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Detection Curb

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1. Introduction

Immunoassay is an analytical method based on antigen-antibody reaction. The antigen-antibody reaction is specific for substrate, and provides tight bondage between them. Though the characteristics of the bondage has not been understood completely, it is considered as morphological interaction between them. It has resemblance to enzymatic reactions which has the interaction like between key and key hole. Beside the morphological interaction it include some interactions such as hydrophilic and hydrophobic interactions. Hydrogen bond is frequently included in the interaction. Such weak interactions are accumulated to form strong bondage. The accuracy of the method is based on the quality of the antibodies. A good antibody has a high specificity for antigen and tight bondage for antigen, resulting good sensitivities. Enzyme-Linked Immunosolvent assay (ELISA) has been developed. Because of its convenient and simple operation, it has been widely spread as commercial reagents or kits to detect chemicals of various field, pesticide, herbicide, endocrine disruptor, antibiotics, and human hormones. While ELISA is a batch system of immunoassay, flow systems have been developed such as Surface Plasmon Resonance (SPR) and Kinexa (will be mentioned in this chapter). In the flow system, the signal for immunoassay directly reflects equilibrium state between antigen and antibody. Theoretically it enables the analysis of the equilibrium between antigens and antibodies In this chapter, based on data obtained by an instrument, Kinexa 3000 from Sapidyne Instruments Inc.(Boise, ID), fundamental issues, such as shape of detection curbs, detection range, detection limit, and inherent error are theoretically mentioned.

2. Detection range and limit with monoclonal antibody

Calibration curbs for detection of analyte was provided with various concentration of antibody and antigen(analyte) where antibody-antigen reaction is at equilibrium. Usually, the concentration of analyte is decided by measuring the concentration of free-antibodies which is not bound for analyte. When total concentration of antibody is known and free-antibody concentration is decided, the analyte binding antibody concentration can be decided. The free analyte concentration can be decided with the concentration of free-antibody, the concentration of analyte binding antibody, and dissociation constant (Kd). Then, the total analyte concentration is decided as sum of free and antibody-bound analyte. A calibration curb provide the relationship between free antibody concentration and total analyte concentration.

Calibration curbs are usually demonstrated on semi log graph. X axe is percentage of free antibody concentration for total antibody concentration, Y axe is logarithmic scale of total antigen concentration. In immunoassay, calibration curbs are available for the $10^{2\text{-}3}$ order of analyte concentration. Then, the logarithmic scale is used. With the curb, total analyte concentration is easily decided from the ratio of free-antibody concentration by total antibody concentration.

2.1 Detection range

The model calibration curb are demonstrated in Figure 1, where antibody is monoclonal one, its concentration is 1 Mol, and antibody has dissociation constant, Kd value of 2M. The curb is drawn with various analyte concentrations. The calculation was carried out under the assumption that an antibody has only one site for binding antigen, though actual antibody has two. Then, free antibody concentration is given for total antigen. The ideal calibration curb shows a sigmoid curb as shown in Fig.1, The steep part of the sigmoid is the detection range, since the other part gave more errors for detection of the concentration.

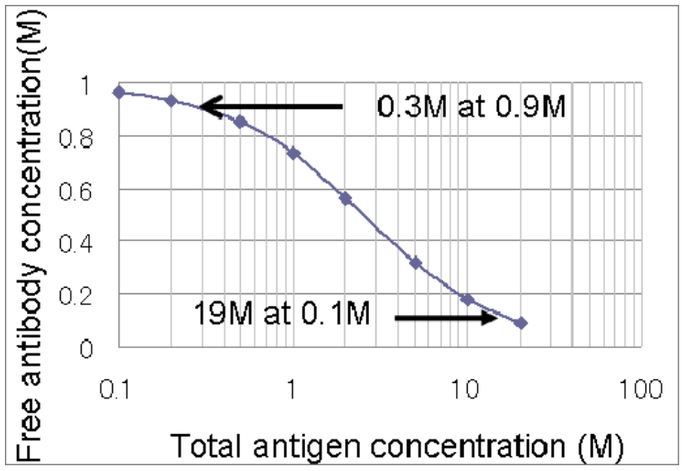


Fig. 1. A model detection curb with a model antibody. The antibody is monoclonal one, and its concentration is 1M. The dissociation constant of the antibody is 2M.

