 1.623 AND CONSTANT (B) = -0.947
CAPACITY (X0) = -0.418, SIGNAL =  0.952 AND FLATNESS (C0) = -0.460
OD-100 = 0.952, [AB] CONC. AT OD-50 =   0.670, MICROGRAMS/ML


                          [Ag] CONC. =   312.50

SL-RANGE(OD) = 0.25 - 0.61, SLOPE (SL) = 1.663 AND CONSTANT (B) = -0.677
CAPACITY (X0) = -0.470, SIGNAL =  0.750 AND FLATNESS (C0) = -0.414
OD-100 = 0.750, [AB] CONC. AT OD-50 = 0.885, MICROGRAMS/ML


                          [Ag] CONC. =  156.25

SL-RANGE(OD) = 0.23 - 0.44, SLOPE (SL) = 2.895 AND CONSTANT (B) = -0.628
CAPACITY (X0) = -0.849, SIGNAL =  0.509 AND FLATNESS (C0) = -0.460
OD-100 = 0.509, [AB] CONC. AT OD-50 =   1.283, MICROGRAMS/ML


                          [Ag] CONC. =   78.12

SL-RANGE(OD) = 0.08 -  0.22, SLOPE (SL) = 8.802 AND CONSTANT (B) = -0.963
CAPACITY (X0) = -2.882, SIGNAL =  0.281 AND FLATNESS (C0) = -0.712
OD-100 = 0.281, [AB] CONC. AT OD-50 =   1.873, MICROGRAMS/ML


                          AFFINITY OF ANTIBODY

 6.820201E+08  4.461169E+08  3.045496E+08 FOR [Ag] RATIO N = 2.00
 5.042557E+08  3.405747E+08 FOR [Ag] RATIO N = 4.00
 3.668088E+08 FOR [Ag] RATIO N = 8.00
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Fig. 1 Shows an example printout from one ELISA plate. For each plate, the user has the option to print the average OD values, Sl-range, linear interpolation formula, the hyperbolic interpolation formula, OD-100 (signal), AB-50 (concentration of antibody at OD-50) for different amount of antigen coated on the plate and affinity of antibody.

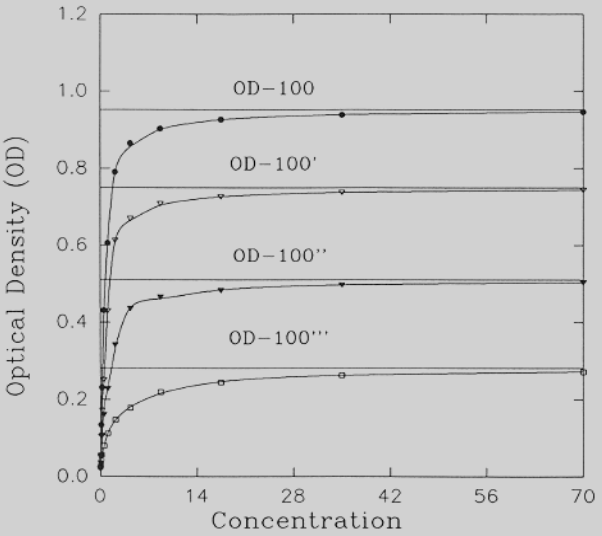

Fig. 2 Experimental ELISA curves for GORK monoclonal antibody at different TNP coated concentrations. The concentration of TNP in the coating solutions were were: 1, 625 ng/ml; 2, 312.5 ng/ml; 3, 156.25 ng/ml; 4, 78.12 ng/ml. The OD-100 for different coating solutions is also shown.

Table 1

Summary of affinity constant K_aff of MAb Gork bound to TNP

Antigen coated ratio = $\frac{[Ag]}{[Ag']}$	Our method		Logit-log method	
	K_aff(mean)	SE(K_aff)	K_aff(mean)	SE(K_aff)
2	6.21	5.65	6.34	12.34
3	4.45	4.34	8.34	8.34
4	5.24	3.43	3.34	5.34

The affinity calculated by our method had higher correlation coefficient 0.98 as compared to 0.96 obtained with competition RIA, where affinity is calculated by Beatty *et al.* [8] method. This shows the affinity calculated by our non-competition ELISA method is more accurate then the previous non-competitive method. The standard error

in affinity calculated by our method is very less in comparison to the earlier method, (Table I), showing thereby that our method is more reliable. The OD-100 calculated in our case is the representation of full ELISA data where in Beatty *et al.* [8] it was average of upper values only.

DISCUSSION

The objective of this study was to describe a simple, reliable and rapid method for determining affinity of monoclonal antibodies by using non-competitive ELISA and to develop a computer program using this strategy. A variety of competitive methods using RIA [1,2,14] and ELISA [3,4,15,16] for determining the affinity of the antibody have been described in literature. These approaches rely on the measurement of bound *vs* free antigen ratio, requiring an additional separation step of free and bound entities. This may intern falsify the analytical results, especially when equilibrium disturbing procedures are included. Another problem in determining the affinity constant of antibody by competitive ELISA method is fluid phase equilibrium disruption by solid phase [7].

To address some of these issues, a method, which does not require any purification of reactants or separation steps at equilibrium has been described earlier [17] for determing the affinity constants of monoclonal antibodies to enzymes. However, this approach has limitations as it can be used for calculating the affinity of only those antibodies which are inhibitory in nature. To understand the impact of affinity upon solid-phase and for determining the affinity by non-competitive ELISA method, Beatty *et al.* [8] have derived a new equation from the principle of law of mass action. This method compares the OD-50 of two sigmoid curves of antibody serial dilutions on plate coated with two different concentrations of the antigen. In this approach the exact value of the antigen adsorbed onto the plate are unknown and the relationship of the antigen adsorptions is therefore assumed. This method [8] for determining the affinity constant of the antibody, by using non-competitive ELISA, is an alternative method of competitive methods described earlier.

