ELISA -A to Z .....from introduction to practice

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Contents.

I. ELISA is an immunoassay method

ELISA (Enzyme-linked immunosorbent assay) is one of immunoassay method using antibodies to capture an antigen and an enzyme labeled antibody to estimate the amount of antigen.

Qualitative detection and quantitative measurement

We sometimes express the presence of a particular substance simply as “+” or “-”. This expression is called qualitative detection and means that the substance is present more than detection limit or less, and the judgment or conclusion is dependent on the sensitivity of the detection method or the certain level set by organization for safety. Quantitative measurement, on the other hand gives information about “how much”, and this kind of information is necessary for more detailed and accurate statistical judgment.

What is immunoassay?

The term “immunoassay” is a combined term of “immuno” (= immunological, practically immunochemical antigen-antibody-reaction) and “assay” (= determination of the purity of a substance or the amount of any particular constituent of a mixture according to Dorland Medical Dictionary). So, immunoassay means a method to measure any particular substance in a mixture using its specific-binding antibody. One of the merits of immunoassay is that we can measure a substance that is present in a mixture of various contaminants, for example, one constituent of blood without any purification process.

Important components in immunoassay

Antibody (antiserum)

In immunoasay, we use antibody as a binding reagent of high specificity and high binding ability, that is, high affinity to the substance to be measured.

Antibody against a particular substance is obtained by immunizing an animal (e.g. rabbit) with the substance. Some substances easily cause antibody production with a minute amount, while others cannot produce antibodies easily, and need the help of an adjuvant like Freund's complete adjuvant that helps immune response.

Antigen and hapten

We call substances that can produce antibody and can bind the antibody "antigens". Those antigens have generally large molecular sizes over 1000 Dalton. Substances with smaller molecular sizes cannot produce antibody by itself, but they can bind antibody if produced. Those substances are called "haptens". In order to get antibody for hapten, it has to be coupled with some carrier proteins of large molecular size. We can get antibody by immunizing an animal with such "immunogen" Antibody appears after the first innoculation is IgM-type which is a pentamer of the basic component of antibody. This type of antibody changes to IgG-type after the repeated innoculations. This is called "class switch", and mostly IgG type of antibodies are used in immunoassay.

Antibodies are also called "immunoglobulins" from their chemical nature, and the basic molecule is composed of two heavy (H) chains and two light (L) chains, molecular weighs are 50,000-70,000, and 23,000, respectively. Immunoglobulins are classified from the structure of H-chain as IgG, IgM, IgA, IgD and IgE. L-chains are classified into 2 types, lambda and kappa. IgG, IgD, and IgE are of basic structure and molecular weights are 150kDa, 170-200 kDa, and 190 kDa, respectively. IgA is a dimer type with the molecular weight of 390 kDa, while IgM is a pentamer type of 900 kDa.

A certain area located at one end of H-chain and L-chain is called variable region (V_H, V_L) because of the variability in amino acid sequences within the same class, while the rest of the chain is called constant region (C_H, C_L). The variable regions are though to be the place for recognition and binding with the corresponding epitope (determinant) of antigen...

Polyclonal antibody and monoclonal antibody

Because of high molecular size of antigen, an antigen generally has several regions (antigenic determinants or epitopes) against which antibody is formed and bound. So, immunization with an antigen caused the production of several antibodies each recognizes and binds different epitopes of the antigen. This means that antiserum we get is a mixture of these antibodies. We call such set of antibodies polyclonal antibody.

With special techniques, we can obtain an antibody that recognizes single epitope in antigen. For example, after immunization of a mouse with an antigen, we take out its spleen, and disperse the antibody producing cells. We fuse these cells with mouse myeloma cells, and dilute the hybridized cells (hybridomas) to a single hybridoma/well and culture them. If cell

culture is successful, the single clone of hybridoma will produce an antibody that recognizes single epitope, and we can get enough monoclonal antibody by trasplantation of such single clone to mouse abdominal cavity. Because a monoclonal antibody recognizes only one place of the antigen, by selecting two monoclonal antibodies, we can easily capture an antigen at two epitopes located at the places we want, for example, at A-chain and B-chain of insulin.

Affinity of an antibody to the corresponding antigen is expressed by association constant (Ka) or dissociation constant (Kd).

When angen and antibody are mixed, they will bind to form antigen-antibody complex.

$$Ag + Ab \Leftrightarrow Ag\text{-}Ab$$

This reaction is reversible, and antigen-antibody complex will separate to each component.

Association constant is defined as:

$$Ka = [Ag\text{-}Ab] / [Ag][Ab]$$

Where $[Ag\text{-}Ab], [Ag], [Ab]$ are concentrations of components expressed in M at the equilibrium state. Kd is defined as 1/Ka

Association constant of antigen-antibody reaction seems to be very large, as shown in the table below.

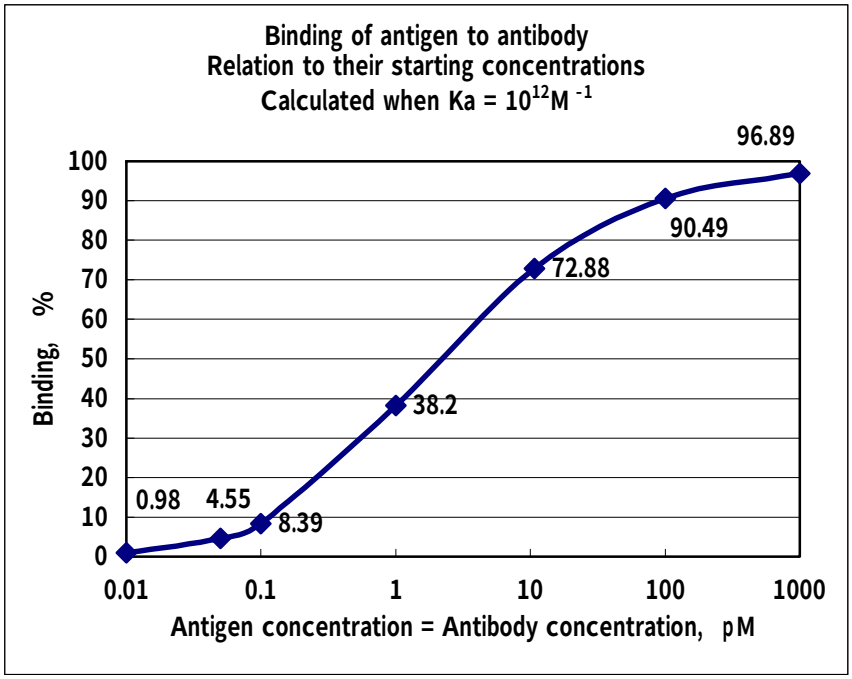
Some instances of affinity of antibodies, hormone receptors, and binding proteins

Binding agents	Association constant (Ka), (M^{-1})	Dissociation constant (Kd) (M)
Antibody		
Anti-ovine LH	1.5×10^{11}	6.6×10^{-12}
Anti-human FSH	4×10^{10}	2.5×10^{-11}
Anti-insulin	5×10^{11}	2×10^{-12}
Receptor (R)		
Estrogen R	10^{10}	10^{-10}
Rat ovarian FSH-R	1.3×10^{10}	7.2×10^{-11}
Testicular FSH-R		
Rat	1.5×10^{8}	6.7×10^{-9}
Rat	1.3×10^{9}	7.8×10^{-10}
White crowned sparrow	1.3×10^{9}	7.8×10^{-10}
Japanese quail	2.4×10^{8}	4.1×10^{-9}
Domestic fowl	6.7×10^{8}	1.5×10^{-9}
Binding protein		
cAMP binding protein	5×10^{8}	2×10^{-9}

(Here, antibodies are polyclonal. Affinity of monoclonal antibody is told to be less than polyclonal antibody.)

However, if concentrations of both antigen and antibody are very low, for example both of them are as low as 1 pM, the binding rate will be only 38% even if association constant is as high as 10^{12} M^{-1}. (If molecular weight of antigen is 30,000, 1pM is 30pg/ml) as shown the figure below. When we think about blood levels of hormones (around 1-10ng/ml), binding reaction will occur at such concentration, and we can understand that the affinity of antibody

is a very important factor.



Standard preparation

Standard preparation is necessary for immunoassay. Using a standard preparation, we draw a standard curve from graded reaction results of various standard concentrations, and by comparison of a sample reaction result with the standard curve, we get assay value of the sample. Assay results are expressed by either absolute amount of the target substance, such as weight and concentration, or comparative amount such as potency (biological unit, enzymatic activity, and officially defined international unit).

Even if the absolute amount of a substance is required, purity of the standard preparation is not necessarily requested if the assay results are not expressed by the amount of the standard preparation employed. If the purity of the standard preparation, i.e., the content of the pure substance in the standard preparation is known, it is enough to be used in assay because assay results can be expressed in terms of pure substance. Sometimes, highly pure preparation is unstable and easily denatured or inactivated, and sometimes might be lost by adsorption to the wall of a container. In such case, some protective substances are indispensable.

Very often, an international organization, like WHO, issues standard preparations in which the amount of the substance to be measured is expressed as International Unit (IU) per vial. This IU does not necessarily express the amount pure substance, and in many cases IU is defined, for example, from the biological potency. So, if this kind of preparation is used, assay results will be expressed like IU/ml, IU/mg, etc.

If a target substance has small molecular size and highly purified preparation is easily obtained and is stable, it would be easy to use such pure substance as a standard preparation, and assay value is expressed like ng/ml, ng/mg, etc.

Labeling materials

In immunoassay, it is necessary to use any marker to know the antigen-antibody binding. For such purpose, we label either antigen or antibody with some materials that do not interefere with the binding. We use radioisotopes, enzyme, fluorescent substance like FITC, chelates of lanthanide elements, and so on, as labeling materials. These are also very important factors in setting up immunoassays.

Beginning of immunoassay…Radioimmunoassay (RIA)

In 1950's Solomon A. Berson and Rosalyn S. Yalow worked on the metabolism of insulin in the blood of diabetic patients to clarify the reason for insulin deficiency. Their hypothesis was that in diabetic patients, insulin might be decomposed by some factor(s) in the blood. So, they labeled insulin with iodine-131 and incubated with sera of diabetic patients. After incubation, the reaction mixture is analyzed by paper-electrophoresis. The radioactive insulin, incubated with control serum obtained from normal human subject, showed a single peak of radioactivity, while that with diabetic serum showed 2 peaks of radioactivity. After examinations, they concluded that the radioactive insulin bound with antibodies which had been formed in diabetic patient by long term therapeutic treatment of the patient with porcine insulin.

Yalow wrote in "Principles of Competitive Protein-Binding Assays"、 Ed. Odell & Daughaday, J.B.Lippincott Co., pp.1-21, 1971,

"…we studied the metabolism of ^{131}I-labeled insulin in diabetes and made the discovery that virtually all insulin-treated diabetics had insulin-binding antibodies.