If detection range is assumed by the range from 0.1M(10%) to 0.9M(90%) of free-antibody concentration, the detection concentration will be from 0.3 to 19 Mol. This range shows that the detection range will be about 10^2 in the model detection curb. If we want to make a calibration curb with more high concentration of analyte, more concentration of antibody is required. When a half of antibody reacts with antigen, the point of half antibody reaction is the center of detection curb. The antibody concentration affects the detection range of the curb. The comparable amount of antibody is used to detect the high concentration of analyte. The detection curbs with high concentration of the antibody are summarized in Fig.2 where X axe is normalized as percentage to compare the detection curbs of various concentration of antibody each other. With the high concentration of antibodies, the detection curbs become steep, For 100 M antibody concentration, detection rage is from 10 to 95 M, this range is within 10 times. When compared with 1M detection curb, 100 M detection curb is almost one tenth of detection range.

The detection range can be calculated from equation (1)., (2), and (3). Dissociation constant is expressed by the equilibrium state between free antibody concentration, free antigen concentration, and complex concentration of antibody and antigen.

$$Kd = [Ab] \times [Ag] / [AbAg] \quad (1)$$

$$[Ab_0] = [Ab] + [AbAg] \quad (2)$$

$$[Ag_0] = [Ag] + [AbAg] \quad (3)$$

[Ab]: free antibody concentration, [Ag]: free antigen concentration, [AbAg]: complex between antigen and antibody, $[Ab_0]$: total concentration of antibody, $[Ag_0]$: total concentration of antigen.

When free antibody is 10% of total antibody, complex formation is 90% from equation 2. In this condition, equation 1 can be written as follow.

$$Kd = 10 \times [Ag]/90 = 1/9 \times [Ag] \quad (4)$$

Then, the [Ag] and $[Ag_0]$ can be presented for 10% antibody complex formation as follow

$$[Ag] = 9Kd \quad (5)$$

$$[Ag_0] = 9Kd + [AbAg] \quad (6)$$

The [AbAg] is 90% of total antibody, then the analyte concentration(total antigen concentration) can be expressed as the function of total antibody concentration, Ab_0,

$$[Ag_0] = 9Kd + 0.9[Ab_0] \quad (7)$$

For the condition 90% free antibody and 10% complex formation. Similar calculation give equation 8.

$$[Ag_0] = 1/9Kd + 0.1[Ab_0] \quad (8)$$

From equation 7 and 8, the detection range is defined for the assumption of the range from 90% free antibody to 10% free antibody. If we use a low concentration of antibody, term $[Ab_0]$ will be negligible, the detection range will be from 1/9Kd to 9 Kd. The width is 81, ca. 10^2 order. On the other hand, when we use a high concentration of antibody, Kd value will be negligible. Then, the detection range will be from $0.1[Ab_0]$ to $0.9\ [Ab_0]$. This width is 9, and steeper curb than the low concentration antibody.

2.2 Detection limit

As shown in Fig.2, for the high concentration of antigen, we can provide the detection curbs with high concentration of antibodies. On the other hand for the low concentration of antigen, we can observe that the detection curbs are superimposed at antibody concentration of 0.01 and 0.1M. The detection curb would not shift to lower concentration any more. It shows that there are limits of detection value. This value is controlled by Kd values.

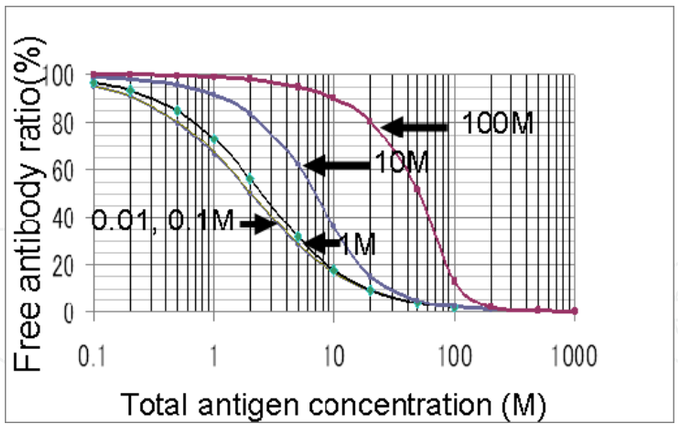


Fig. 2. Theoretical detection curbs for various antibody concentration. A model antibody has Kd values of 2M, Antibody concentration is changed from 0.01 to 100M.