In this manuscript, we have utilized the similar approach as described earlier [8], which calculates the affinity of antibody using ELISA. However, in our approach contrary to logit-log method used by Beatty *et al.* [8], the ELISA data is fitted by graphical method for calculating the signal (OD-100) and the total Ab concentration at OD-50. It has been shown that the most reliable representation of ELISA data is obtained by graphical method rather than logit-log method[10,11,12]. In graphical method ELISA data, falling in Sl-range is fitted by linear regression while that is falling beyond Sl-range, is fitted by hyperbolic curve fitting method. The main drawback in the approach of the Beatty et al. [8] was that it calculates the maximum OD (OD-100) by taking the average of upper values of OD. It was also assumed in the derivation that the OD-100 is the maximum value when free concentration of the antigen approaches to zero. Accordingly, the value calculated by taking average would always be less than OD-100. The OD-100 calculated is directly affected by upper values (saturated portion of the curve). It may be pointed here that normally the errors occur in saturated portion of the curve. Therefore, calculation based on saturated portion of the curve may not necessarily always reflect the actual data of the curve. In the present report, the above problems of calculating OD-100 have been eliminated by fitting the ELISA data by graphical method and the signal (OD-100) of the curve is calculated, which is the asymptote of hyperbola. As OD-100 calculated in our method represents the actual ELISA data and the small errors occurring in the upper portion of the curve will not affect accuracy significantly in calculating the affinity. The accuracy has further been increased by calculating the antibody concentration at OD-50 by using graphical method [12].

In conclusion, it may be stated that we have improved the non-competition ELISA method described earlier by Beatty et al. [8] for calculating the affinity. This method is ideal for screening and testing hybridoma products, determining the affinity and avidity. Further, the data analysis was simplified by a computer program. The principles employed in this method should have application in a broad range of immunoassays.

The computer program was developed to run on an IBM compatible microcomputer. It is written in the GW-BASIC. The program is simple to use, has been designed to be run by users with little knowledge of mathematics or computers. Program provides the data storage facility on Disk. The Ab_affi program is freely avaliabe from authors on request. The Authors will prefer to distribute the source code via e-mail (contact Raghava@imtech.ernet.in).

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REFERENCES

1 Muller, R. Calculation of average antibody affinity in antihapten sera from data obtained by competition radioimmunoassay. J. Immunol. Methods 1980; 34: 345-52.

2 Larsson, A. and Axelsson, B. Calculation of affinity constants directly from homologous displacement curves. J. Immunol. Methods 1991; 137: 253-59.

3 Macdonald, R.A., Hosking, C.S. and Jones, C.L. The measurement of relative antibody affinity by ELISA using thiocyanate elution. J. Immunol. Methods 1988; 106: 191-4.

4 Luxton, R.W. and Thompson Affinity distributions of antigen-specific IgG in patients with multiple sclerosis and in paitents with viral encephalitis. J. Immunol. Methods 1990; 131: 277-82.

5 Peterfy, I., Kaasela, P. and Makela, O. Affinity requirements for antibody assay mapped with monoclonal antibodies. J. Immunol. Methods 1983; 138: 1809-13.

6 Griswold, W.R. and Chalquest R.R. Theoretical analysis of the accuracy of calibrated immunoassay for measuring antibody concentration. Mol. Immunol. 1991; 28: 727-32.

7 Hetherington, S. Solid phase disruption of fluid phase equilibrium in affinity assay with ELISA. J. Immunol. Methods 1990; 131: 195-202.

8 Beatty, J.D., Beatty, B.G. and Vlahos, W.G. Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. J. Immunol. Methods 1987; 100: 173-9.

9 Beatty, J.D., Beatty, B.G., Vlahos, W.G. and Hill, L.R. (1987) Method of analysis of non-competitive enzyme immunoassays for antibody quantification. J. Immunol. Methods 1987; 100: 161-72.

10 Studnicka, G.M. Hyperbolic regression analysis for kinetics, electrophoresis, ELISA, RIA, Bradford, Lowery, and other applications. Comput. Appl. Biosci. 1987; 3: 9-16.

11 Studnicka, G.M. ELISA assay optimization using hyperbolic regression. Comput. Appl. Biosci. 1991; 7: 217-24.

12 Raghava, G.P.S., Joshi, A.K. and Agrewala, J. N. Calculation of antibody and antigen concentrations from ELISA data using a graphical method. J. Immunol. Methods 1992; 153: 263-4.

13 Agrewala, J.N., Raghava, G.P.S., Mishra, G.C. Measurement and computation of murine interleukine-4 and interfron-γ by exploiting the unique abilities of these lymphokines to induce the secretion of IgG1 and IgG2a. Journal of Immunoassay 1993; 14: 83-97.

14 Heyningen, V.V., Brock D. J.H. and Heyningen, S.V. A simple method for ranking the affinity of monoclonal antibodies. J. Immunol. Methods 1983; 62: 147-53.

15 Friguet, B., Chaffotte, A.F., Djavadi-Ohaniance, L. and Goldberg, M. E. Measurement of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J. Immunol. Methods 1985; 77: 305-19.

16 Rath, S., Stanley, C. M. and Steward, M. W. An inhibition enzyme immunoassay for estimating relative antibody affinity and affinity hetrogeniety. J. Immunol. Methods 1988; 106: 245-9.

17 Ehle, H., Godicke, C. and Horn, A. A new graphical method for determining the affinity constants of monoclonal antibodies to enzymes. J. Immunol. Methods 1989; 117: 17-23.