Our attempts to disseminate this information may be of some interest. The first journal to which we submitted the paper rejected it after many months with a comment by a referee to the effect that everyone knows that insulin does not make antibodies. We were able, however, to present the work before the Society of Nuclear Medicine at its first annual meeting in Portland, Oregon in June, 1955. after which the Seattle group under Robert Williams provided confirmation. "

Their first publication was:

"Insulin-I^{131} metabolism in human subjects: Demonstration of insulin binding globulin in the circulation of insulin treated subject.

Berson, S. A., Yalow, R. S., Bauman, A., Rothchild, M. A. and Newerly, K.

J. Clin. Invest. 35, 170-190, 1956

They found that the radioactivity of the antibody-bound spot decreased by addition of non-radioactive insulin, while radioactivity of free insulin spot increased, and the ratio of bound to free insulin decreased in hyperbolic manner with non-radioactive insulin added. They noticed that insulin could be measured by employing this fact. After various detailed examinations, they published a report where radioimmunoassay (RIA) was first established.

Quantitative aspects of the reaction between insulin and insulin-binding antibody.

Berson, S. A. and Yalow, R. S.

J. Clin. Invest. 38, 1996-2016, 1959

In the presence of fixed amounts of anti-insulin antiserum and iodine-131 labeled insulin, non-radioactive insulin added to this system binds anti-insulin antibody in competition with the labeled insulin if the amount of the antibody is relatively small enough. In the absence of non-radioactive insulin, the amount of radioactive insulin bound to antibody, B/F is maximal where B and F are radioactivity of bound and free insulin, respectively. If the amount of non-radioactive insulin increased, the specific radioactivity of the insulin mixture (radioactive and non-radioactive) is decreased by isotope dilution. So, if the amount of antibody-bound insulin remains unchanged, B/F would show a hyperbolic curve. Very often B/Bo (Bo means radioactivity in the absence of non-radioactive insulin) is also used instead of B/F. Using this expression the standard curve will be expressed as $Y = A / (X+A) \times (b/bo)$

A: concentration of radioactive insulin (or radioactive ligand)

X: concentration of non-radioactive insulin (or non-radioactive ligand)

bo: concentration of antibody bound insulin (or ligand) when $X = 0$

b : concentration of antibody bound insulin (or ligand) when $X \neq 0$

Because the antigen-antibody binding is a reversible reaction and the amount of antigen bound to a fixed amount of antibody increases with increasing amount of antigen, b/bo will increase if X increases, and the extent of increase is inversely related to Ka. This member b/bo should be called “binding-increasing effect”, on the other hand $A / (A+X)$ will decrease if X increases, and this member should be called “isotope dilution effect”. The sensitivity of the standard curve always worse than the curve $Y = A / (A+X)$ due to b/bo, and the shape of the standard curve apparently looks like hyperbolic but strictly, not.

The principle of RIA is called “competitive binding” which is the first principle used in immunoassays. The assay methods using competitive binding principle are called “competitive assays”, and those methods in which radioisotope are used are called “competitive radioassays”.

In competitive assays, association constant Ka influences b/bo (larger Ka causes lower b/bo, to make sensitivity better), and the amount of antibody used in the assay also influences b/bo (larger amount of antibody increases b/bo causing bad sensitivity). Amount of labeled antigen influences $A/(A+X)$, and large amount of labeled antigen minimize isotope dilution effect, moving the standard curve to the right.

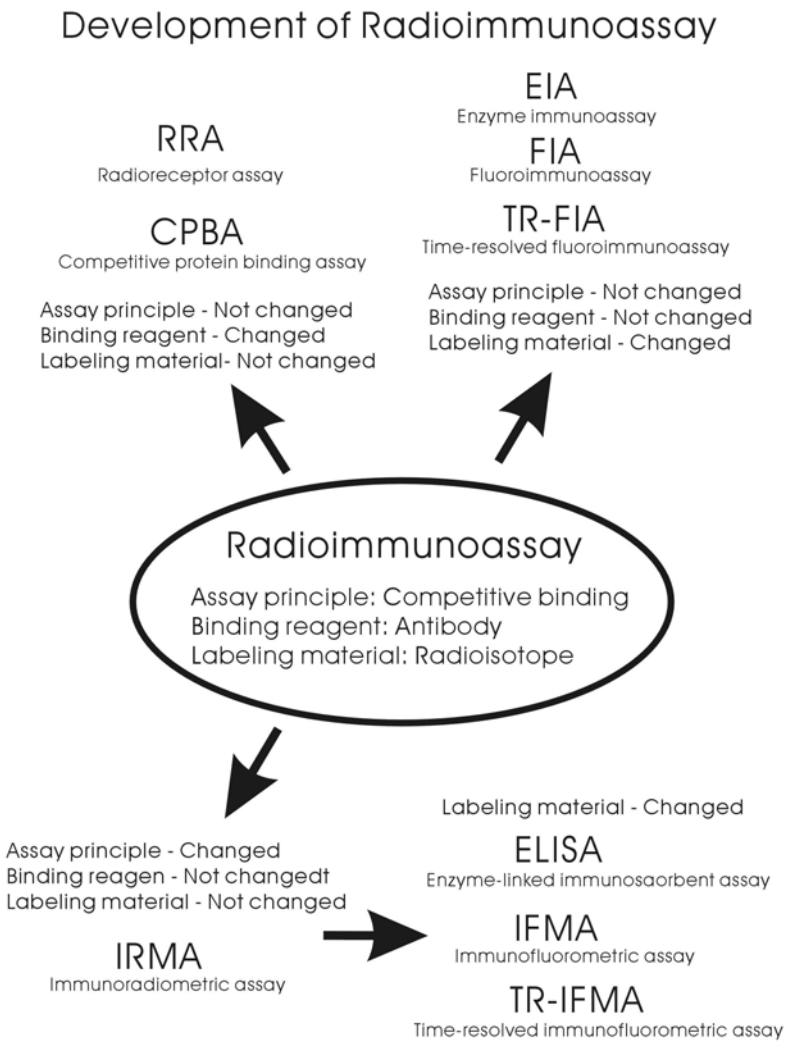
The sensitivity of insulin radioimmunoassay was enough to measure circulating insulin levels. So, many scientists started to apply this method to various other hormones. In RIA, highly purified antigen is necessary for antiserum production and for radioiodination. RIA systems in 1960's were established mostly for hormones of animals such as sheep, pig and bull. Use of antibody caused some difficult problems because of the strict specificity of antibody, that is, species specificity. One RIA system established for a hormone of one species of animal,

in many cases, cannot be applied to the same hormone of other animal species. It took some years until most human hormones came to be measured by RIA. Significance of measurement of human hormones in blood has been so great both in clinical diagnosis and clarification of endocrine physiology. Yalow was awarded the Novel prize in 1977.

Development of immunoassay

Since the establishment of radioimmunoassay, other assay methods have been searched for by changing the components of RIA, namely assay principle, binding reagent, and labeling material.

It has been very difficult to find alternative binding reagents, and as the results of such efforts, RRA (radioreceptor assay) and CPBA (competitive protein binding assay) were established where only antibody has been replaced for hormonal receptor and binding protein, while assay principle and labeling material remained unchanged. But affinity of these reagents for target substances were proved to be not enough (see those data shown in the table in antibody section), and good sensitivity was not obtained. So, they failed to be popular



assay methods. RRA, however, was found to be useful in analyzing hormone receptors.

Next efforts were finding of proper labeling materials other than radioisotope without changing assay principle and binding agent, antibody. Radioisotope has many merits such as simple labeling procedure, easy counting of radioactivity, and simple assay procedure, however, it needs a special laboratory from the point of protection from radiation exposure, though very small, and short half-life of radioisotope (^{125}I: 60days, ^{131}I: 8 days, ^{3}H: 12years). Tritium has a comparatively long half life, and "long half life" means "low specific radioactivity", resulting in inferior assay sensitivity. In some countries, like Japan, regulation for radioisotope is very strict. As the results of searching for a good labeling materials, there found enzymes, fluorescent compounds, lanthanide elements, luminescent compounds, spin reagents (free radicals) and so on.

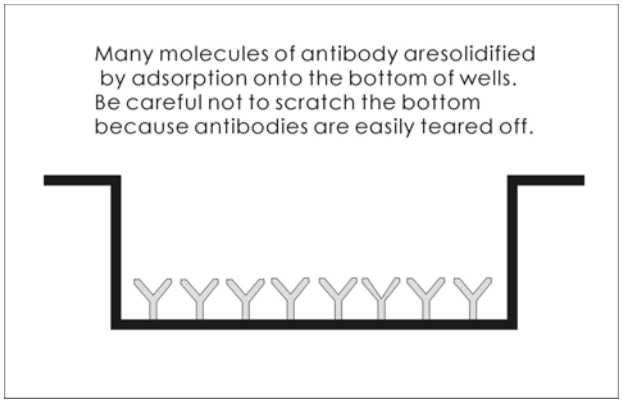
A crucial defect of competitive assays is that the antigen-antibody binding has to be done at very low concentrations of both antibody and antigen (including labeled and unlabeled antigen). So, the sensitivity is quite limited, and also assay variation is large in lower area of the standard curve.

Different assay principle has been also a target of researchers, and a second assay principle, sandwich binding principle, depends upon multi-valency of high molecular size antigen. If an antigen is large enough and has two epitopes, this antigen can be caught by two different antibodies which bind to different epitopes, and the complex looks like sandwich, a slice of ham (antigen) between two pieces of bread (antibody).

The assay method using this principle without changing labeling material and binding reagent is IRMA (immunoradiometric assay), and as in the case of RIA, labeling has been made with enzymes, fluorescent compounds, lanthanide elements, etc., as shown in the figure. This principle has possibility to be more sensitive than competitive binding principle.

II. What is ELISA?

The name ELISA derived from enzyme-linked immunosorbent assay. This assay method utilizes enzyme as a labeling material, and solidified antibody to capture target antigen.