If the 50% of antibody is bound to antigen, free antibody concentration is equal to Antibody-antigen complex concentration. Equation 2 can be written as follow.

$$[Ab_0] = [Ab] + [AbAg] = 2[Ab] = 2[AbAg] \tag{9}$$

Then, equation 1 becomes more simple one.

$$Kd = [Ag] \tag{10}$$

In this condition, equation 3 becomes as follow

$$[Ag_0] = [Ag] + [AbAg] = Kd + 1/2[Ab_0] \tag{11}$$

Equation 11 shows that at the center of the detection curb, where 50% of antibody is free,

analyte concentration (Ag_0) is expressed as the function of Kd values and initial antibody concentration (Ab_0). From equation 11, it is also observed that by dilution of antibody, the detection curb shift toward lower concentration of analyte. However, the detection curbs would not shift to beyond Kd value at the center of the curb. This is experimentally proved by Ohmura et al in 2001. In fig. 3, detection curb of estradiol is prepared with a estradiol antibody. The measurement was done in solution phase with KinExa 3000. The Kd value of the antibody is 28 pM. The detection curbs would not shift to lower concentration of estradiol, when the antibody is less than 10 pM (Fig. 3A, symbol of solid circle). If the center points of detection curbs (50% of free antibody) are plotted versus antibody concentration, the value of the center point approaches to Kd value (solid line in Fig.3B), by the decrease concentration of antibody (Fig.3B). This is a detection limit of immunoassay using monoclonal antibodies. If the 90% of free antibody concentration is the detection limit of immunoassay, 1/9 Kd is the limit as shown in equation 8.

3. Detection curb with multiple antibodies

In the previous paragraph, it is shown that antigen-antibody reactions are governed by affinity of antibody (Kd values). As every antibodies have their own affinities, in the mixture of multiple antibodies and antigens, it is expected that each antigen-antibody

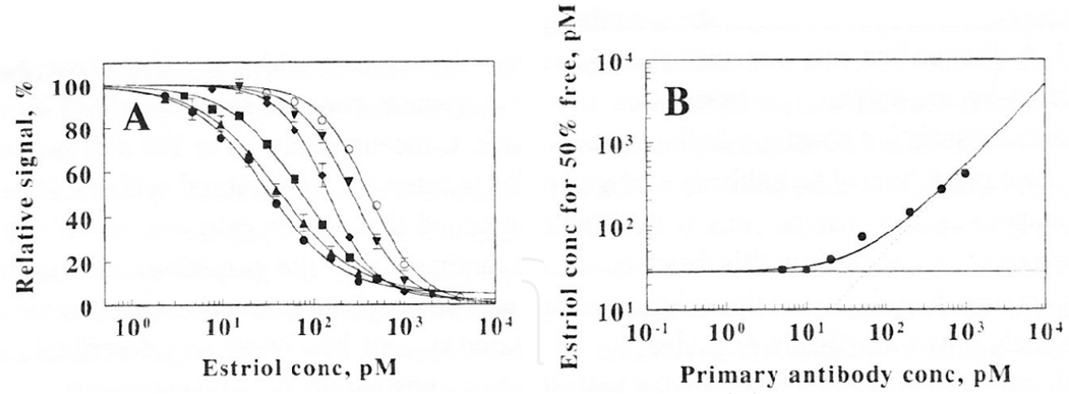


Fig. 3. Detection limit of antibody controlled by dissociation constant, Kd. A; response plotted as a function of estriol concentration. The concentration of antibody are 10(●), 20(▲), 50(■), 200(◆), 500(▼), and 1000(○) pM. B; detection limit of the assay. The y axis shows estriol concentration at 50% of free antibody, while the x axis total antibody concentration. All plots are derived from Fig.A. (cited from Analytical Chemistry vol.73, pp3392-3393,(2001))

reactions occur simultaneously. If these multiple antigen-antibody reactions are mutually independent, we can use multiple antibodies with variety in single immunoassay. It can be applied to detect multiple analyte in an immunoassay or to expand dynamic range of immunoassay, using a mixture of antibodies. In this paragraph, expansion of dynamic range is focused on.