In ELISA a plate with 96 wells (well-plate) is used, and wells are coated with antibodies. These antibodies are called "capture antibodies", the role of which is to capture the target antigen molecules in the sample. Coating is carried out by adsorption on the surface of bottom area. The well-plate is made of polystyrene which is modified for highly efficient adsorption. Because the concentration of antibody is related to

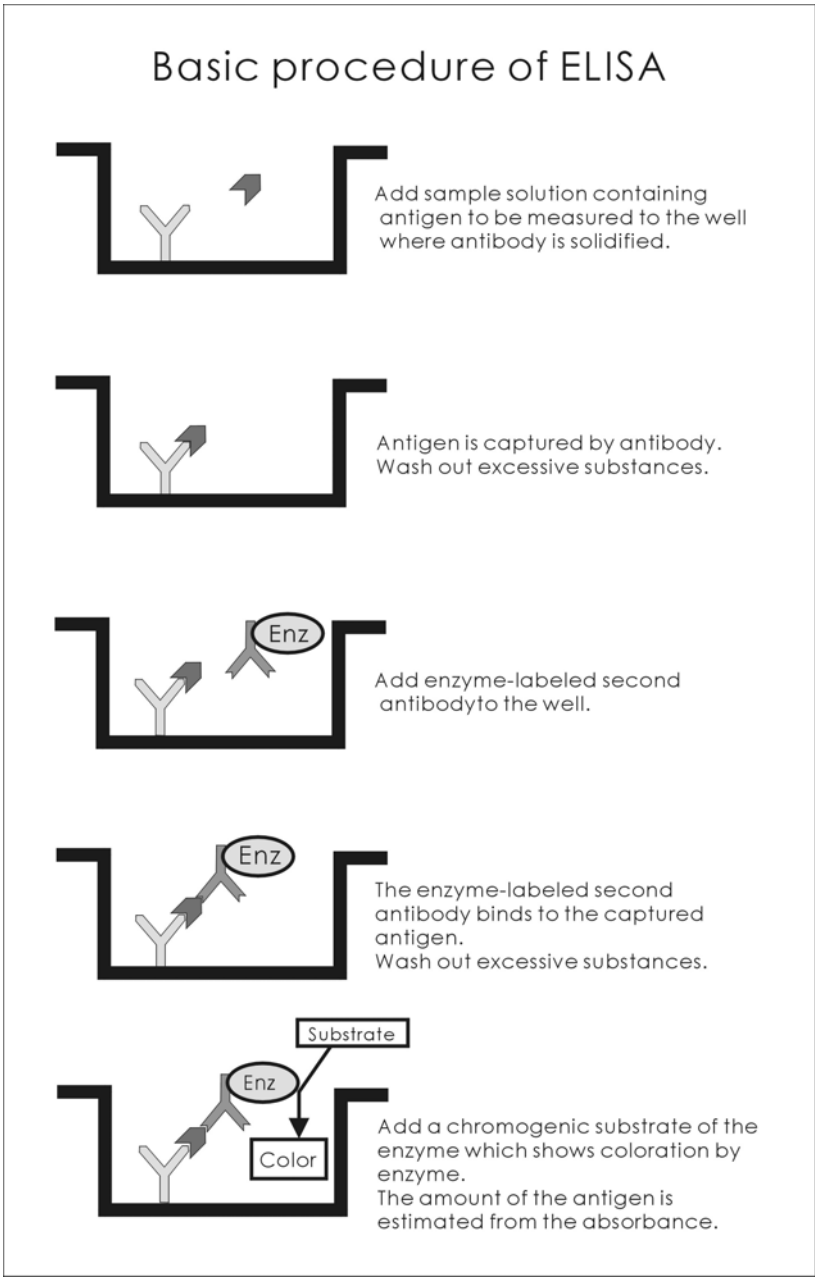
efficiency of capturing antigen for excellent sensitivity, antibody preparation is used as IgG fraction, or monospecific antibody fraction obtained by an affinity chromatography rather than crude gamma-globulin fraction prepared by ammonium sulfate fractionation.

A basic procedure of ELISA (See the illustration below)

1) Standard solutions or assay samples are added to the antibody-coated wells, and incubated for several hours so as to the antigen molecules are captured by “capture antibody”.

2) After this binding reaction, the reaction mixture is discarded, and wells are washed to remove excessive materials.

3) The second antibody which recognizes another epitope in antigen is added. This second



antibody has been labeled with an enzyme such as horseradish peroxidase (HRP).

4) The enzyme-labeled second antibody will bind to the antigen which is bound to the capture

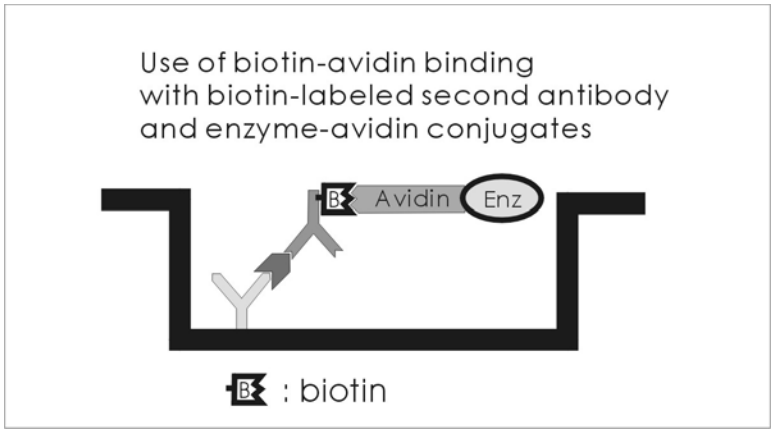
antibody on the bottom area of wells. This means that the enzyme (HRP) is also fixed on the bottom of wells. The amount of the antigen captured is proportional to fixed enzyme.

5) Enzyme activity is measured by adding a chromogenic substrate of this enzyme. In the case of HRP, tetramethylbenzidine (TMB) is often used. After incubation for some period, the chromogenic substrate is changed to a colored product. The reaction is stopped by adding a reaction stopper, e.g. diluted sulfuric acid, and absorbance is measured using a plate reader.

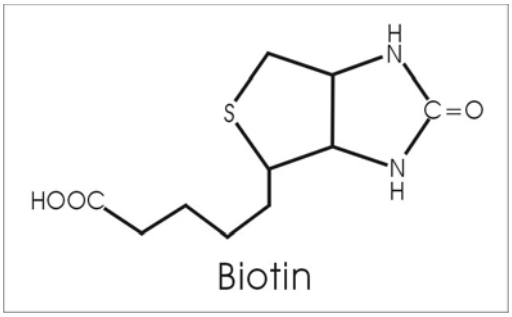
6) The standard curve is prepared from the concentration of standard solutions and their absorbance. And the sample assay values are obtained from the absorbance using the standard curve (calibration curve).

A modified procedure of ELISA using biotin-avidin binding

As the molecular size of enzyme is very large, sometimes the enzyme labeling will interfere with antigen-antibody binding reaction. To avoid such interference, the second antibody is often labeled with a very small molecular substance, biotin (MW=244.31), and a specific binding protein for biotin, avidin is conjugated with enzyme such as HRP. So, the final reaction product of this type of ELISA is shown below.



Note: Biotin and avidin



Biotin: A kind of growth factor present in every cells and belongs to vitamin B complex, and is also called vitamin H. As it acts as co-factor of various enzymes related to carboxylation reactions, it is also called coenzyme R. Biotin is abundant in liver, kidney, pancreas, yeast and milk. It is important in fatty acid and carbohydrate metabolism, and its deficiency causes skin lesion.

Avidin: A basic glycoprotein present in raw egg white. Produced in oviducts of avians and amphibians. It is tetramer of essentially same single chain subunit of 128 amino acids

(N-terminal amino acid is alanine and C-terminal glutamic acid). Molecular weight is about 66,000. It is destructed by heat treatment (cooking) and irradiation.

Avidin binds firmly with biotin and inactivates it. Each subunit can bind one biotin. The dissociation constant, Kd, is 10^{-15}M. Feeding of a large amount of egg white to rat and chicken makes biotin deficient and causes skin lesion and growth retardation. This effect is reversed by biotin administration.

Enzyme and chromogenic substrate

In Shibayagi's ELISA kits, horseradish peroxidase (HRP) is used as an enzyme for labeling second antibodies or avidin.

Horseradish peroxidase, HRP	
Reaction	Chromogenic substrate + H_2O_2 ⇒oxidized chromogen+H_2O
Origin	Horseradish
Molecular size	40,000
Optimal pH	pH 6.5
Substrate	Peroxides: H_2O_2, CH_3OOH, C_2H_5OOH
	No specificity for chromogenic substrate as hydrogen donor
Inhibitors	CN^-, S^{--}, F^-, N_3^-
Stability	Several years in dried, and cooled state. ~1 year in solution

ELISA for measurement of antibody

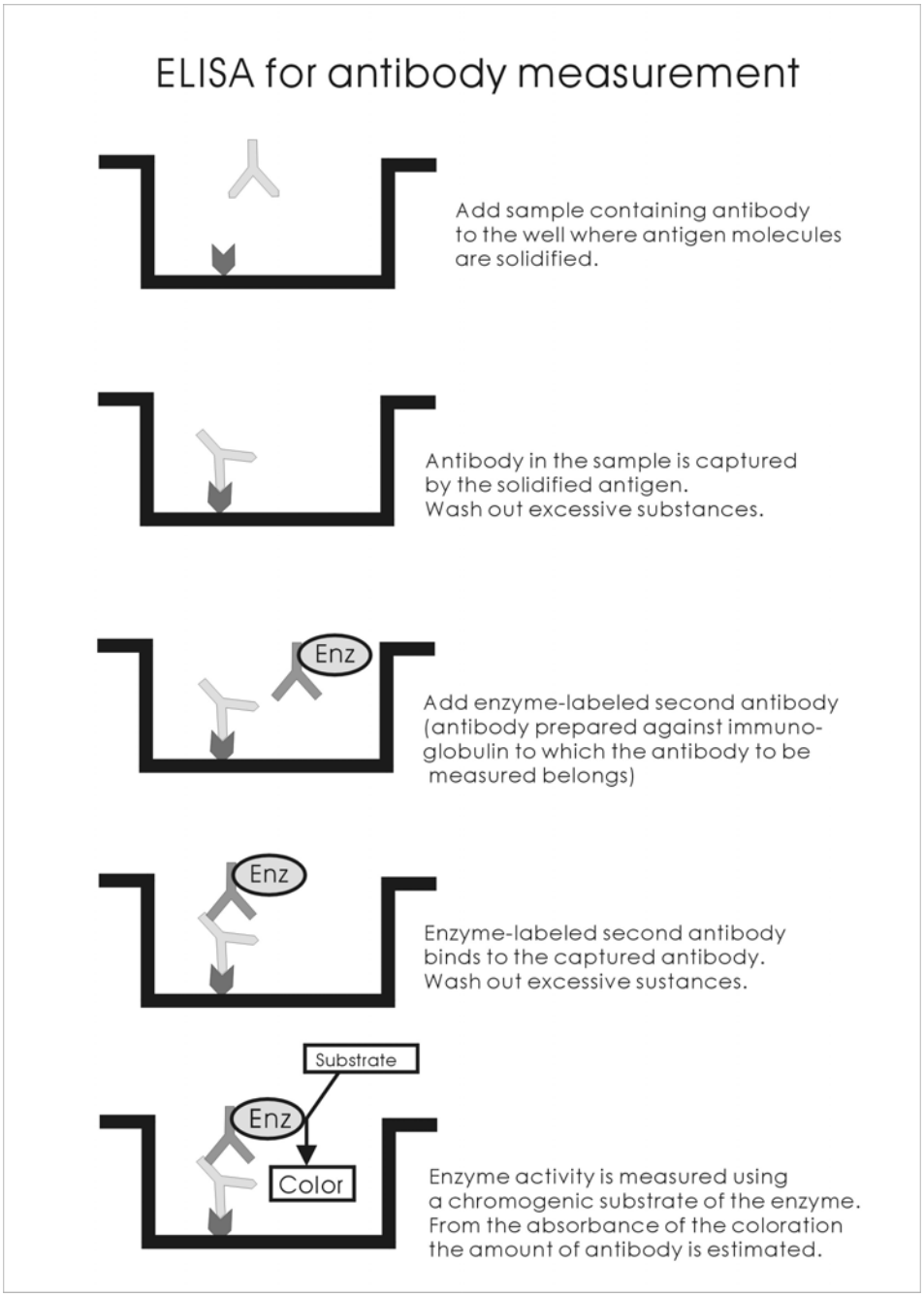
ELISA system can be also set up for antibody measurement. As shown in the figure.

In this system, antigen molecule, specific to the antibody to be measured, is adsorbed on the bottom of wells, and samples containing antibody are added to the wells.

For estimation of the captured antibody, the second antibody* labeled with enzyme is added and washed out after the binding reaction. Then by incubation with chromogenic substrate, the coloration is measured from the absorbance.

Various modifications have been made on ELISA systems to fit the requests of researchers.

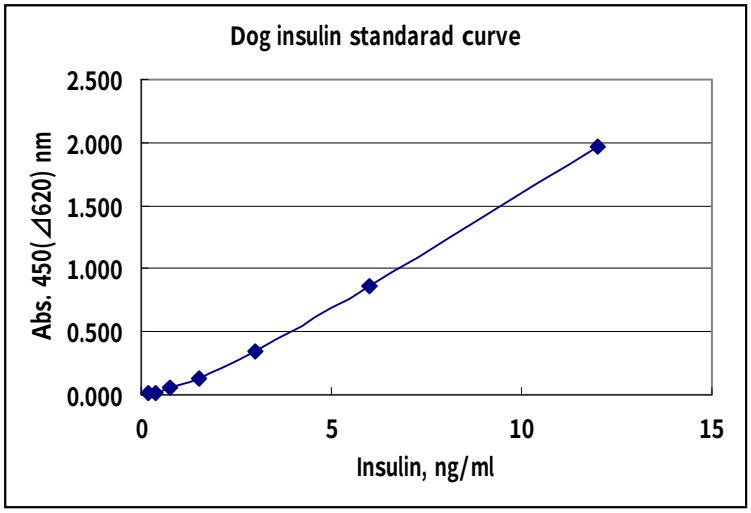
Sandwich binding principle cannot be applied to small molecular substances, haptens such as steroids, olgo-peptides, neurotransmitters, etc. Small molecular substances cannot have plural antigenic determinants to form sandwich. For these substances, competitive assay with an enzyme is applied. Sometime it is called competitive ELISA. But the author thinks that EIA (enzyme immunoassay, or enzymoimmunoassay) would me proper because of its competitive binding principle like RIA (radioimmunoassay).



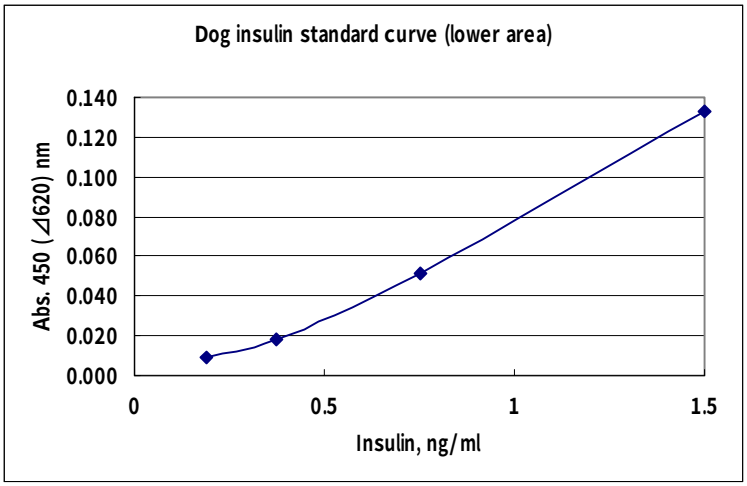
III. Standard curve of ELISA

1. Shapes of standard curves depending on scales in X- and Y-axes.

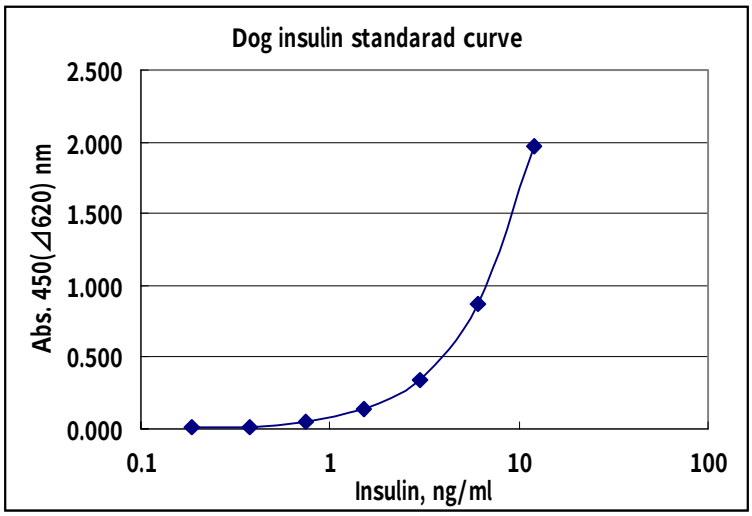
Standard curve of ELISA prepared by plotting standard concentration on X-axis and absorbance on Y-axis, both in normal scale, looks like a linear line except for lower concentration area. In much higher concentration area, it becomes a curve linear with gradually decreasing slope (not seen in this figure). But this standard curve is very inconvenient, because the lower standard points are very close. Especially in manual reading is impossible in this area. Here I show an example of insulin ELISA kit.



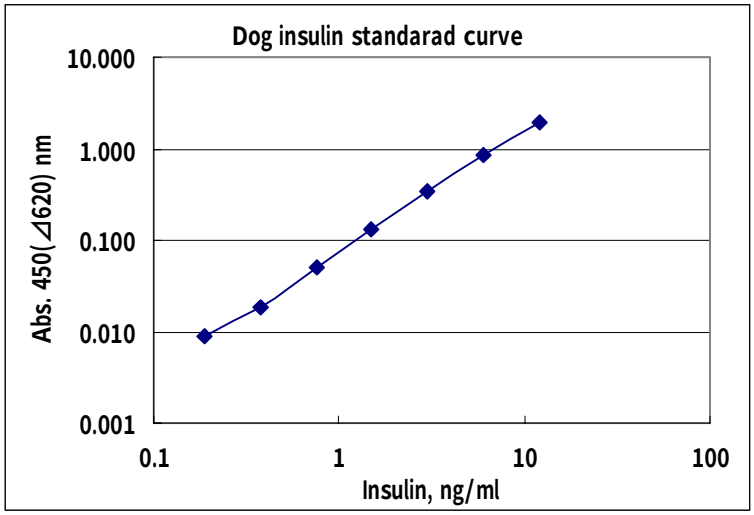
If you plot only lower concentration area, you can see the detail of the standard curve, which is shown below. But still the lowest part is rather difficult to use for manual reading.



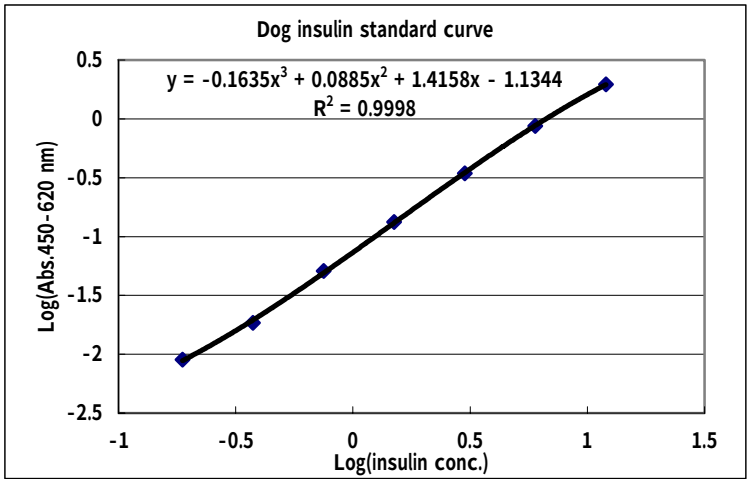
If the scale of X axis is changed to logarithmic scale as in the figure shown below, all the standard points become distinguish, however, manual reading is still difficult owing to the distances in Y-axis are close in low concentration area.



When both X- and Y-axes are transformed to logarithmic scales, the standard curve looks nearly linear, though still curve linear strictly. By such plotting all the standard points are located with enough intervals with regard to both axes, and also third order regression fits very well due to the slight curve. Manual reading is also easy.



But in EXCEL, simple change of to logarithmic scales is not suitable for calculation of regression, because data themselves are not transformed to logarithm in EXCEL. You can only see how the standard curve looks like. If you try to get regression equation without transformation, you will obtain very poor results.



In order to get a best-fit third order regression equation in EXCEL, firstly transform standard concentration and absorbance to logarithmic value, then draw the curve. As you can see in the above figure, you can obtain a third order regression equation with excellent fitness to all the standard points (please, see R^2 in the figure). . For calculation of sample assay values, refer to the part "VI. How to calculate ELISA assay value by EXCEL" on page 38.

2. Influence of affinity and amount of capture antibody on standard curve

Here I would like to try to simulate ELISA standard curve using a very simple hypothesis

that antigen-antibody binding is performed in solution (not solidified system), and that antigen and antibody bind at 1 to 1, following the low of mass action as is described in former section “Important factors in immunoasssay”. Though, in ELISA, antibody is fixed in on the wall of wells, at least this simple postulation is useful in understand how standard curve changes depending on basic factors, association or dissociation constant, and capture antibody concentration. Later on, I will show what’s going on practically.

In the former section, log-log transformed standard curve looks a slight sigmoid curve. Why is not it linear?