3.1 Combination of plural antibodies

In an ideal antigen-antibody reaction, detection range is within an order of 100. If we use more than two antibodies, we can expand the detection range. The example is shown in Fig.4. Two kinds of antibodies are used to provide individual and combined detection curbs. The measurements were done in solution phase with KinExa 3000 instrument from Sapidyne Instruments Inc.(Boise, ID). A detection curb was made with an anti-estrogen antibody of 10 pM, which has a dissociation constant of 22 pM. It is expressed dashed line with symbol of ○. Another detection curb was made with the antibody of 40pM, dissociation constant of 89pM, shown with symbol △. Then, the two antibodies were mixed and detection curb was made as solid line with symbol of □. Signal % is as the same as the ratio of free antibody. When two antibodies were used independently, the half maximum signals were obtained at 55 pM and 140 pM, respectively. When, these antibodies were used together, the half signal was obtained at 115pM. This shift indicates that two antibodies to same antigen works independently, and the affinity of each antibody is reserved even in a mixture. Because of similar affinities of two antibodies, the overlap of two detection curbs result in little change in dynamic range. Then, the mixture of two antibodies which have large different affinities would give the larger dynamic range. A high affinity of antibodies would bind to low concentration of antigen, whereas a low affinity of antibodies would give a good detection curb at high concentration of antigen. In Fig. 4B, the antibody which has Kd of 3 nM, is used instead of the antibody with Kd of 89pM. It is expressed dashed line with symbol of △. This antibody is sensitive to the antigen in the range between 1nM and

1 μM, whereas the other antibody is sensitive between 10pM and 1nM. Then, the mixture of the two antibodies give the expansion of dynamic range. This detection curb is shown as a solid line with symbol of ○ (Fig. 4B). The dynamic range is the sum of two antibodies. To achieve the expansion of dynamic range, the overlap of two detection curb is necessary. If the overlap does not exist, the mixture of the antibody would not give a continuous change in the detection curb as sum of two detection curbs. Though there are no commercial kits that use more than two antibodies simultaneously, it will be possible to give the expansion of dynamic range. The combinational use of antibody can be also applied for the detection of multiple analyte., This application is not mentioned n this chapter.

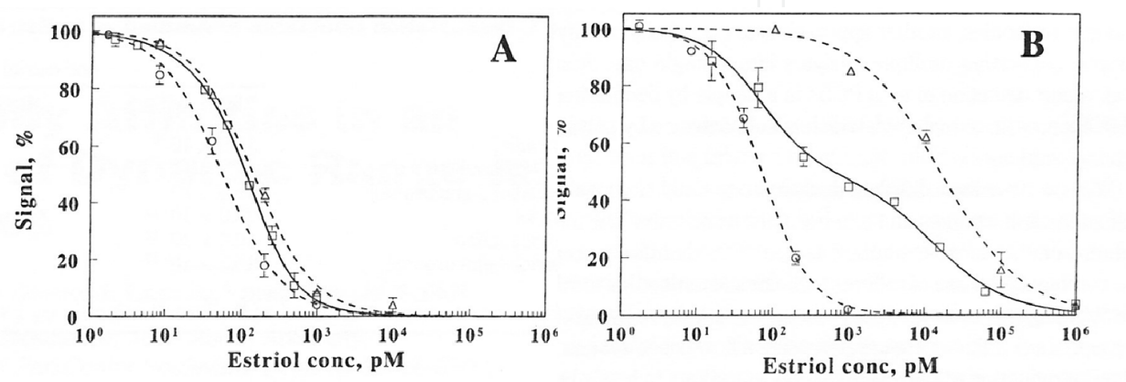


Fig. 4. Coordinative work of two antibodies, A. Two dashed lines are curb with antibody of Kd 22 pM (lefts side, ○, concentration of 10pM) and with antibody of Kd 89 pM (right side, △, concentration of 40 pM). Solid line is a detection curb made by a mixture of them. B. Dashed lines are with antibody of Kd 22 pM (lefts side, ○, concentration of 10pM) and antibody of Kd 3.1 nM (right side, △, concentration of 100 pM). Solid line is a mixture of them. (cited from Analytical Chemistry vol.75, pp104-110,(2003))

3.2 Polyclonal antibody

Polyclonal antibodies can be regarded as a mixture of huge amount of monoclonal antibodies which have different specificity and dissociation constant. If, so, the detection curb would become a straight line in a semi-log graph like a dashed line in Figure 5. This is a speculated detection curb of polyclonal antibody based on an assumption that the distribution of Kd consisted of Bolzman distribution. As shown in previous paragraph, mixing monoclonal antibodies which have different Kd's will give the extension of detection range. The shape and slope of the detection curb depends on the distribution of Kd's of polyclonal antibody. However, actual polyclonal antibodies, which we can obtain from reagent companies, gave the detection curb that was identical to those of monoclonal antibodies. As shown in Figure 5, solid line is a detection curb obtained from a polyclonal antibody. It was identical to the monoclonal antibody which has kd values of 0.1 nM. Probably, this is because of their use in a purified form. Polyclonal antibodies are usually sold after being purified by affinity column chromatography. After that process, the broad range of Kd values of polyclonal antibody may become narrower, though the distribution of kds of polyclonal antibody has not been investigated.