From the equation of antigen-antibody reaction, below,

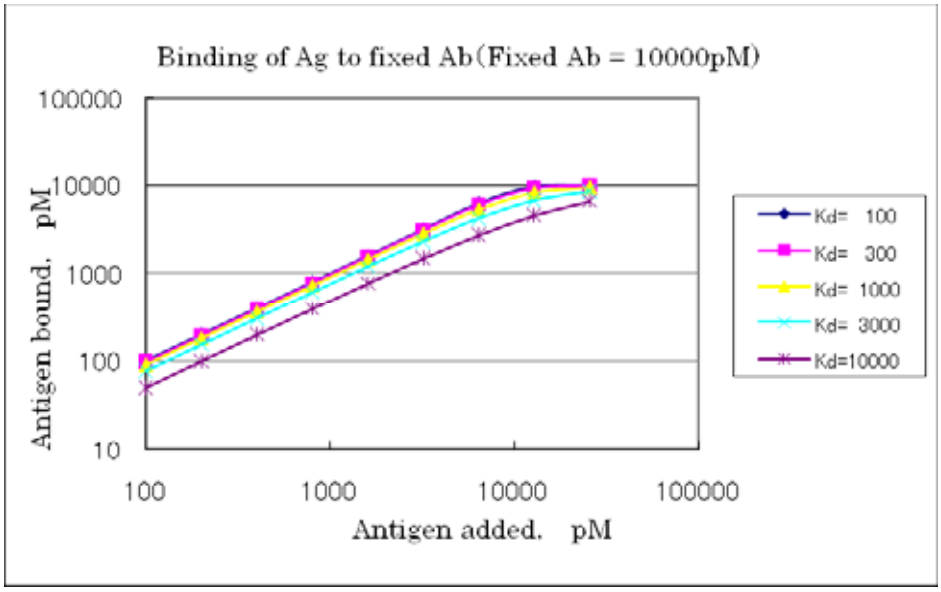
where initial antigen concentration: H, antibody concentration: R ,

dissociation constant: Kd, and bound antigen concetration: b (unit:M),

$Kd=(H-b)x(R-b)/b$

$b2-(Kd+H+R)b+RH=0$

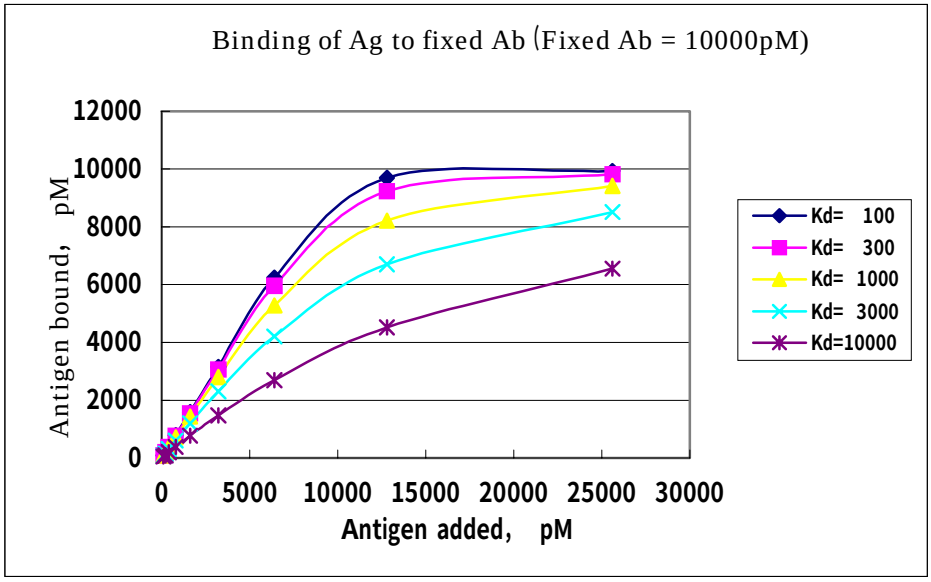
$$b=\frac{Kd+H+R-\sqrt{(Kd+H+R)^2-4HR}}{2}$$



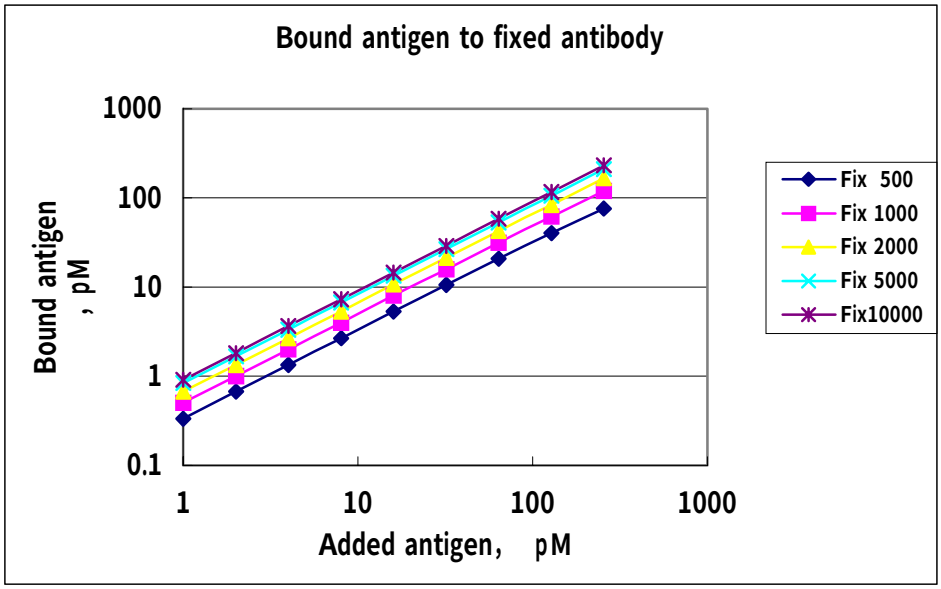
Above graph shows changes of bound antigen concentration to capture antibody (10000pM) with various Kd when initial 5-1280pM antigen is added. If the results are plotted with log-log scales, the binding goes up straightly to some points then bends.

It is shown that the standard curve move toward right depending on Kd, indicating that assay sensitivity is related to Kd. If Kd is small (that is, affinity constant is large) the sensitivity becomes excellent.

When the results are expressed with normal scales, like the graph below, each binding curve shows different slopes depending on Kd.



If Kd is constant, the standard curve (binding curve) moves to the right when fixed antibody is increased.



In this simulation, we can understand the changes of standard curve, especially, that of sensitivity due to Kd and capture antibody. But the lower standard concentration area is linear, and different from the real ELISA standard curve in which lower area is curve linear.

3. Simulation of ELISA standard curve

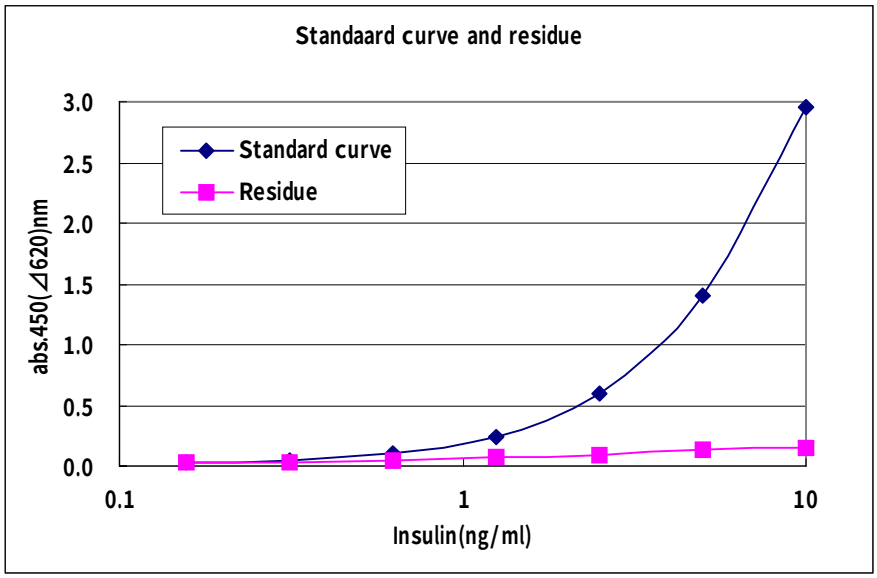
So, let us try to estimate how much antigen is bound to fixed capture antibody using real ELISA kit.

Amount of bound antigen to fixed capture antibody can be estimated by measuring the residual antigen after incubation with fixed antibody in the well.

So, the author prepared two insulin ELISA well-plates (plate A, and plate B) of Shibayagi's rat insulin ELISA Kit. Then added standard series of insulin solutions to plate A, and

incubated for 2 hours. After incubation 100μl of the reaction mixtures were transferred to plate B and incubated for 2 hours. Plate A and B were washed and treated through the rest of the assay procedure. A standard curve was prepared using plate A, the amounts of insulin in plate B were calculated which indicated residual insulin.

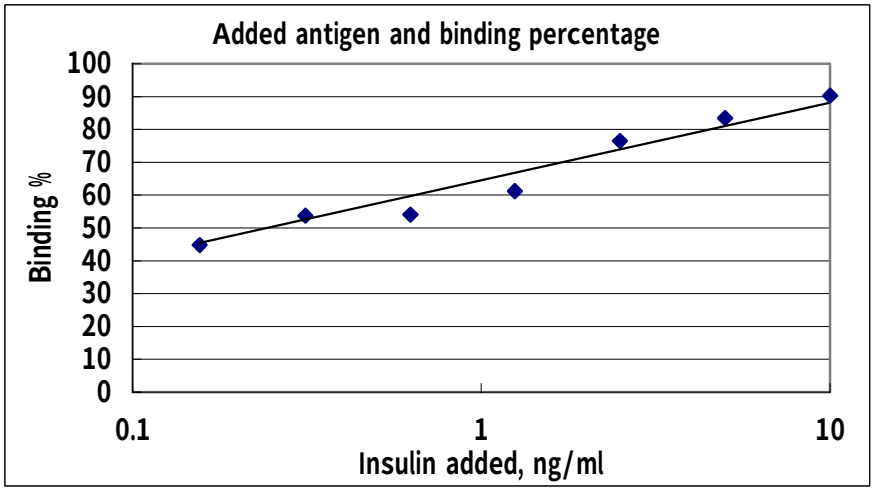
Results are shown in the following figure and table.



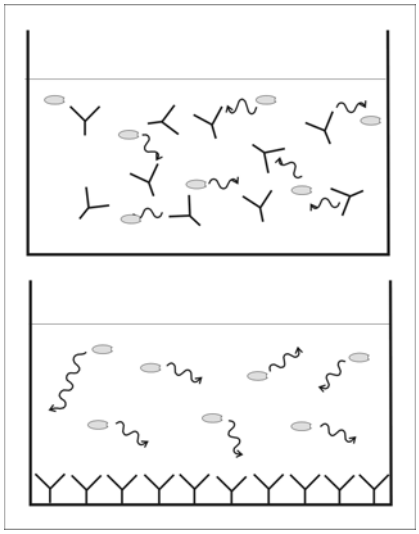
Added ng/ml	Residue ng/ml	Residue (%)	Bound indulin(%)
10	0.9672	9.7	90.3
5	0.8273	16.55	83.45
2.5	0.5880	23.52	76.48
1.25	0.4844	38.75	61.25
0.625	0.2868	45.89	54.11
0.3125	0.1444	46.21	53.79
0.156	0.0860	55.13	44.87

It is shown that binding percentage decreases if added insulin is less. If 10ng/ml insulin is added, about 90% is bound to the capture antibody, while binding percentage is about 45% when 0.156ng/ml is added.

The figure below is obtained by plotting the logarithmic concentration of added insulin against binding percentage. It seems linear, indicating that binding percentage is proportional to logarithmic concentration of added insulin.



This may be explained from the retardation of the antigen-antibody reaction in solid phase antibody system. Solidified antibody cannot move around the solution and only antigen can move to fixed antibody. If the concentration of antigen is low, the efficiency of finding the partner and binding would be decreased, and takes more time. Incubation for 2 hours may not enough for the low population of antigen.



The author calls this effect “binding decreasing factor 1(BDF-1)”.

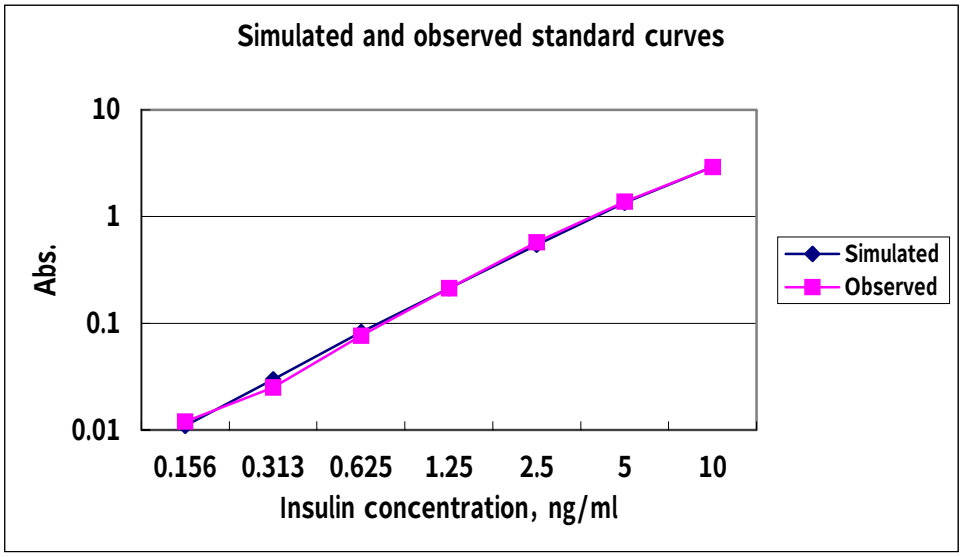
In ELISA, we have to take another factor into consideration. It is the binding of labeled antibody to the antigen captured on solidified antibody. If the all the captured antigen molecules bind the enzyme labeled second antibody the specific enzyme activity (enzyme activity/bound antigen) must be constant. The author found that the specific enzyme activity that was expressed by absorbance/bound insulin was not constant but also proportional to added insulin concentration. The author calls this factor “binding decreasing factor 2 (BDF-2)”.

In order to simulate the ELISA standard curve composed of antigen concentration and absorbance, bound antigen calculated from the antigen-antibody reaction in liquid phase should be multiplied by BDF-1 and BDF-2 for correction.

The author tried the simulation for rat insulin ELISA Kit by estimation of bound insulin in liquid phase with postulation of solid antibody concentration of 10000pM and dissociation constant of 1000pM, and standard insulin 0.156-10ng/ml (27-1724pM). Then using the equations of BDF-1 and BDF-2. The results obtained are shown in the figure below.

The simulated standard curve was compared with the real standard curve.

Both curves coincided very well.



4. How does variation of absorbance influence on assay value variation?

In competitive assays like RIA and competitive enzyme immunoassay (sometimes called "competitive ELISA"), relative assay variations in low concentration area are very large owing to the shape of their standard curves.

Then, how about in ELSA?

The author shows an example indicating the nature of variation in ELSA.

Usually, we draw standard curve of ELISA by plotting standard concentration against absorbance of coloration of enzymatic reaction product.

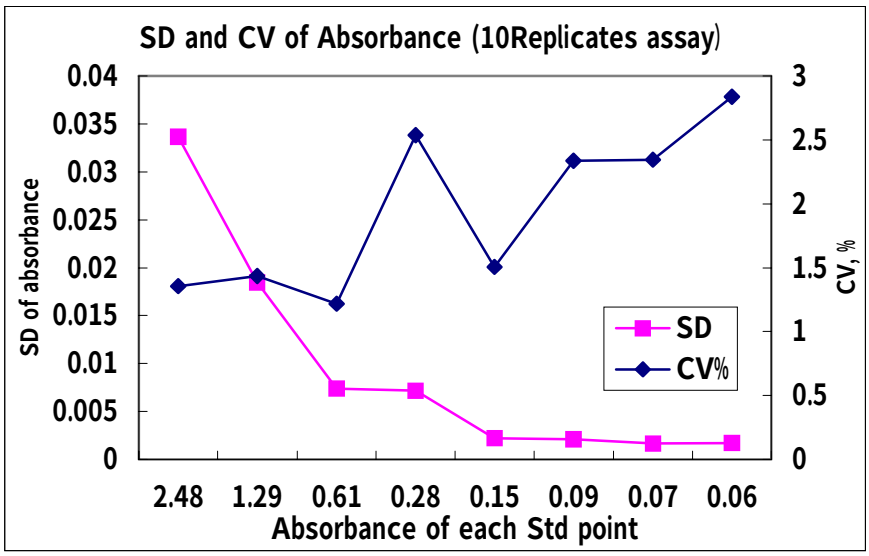
Variation in absorbance, of course, depends upon proficiency of a technician who performs ELISA.

Here we present the results of our trials in Shibayagi Co. Ltd. Then influence of the variation on assay value will be estimated.

Absorbance obtained by ten replicates assay of standard solutions of a kit are shown below..

1	2	3	4	5	6	7	8	9	10	Mean	SD	CV%
2.398	2.484	2.492	2.464	2.511	2.513	2.486	2.505	2.5	2.492	2.4845	0.0336	1.3545
1.286	1.271	1.272	1.263	1.279	1.3	1.299	1.306	1.285	1.323	1.2884	0.0184	1.4349
0.61	0.604	0.609	0.612	0.61	0.602	0.597	0.606	0.602	0.624	0.6076	0.0073	1.2173
0.275	0.292	0.283	0.286	0.284	0.284	0.271	0.29	0.273	0.287	0.2825	0.0071	2.5375
0.147	0.147	0.144	0.152	0.146	0.149	0.146	0.146	0.149	0.148	0.1474	0.0022	1.5068
0.086	0.088	0.091	0.091	0.092	0.092	0.088	0.089	0.089	0.092	0.0898	0.0021	2.3358
0.07	0.071	0.07	0.075	0.069	0.071	0.071	0.07	0.07	0.072	0.0709	0.0016	2.3460
0.06	0.064	0.06	0.061	0.06	0.06	0.061	0.061	0.061	0.057	0.0605	0.0017	2.8362

The data were expressed in a graph.



In this figure, data are arranged from the largest standard concentration to the least.

The absolute values of SD of standard points increase in parallel with the standard concentration, however, CV (RSD) shows an opposite tendency, i.e. CV at the least standard point is about twice of that at the largest point.

Increase of CV in lower concentration area is possibly influenced by variation of a plate-reader, heterogeneity of well-plate (such as thickness of the bottom and flaw) and non-specific adsorption of labeled antibody.

Variation in absorbance and variation of assay values

In order to obtain assay values, we have to read assay values from absorbance using a standard curve. Then what would happen with variation?

If absorbance of each standard point in increased by 2%, how much difference in assay value would be? Please, look at the table shown below.

STD.	Absorbance	Calc. Std.	Absorbance+2%	Calc. value	⊿%
0.1	0.063	0.1004	0.06426	0.1047	4.28
0.25	0.105	0.2496	0.1071	0.2572	3.04
0.5	0.199	0.5018	0.2034	0.5124	2.11
1	0.426	0.9977	0.4345	1.0159	1.82
2.5	1.173	2.5119	1.1965	2.5612	1.96
5	2.206	4.9683	2.25	5.0902	2.49
10	3.558	10.0479	3.629	10.4286	3.79

We used a standard curve with concentration from 0.1 to 10ng/ml.

Calc. Std. is the value obtained from a regression equation with good fitness (so called trueness), then we increased each absorbance by 2%, and calculated concentration (Calc. Value). ⊿% is the percentage of the difference of Calc. Std. and Calc. Value. This will give relative variation in assay values for 2% variation in absorbance.

Change of absorbance by 2% will cause nearly 2% change around 0.5 to 2.5 ng/ml (in the middle area of the standard curve), and shows a tendency to increase outside this area. The lowest and the highest standard show CV nearly 4%. This is due to the difference in slope of the standard curve. But from our experience, influence of variation in absorption is far smaller than that of competitive assay, especially in lower concentration area. The slope of ELISA standard curve expressed by bi-logarithmic scale is steeper than that of competitive assay.

IV. Procedure of ELISA ...Step by Step

In this section the author will introduce assay procedures of two typical assay systems provided by Shibayagi, one is a basic procedure using HRP-labeled second antibody, and the other is the procedure using biotin-labeled second antibody and HRP-labeled avidin.

Detail techniques will be explained in the section IV.

1. An example of kits with HRP-labeled second antibody ...Rabbit CRP ELISA KIT

Components of the kit

	Reagents	Amounts
(A)	Anti-CRP-coated microplate	96 wells(8x12) / 1 plate
(B)	Standard CRP solution (2μg/ml)	200μl / 1 vial
(C)	Buffer solution	60ml/1 vial
(D)	Peroxidase-conjugated anti-CRP antibody	200μl/ 1 vial
(F)	Chromogenic substrate reagent(TMB)	12ml/ 1 vial
(H)	Reaction stopper (1M H_2SO_4)	12ml/ 1 vial
(I)	Concentrated washing buffer(10x)	100ml/ 1 bottle

○Standard solution (B) is provided as an original concentrated solution. A series of standard solutions of various concentrations are prepared by dilution of this original solution with buffer (C), in most cases, by serial dilution, while others by proportional dilution.

An example of preparing standard solutions

Dilute the original standard solution (B) with the buffer solution to prepare 200ng/ml, then prepare lower standard solutions by a dilution program shown below.

Concentration, ng/ml	200	100	50	25	12.5	6.25	3.13	0
Standard solution, μl	50**	200*	200*	200*	200*	200*	200*	0
Buffer, μl	450	200	200	200	200	200	200	200

** Original standard solution, *One rank higher standard solution

○ Horseradish peroxidase (HRP)-second antibody conjugates (D) is provided as an concentrated solution, and is used after dilution with the buffer (C), in most cases, 1:100..