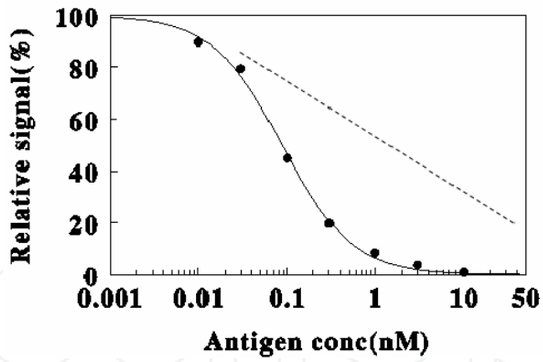


Fig. 5. Detection curb with polyclonal antibody. Dashed line; speculative detection curb under the assumption of that Kd distribution is Bolzman distribution. Solid line: Antibody is Cy5-conjugated AffiniPure Goat Anti-Mouse IgG (Jacson Immuno Research Laboratories, INC.) Code Number:115-175-008, The data was taken with Kinexa 3000 by Dr. Ohmura of Central Research Institute of Electric Power Industry.

4. Inherent error of immunoassay with antibody

For usually immunoassay, an antibody is used as detection prove. In most cases, free antibody concentration is decided by capture with an antigen which are usually fixed on a solid phase. Antibody has two binding sites. We used it under the assumption that they have equal binding affinity, and that each one site does not affect the other when binding with the antigen. Then, we use antibodies in the condition that the concentration of binding site is twice of antibody concentration.

Under these assumptions, the fraction of the binding site which binds to an antigen is demonstrated as F. F can be changed from 0 to 1. The fraction of a free binding site will be 1-F. Since the right side binding site reacts with an antigen as the left side, both site occupied antibody has the fraction of left side times right, i.e. F^2 from a mathematical independence. Therefore, all fractions will be presented both free antibody, right filled antibody, left filled antibody, and both filled antibody as $(1-F)^2$, (1-F)F, F(1-F), and F^2, respectively. The sum of all fractions will be total fraction and 1. From these values, the fraction of the antibodies which is half occupied by antigen, will be calculated by the following two equations, that should give same solution.

Both free and occupied fractions are subtracted from total fraction to give the half occupied fraction/

$$1 - (1-F)^2 - F^2 = 2F - 2F^2 \tag{12}$$

The sum of fractions of either right or left occupied fraction is

$$F(1-F) + (1-F)F = 2F - 2F^2 \tag{13}$$

In an immunoassay, the detection signal is directly reflected to the fraction of free antibodies, the antibodies of half binding site occupied will captured on the solid phase of the detection system by the 50% possibilities when compared with both free antibodies, to give the 50% strength of signal for detection. The ideal condition can be stated as

$$P_{c2} = 2P_{c1} \quad (14)$$

Where P_{c2} presents the probability of capture of antibodies with two binding sites, and P_{c1} the probabilities of capture of half-occupied antibodies.

The above equation shows an ideal probability. This probability, Pc2 can be calculated for divalent antibody in the other way. The probability of capture of a single binding site to the solid phase is defined as P. In this case, the probability of not capturing a site is 1-P. For a bivalent antibody where both sites are not captured, the probability of not capturing can be calculated as left side possibility times right side possibility, i.e., (1-P) x (1-P), or $(1-P)^2$. The probability of bivalent antibody capturing is expressed by subtraction of the possibility of not capturing from total probability:

$$1 - (1-P)^2 = 2P - P^2 \quad (15)$$

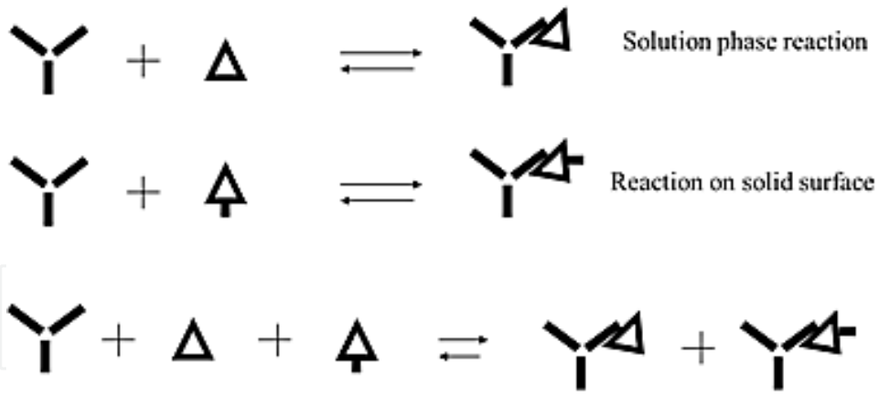
In the equation 15, P is regarded as probability of capturing half-occupied antibody. The probability of capturing antibody with two free sites can be written as follows.