○Concentrated washing buffer (I) is used after dilution with purified water to 1:10.

○Chromogenic substrate solution (F) and reaction stopper (H) are used as they are.

All the reagent solutions should be used after getting back to room temperature (20-25C).

Assay procedure

Remove the cover sheet of the microplate after getting back to room temperature.

(1) Rinse the anti-CRP coated wells (A) by filling the washing buffer and discard 3 times, then strike the plate upside-down onto folded several sheets of paper towel, and remove the excess buffer.

(2) Pipette 50μl of sample solution to the wells for samples.

(3) Pipette 50μl of the standard solution to the wells for preparing standard curve.

(4) Shake the plate gently on a plate shaker for 10-15 seconds.

(5) Incubate for 1 hour at room temperature (20-25C).

(6) Discard the reaction mixture, and then wash wells as described in (1).

(7) Pipette 50μl of peroxidase-conjugated anti-CRP solution to all wells. Then shake gently on a plate shaker for 10-15 seconds.

(8) Incubate the plate for 1 hour at room temperature.

(9) Discard the reaction mixture, and then wash the plate as (1).

(10) Pipette 50μl of chromogenic substrate solution to wells, and shake as (4).

(11) Let the plate stand for 30 minutes at room temperature.

(12) Add 50 μl of the reaction stopper (H) to all wells and shake.

(13) Measure the absorbance of each well at 450 nm (sub-wave length, 620nm) by a plate reader within 30 minutes.

Summary of Assay Procedure

Antibody-coated 96 well microplate

↓Washing 3 times

Sample or Standard 50μl

↓Shaking and reaction for 1 hour at room temp.

↓Washing 3 times

Peroxidase-conjugated anti-CRP 50μl

↓Shaking, and reaction for 1 hr. at room temp

↓Washing 3 times

Chromogenic substrate solution 50μl

↓Shaking, and reaction for 30 mins. at room temp

Reaction stopper 1M H_2SO_4 50μl

↓

Shaking and measurement of absorbance
at 450nm(sub. 620nm)

Room temp.: 20~25C

2. An example of kits with biotin-labeled second antibody and HRP-labeled avidin.

...Mouse leptin ELISA KIT

Components of the kit

	Reagents	Amounts
(A)	Anti-leptin-coated microplate	96 wells(8x12) / 1 plate
(B)	Standard mouse leptin solution (5,000pg/ml)	500µl / 1 vial
(C)	Buffer solution	60ml/1 vial
(D)	Biotin-conjugated anti-leptin	200µl/ 1 vial
(E)	Peroxidase-conjugated streptavidin	200µl/ 1 vial
(F)	Chromogenic substrate reagent (TMB)	12ml/ 1 vial
(H)	Reaction stopper (1M H_2SO_4)	12ml/ 1 vial
(I)	Concentrated washing buffer (10x)	100ml/ 1 bottle

○Standard solution (B) is provided as an original concentrated solution. A series of standard solutions of various concentrations are prepared by dilution of this original solution with buffer (C), in most cases, by serial dilution, while others by proportional dilution.

An example of preparing standard solutions

Std. Conc. (ng/ml)	5000	2000	500	100	50	25	10	0
Std. sol.(µl)	Orig. sol.	100**	100*	100*	200*	200*	200*	0*
Buffer (µl)	0	150	300	400	200	200	300	300

**Original standard solution, *One rank higher standard solution

○ Biotin-conjugated second antibody solution (D) is provided as an concentrated solution, and is used after dilution with the buffer (C), in most cases, 1:100..

○Horseradish peroxidase (HRP)-conjugated avidin (E) is provided as an concentrated solution, and is used after dilution with the buffer (C), in most cases, 1:100..

○Concentrated washing buffer (I) is used after dilution with purified water to 1:10.

○Chromogenic substratee solution (F) and reaction stopper (H) are used as t hey are.

All the reagent solutions should be used after getting back to room temperature (20-25C).

Assay procedure

Remove the cover sheet of the microplate after getting back to room temperature.

(1) Rinse the anti-leptin coated wells (A) by filling the washing buffer and discard 4 times, then strike the plate upside-down onto folded several sheets of paper towel, and remove the excess buffer.

(2) Pipette 40µl of buffer solution into the wells for samples, then add 10µl of sample to

each well. Alternatively, if you use larger sample volumes (X μl), the volumes of buffer(C) should be (50 – X) μl to adjust the final volume to 50 μl.

It would be also convenient to dilute the assay samples first in test tubes, and pipette 50μl of the diluted sample to a well.

(3) Pipette 50μl of the standard solution to the wells for preparing a standard curve.

(4) Shake the plate gently on a plate shaker.

(5) 50μl of biotin-conjugated anti-leptin solution to all wells. Then shake gently on a plate shaker.

(6) Incubate the plate for 2 hours at room temperature.

(7) Discard the reaction mixture, and then wash the plate 4 times as described in (1), and remove excess washing buffer remaining in the wells as (1).

(8) Pipette 100μl of HRP-conjugated avidin solution to all wells, and shake as (4).

(9) Incubate for 30 minute at room temperature.

(10) Discard the reaction mixture, and then wash the plate 4 times as (1), and remove excess washing buffer

(11) Pipette 100μl of chromogenic substrate solution to wells, and shake as (4).

(12) Let the plate stand for 30 minutes at room temperature.

(13) Add 100 μl of the reaction stopper (H) to all wells and shake.

(14) Measure the absorbance of each well at 450 nm (sub-wave length, 620nm) by a plate reader within 30 minutes.

Summary of Assay Procedure

Antibody-coated 96 well microplate

↓Washing 4 times

Sample* or Standard 50μl

↓

Biotin-conjugated anti-leptin antibody 50μl

↓Shaking and reaction for 2 hour at 20~25C

↓Washing 4 times

Peroxidase-conjugated avidin 100μl

↓Shaking, and reaction for 30 min at 20~25C

↓Washing 4 times

Chromogenic substrate solution 100μl

↓Shaking, and reaction for 30 min. at 20~25C

Reaction stopper 1M H_2SO_4 100μl

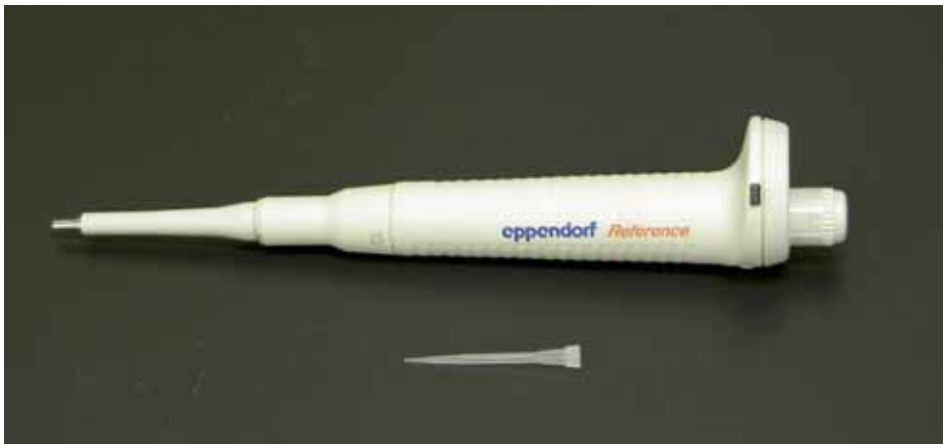
↓

Shaking and measurement of absorbance

at 450nm(sub. 620nm)

V. Fundamental techniques for performing ELISA

1. How to use a tip-exchange type pipette

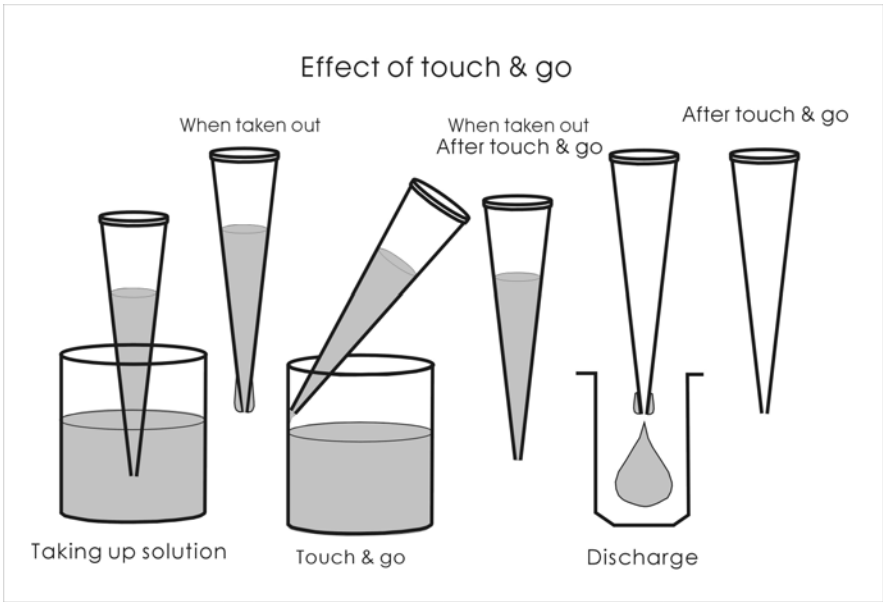


a. "Pre-wetting" method(A technique used most generally)

○ Set a new tip on a the pipette, then push down the plunger to the first stop in the sample solution and repeat filling and discharging the solution for two or three times within the range of the first stop to wet the inside of the tip, then finally fill the solution.

○ When taking out the pipette, touch the inner wall of the container of the sample solution with the tip to remove excess solution outside of the tip (touch and go).

○ When discharge the solution to a well, push down the plunger to the end to deliver the solution completely (blow out), then touch and go.



○ Exchange the tip for the next new sampling.

○ In case to deliver the same solution to several wells in the same volume, you can use the same tip without pre-wetting from the second well.

b. "Co-washing" method

This technique can be used only in the case when some buffer or solution is already in the

well. If there is no liquid in the well, use pre-wetting method.

○ Set a new tip, and pushdown the plunger to the first stop, and fill up the solution to be delivered.

○ Take out the pipette after “touch and go” at the inner wall of the container.

○ Dip the top of the tip in the buffer of the well and deliver the solution to the well by pushing the plunger to the first stop, then within the range of the first stop, repeat filling and discharging of the liquid in the well 2 or 3 times (co-washing), and finally blowout the solution in the tip by pushing down the plunger to the end.

○ Take out the pipette after touch and go. Then exchange the tip.

These methods are described according to the instruction paper for Eppendorf pipette.

● Pipetting should be carried out using one of the methods described above. But do not mix up these two methods.

● If possible, we recommend “Co-washing” method for serum and plasma samples because of high protein concentration with considerable viscosity.