$$P_{c2} = 2P_{c1} - (P_{c1})^2 \quad (16)$$

When compared with equation 14, equation 16 shows the difference from ideal antibody behavior. The term $(P_{c1})^2$ is inherent error on bivalent antibody. This error demonstrates that the probability of antibody is detected at lower level than an actual concentration by possibilities, $(P_{c1})^2$. The meaning of error, $(P_{c1})^2$ is the probability that both sites are captured on the solid phase. To reduce the error, it is necessary that absolute value of Pc1 is small. To reduce P_{c1}, flow detection systems are desired such as SPR and Kinexa. In the flow systems, the contact time of antibodies to the solid phase is limited to less than one second , then the probability of capturing on a solid phase will be decreased to less than 10%. For these systems, the contribution of $(P_{c1})^2$ will be less than 1%. On the other hand, in batch system such as competitive ELISA, the error is inevitable and becomes large. In this case, the detection curb will be far from ideal curb. This error is inherent to bivalent antibodies. On the other hand, monovalent receptors such as receptors of chemicals or fragment of antibody which have only one site for binding are free from the inherent errors.

5. Kinetic exclusion assay

Immunoassay is based on the equilibrium between antibody and antigen. Analyte concentration is usually determined from the concentration of free antibodies which is in equilibrium with antigen. To determine the free antibody concentration, another antigen is used, it is usually fixed on a solid phase. Free antibodies are trapped by the antigens on a solid phase, and then, the concentration of trapped antibody is determined by a secondary antibody which is labeled with dyes for spectroscopy or enzymatic reaction. In this scheme, a simple equilibrium does not exist any more, since there are two kinds of equilibrium. One is based on equilibrium between solution phase antigen and antibody. The other is based on solid phase antigen and solution phase antibody. As a result, a mixture of two kinds of reaction for the same antibody can occur. The ligand exchange can also occur. In this case, the shape and range of detection curb would not become that of an ideal curb.



Y:antibody, △ : antigen, ▲:antigen on solid phase

To avoid the influence of the reaction between antibody and solid phase antigen, it is necessary that flow system is adopted. In the flow system, the mixture of antigen and antibody which is in equilibrium state is drawn to the solid phase where another antigen is fixed on solid phase. The mixture will be exposed on the solid phase antigen in very limited time, so that the influence of the reaction between antibody and solid phase antigen is limited on the solution phase antigen-antibody reaction. Steve, inventor of machine, KinExa, named it Kinetic Exclusion Assay. In the flow system, influence of the reaction between antibody and solid phase antigen would be kinetically exclusive. Then, the equilibrium of original antibody-antigen reaction would be kept.

One of the flow systems is KinExa series (Sapidyne Instruments Inc., ID). The schematic diagram is shown in Fig. 6. The system consists of a capillary flow cell fitted with a microporous screen, which is integrated into an epi-illumination filter fluorometer system and through which flowed selected solutions under negative pressure created by a syringe pump. The beads were packed into the flow cell by drawing a beads' suspension through the cell, washing it by phosphate buffer solution(PBS, pH7) , and allowing it to settle for 15 seconds, thus creating a uniform bed. On a bead, antigens were immobilized to capture antibodies which were present in equilibrated mixtures of antibody, antigen, and antibody-antigen complexes. The mixture was drawn though the beads to accumulate unbound antibody. Excess antibody, antigen, and antibody-antigen complexes were washed out of the flow cell by drawing PBS though the cell. After the wash, Cy5-conjugated secondary antibody solution was drawn though the beads pack to label the captured primary antibody. Unbound secondary antibody was removed by washing the pack with PBS. All steps, including beads preparation, were accomplished though the combined function of a variety of pumps and valves within a computer controlled system. Throughout the experiments, fluorescence was sampled at a rate of one measurement per second, and the data were stored as voltages on a computer.

Typical fluorescence signals, along with schematic diagrams of the binding events occurring during each assay step, are illustrated in Figure 7. In the first step, the binding mixture was drawn through the beads pack, capturing unbound antibody, and then excess primary antibody and antigen were removed by a buffer wash. The fluorescence signal recorded during this period provided the base line fluorescence response of the beads pack prior to the addition of fluorescent tracer. In the second step, the steepest portion of fluorescence trace was elicited when Cy5-conjugated secondary antibody was drawn into the flow cell. This signal reflects the presence of both unbound secondary antibody filling the interstitial regions

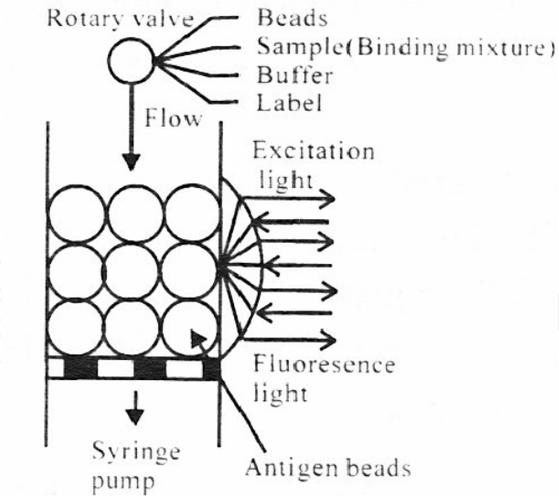


Fig. 6. Schematic diagram of an automatied immunoassay instrument. The system consist of capillary flow cell fitted with a micro porous screen, where micro beads are packed. Flow cell is illuminated by excitation light. Fluorescence light is used to detect free antibody which is tapped on a beads surface and labeled by secondary sntibody. (cited from Analytical Chemistry vol.75, pp104-110,(2003))

between the beads and that bound to the primary antibody. In the third step, unbound secondary antibody was washed out of the cell with PBS, thus enabling quantification of remaining antibody bound in the beads pack. The difference in fluorescence intensity between the base line and the plateau phase was considered to present the bound signal.

With Kinexa 3000, the kinetically exclusive assay was investigated. effects of reaction of solid phase were investigated on the equilibrium between antigen and antibody. For the theoretical detection curb, binding of the free antibody to the immobilized ligand does not lead to a shift in the equilibrium between the antibody and antigen during the detection, and the binding of the immobilized antigen and soluble one should be mutually exclusive. The beads pack provided high ligand capacity, which lead to that the same percentage of antibody is captured on the ligand of the beads regardless of absolute concentration of free antibody. As shown in Fig7, when antibody concentration became higher, the captured amount of antibody became larger. However, the percentage of the antibody captured remained constant. The average amount of the captured was 1.4%. The binding of free antibody to the immobilized ligand during the limit contact was a pseudo-first-order process as a result of excess solid phase ligand. The small percentage of capturing free antibody ensured that the perturbation of capturing the free antibody on the equilibrium between antibody and antigen would be negligible. The equilibrium dissociation constant(Kd) is expressed as dissociation rate constant by association rate constant. If dissociation constant (Kd) is 10^6, it means that the dissociation rate is 10^6 times slower than the association rate. The small percentage of capturing free antibody also indicates that any shift based on the very slow dissociation of antigen-antibody complex would not occur in the equilibrium in a brief contact time, the contact time was 480 ms for the lowest flow rate with KinExa 3000. This brief contact time and kinetic slower rate also inhibits competition reactions. This measurement method provided that the perturbation reactions are kinetically exclusive, and that immunoassay based on theoretical equilibrium can be carried out.

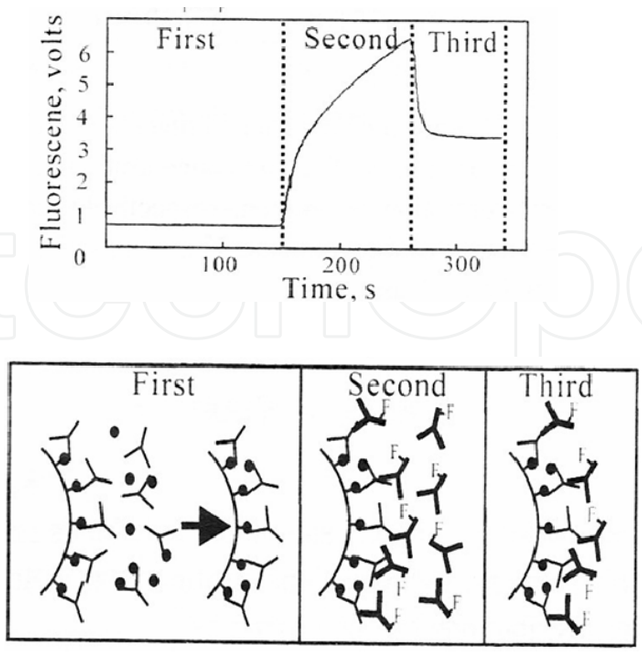


Fig. 7. signal transformation in immunoassay. Upper: change of fluorescence intensity during the three assay steps; step1, an equilibrated mixture of antigen and primary antibody is drawn through the beads pack, followed by buffer wash; step2, Cy5-labelled secondary antibody fills the beads pack and binds to the primary antibody bounds to the beads; step3, excess secondary antibody is removed by buffer wash, and remaining fluorescence intensity is recorded as the response signal. Lower: schematic images depicting events at each step. (cited from Analytical Chemistry vol.75, pp104-110,(2003))

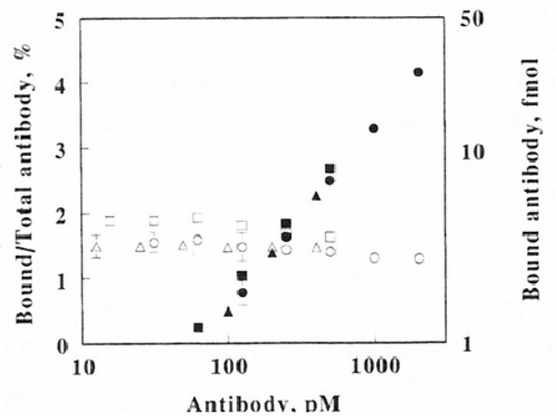


Fig. 8. small ratio of captured free antibodies. Cy5-labelled anti-estriol antibodies (1ml, each) at various concentrations were drawn through the flow cell containing beads coated with BSA-conjugated estriol at 0.25mL/min. Closed and open symbols were shown as a portion of the bound antibody in the entire beads pack and the percentage of antibody bound, respectively. Two different Cy5-labelled antibodies were used. They were shown as triangle and square. (cited from Analytical Chemistry vol.75, pp104-110,(2003))

6. Summary

In this chapter, an ideal detection curb was mentioned. The ideal curb was identical to the curb based on the equilibrium between antibody and antigen. From a view point of analysis, ideal detection curb is not always necessary. For instance, ELISA system always provide the detection curbs which is far from ideal one. Though the detection curb is far from ideal one, the curbs is practically usefull for an analysis and the reproductivities of the analytical data are always obtained. The system fulfilled the elemental requirements of chemical analysis. The ideal curb is especially important for kinetic analysis of antigen-antibody reaction, or determination of dissociation constants. For immunoassay, ideal detection curb is desirable for the accurate detection and for the detection low antibody levels. On the other hand, when unexpected side reaction such as matrix effects or cross reaction is involved in an immunoassay, it will appear as the change of shape with the detection curb from the ideal curb. Ideal detection curbs are important not only for theoretical analysis of antibody-antigen equilibrium, but also for accurate detection of analyte in an immunoassay.

7. Acknowledgement

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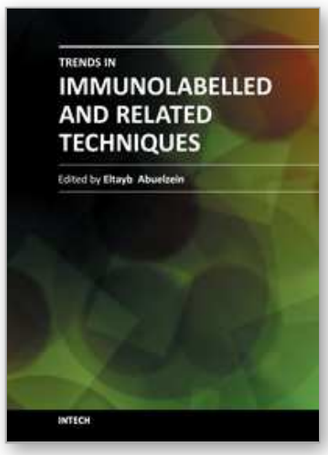

Trends in Immunolabelled and Related Techniques

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The book is coined to provide a professional insight into the different trends of immunoassay and related techniques. It encompasses 22 chapters which are grouped into two sections. The first section consists of articles dealing with emerging uni-and-multiplex immunolabelled methods employed in the various areas of research. The second section includes review articles which introduce the researchers to some immunolabelled techniques which are of vital significance such as the use of the conjugates of the Staphylococcus aureus protein "A" and the Streptococcus Spps. protein "G" in immunolabelled assay systems, the use of bead-based assays and an overview on the laboratory assay systems. The book provides technological innovations that are expected to provide an efficient channel for developments in immunolabelled and related techniques. It is also most useful for researchers and post-graduate students, in all fields, where immunolabelled techniques are applicable.

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