● “Co-washing” method is also recommended for small sample volumes less than 10mcl.

● When sampling volume is rather large, like 50-100mcl, “Pre-wetting” method would be suitable.

● Please, pipette every sample and reagent solution after they return to room temperature. Operation under higher or lower temperature than room temperature may cause inaccurate pipetting, and increase assay variation.

● Variation of temperature of the reaction mixtures would also influence reaction velocity.

● Generally, after freezing and thawing, solutes would be concentrated to the bottom part of the liquid. So, any frozen samples should be stirred by Vortex-type mixer and made homogeneous after thawing.

2. How to prepare standard solution series

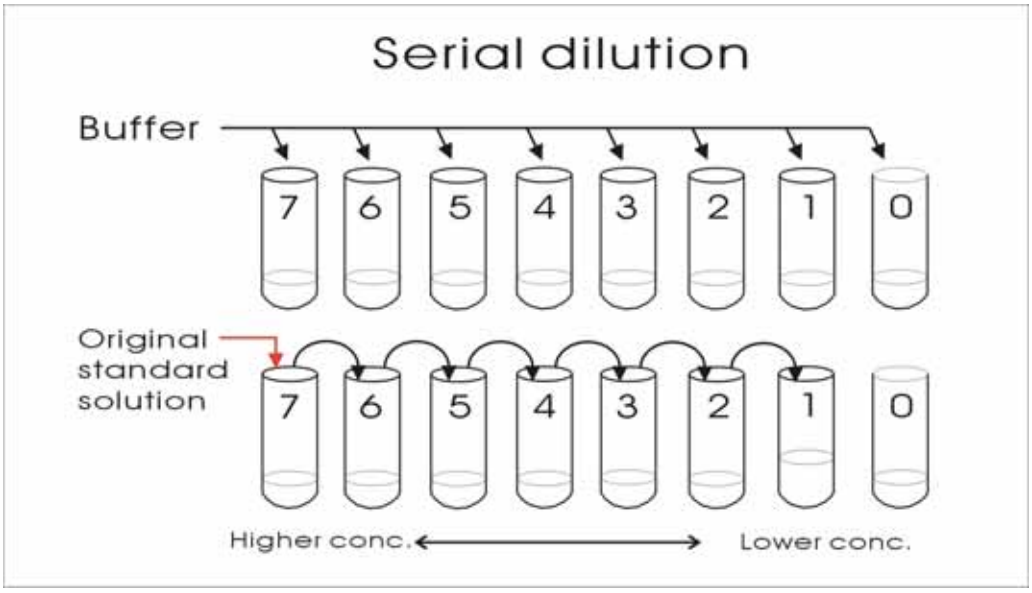
a. Serial dilution

Prepare small test tubes as many as the standard solutions including zero point, and write numbers on them.

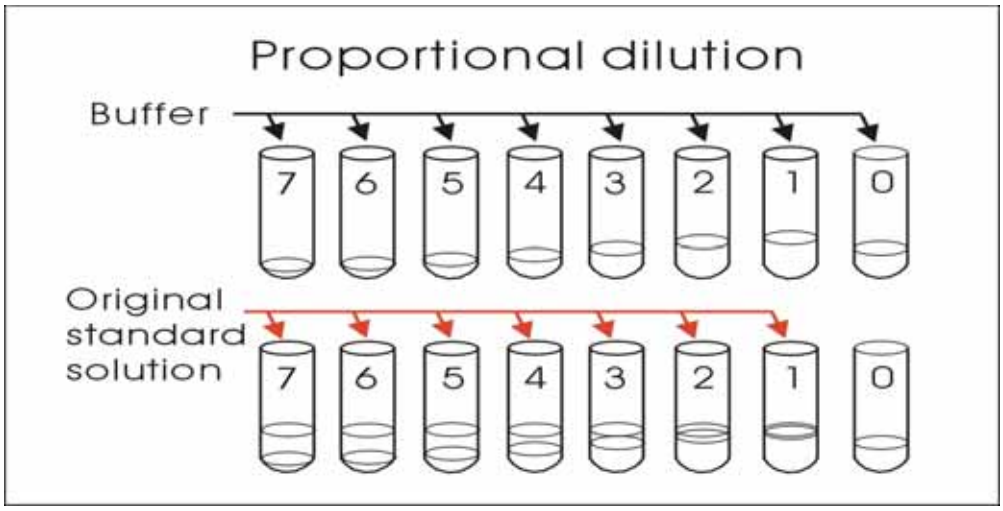
First, add buffer of indicated volume to all the tubes.

To the tube of the highest standard, add the indicated volume of the original standard solution, and mix well.

Then take the indicated volume of the mixture, and add it to the second tube, and mix well. Then from the 2nd tube, transfer the indicated volume of the mixture to the 3rd tube, and so on to the No. 1 tube. No.0 should contain only buffer.



b. Proportional dilution



Prepare small test tubes as many as the standard solutions including zero point, and write numbers on them.

Add the indicated volumes of buffer to the tubes. Generally, the volumes are different from tube to tube. So, take enough care.

Add indicated volumes of the original standard solution to the tubes, and mix well.

Tube No. O should contain only buffer. In some cases, the original standard solution itself is used as the highest standard

3. Dilution of labeled (enzyme, biotin) antibody and HRP-conjugated avidin solutions

Labeled antibody solution and HRP-conjugated avidin solution are provided in small vials.

The amount of the solution provided is enough to take out the volume as indicated in the instruction paper.

Dilution should be carried out in the following way.

First, put the calculated volume of buffer solution to a vessel.

Then from the small vial containing labeled antibody solution or HRP-avidin solution, take

out the volume as indicated in the instruction paper, and add it to the vessel, and mix well.

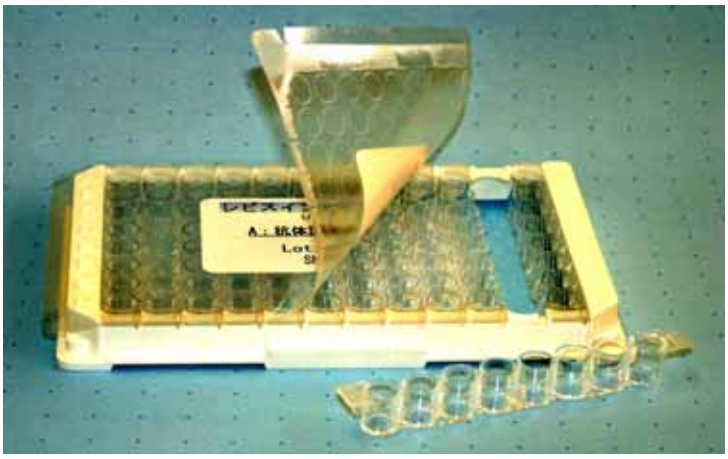
4. Dilution of concentrated washing buffer

To a 1000ml beaker, add all the content of the bottle containing concentrated washing buffer, then add a little less than 900 ml of purified water.

Make the mixture to 1000ml by adding purified water little by little. Then mix well.

Transfer this washing buffer to a plate washer, or to a 500ml washing bottle with a jet nozzle.

5. Structure of antibody-coated microplate and treatment



12 strips of 8 wells connected each other are set in a microplate frame.

The surface of the plate is covered with a seal to avoid dryness. Remove the seal after the plate gets to the room temperature. Remove the seal just before starting assay.

If you want to use only a part of the wells, cut the part of the seal, and take out the strips and transfer them to another frame, and remove the seal.

In this case, please, store the rest of the wells at 2-8C, and reagents

and use them within 3 days.

6. How to wash a microplate

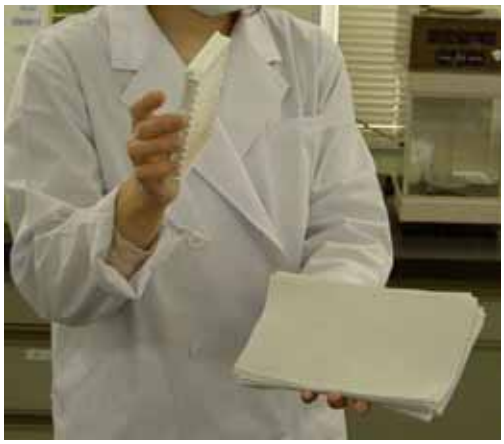
The antibody-coated plate should be washed before assay and after each reaction.

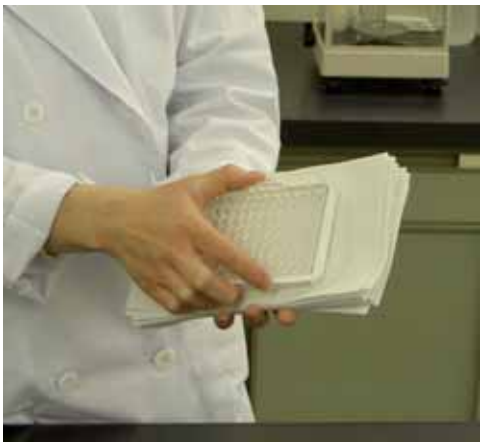
a. Before assay, fill the wells with washing buffer using a washing bottle with a nozzle. Then shake off the buffer from the plate onto a sink. Repeat filling and shaking off as many times as indicated in the instruction paper.

Complete Removal of residual liquid after washing and pouring off.

After final shaking off the buffer from the plate, strike the plate on to some sheets of paper towel for several times to remove the residual buffer.

After confirming that no liquid remains in the wells, start pipetting of the reagent solution of the next step.





b. Washing after the first reaction: The first washing after the first reaction with standards or samples: Shake off the reaction mixture from the plate onto a sink.

Then add washing solution using a multi-delivery pipette set at 250mcl to avoid carry-over caused by flowing out the buffer to other wells. Then shake off the buffer. From this step on, it is not necessary to be afraid of carry-over.

Add new washing buffer from the washing bottle with nozzle, and shake off.

Repeat filling and shaking off as many times as indicated. Then completely remove residual washing buffer as shown above.

c. Washing after other reaction: Shake off the reaction mixture from the plate into a sink.

Add new washing buffer from the nozzled washing bottle, and shake off.

Repeat filling and shaking off as many times as indicated. Then completely remove residual washing buffer as shown above.

d. Automatic plate washers are also commercially available

Caution about washer: Adjust the washer so as to give proper strength of operation. Strong injection and suction of the buffer may remove coated antibody, and may cause a large assay variation and poor color generation.

7. How to add reagent solution using repeating dispenser

For pipetting reagent solutions common to all the wells, a multi-delivery type pipette (e.g. Eppendorf multipette plus) is suitable.

We should be careful in using this type of pipette;

○After filling of the liquid, remove the air, and deliver the first one or two strokes back to the container.

○When deliver the liquid to wells, avoid too strong strokes. A strong stroke may cause splashing out of the liquid.

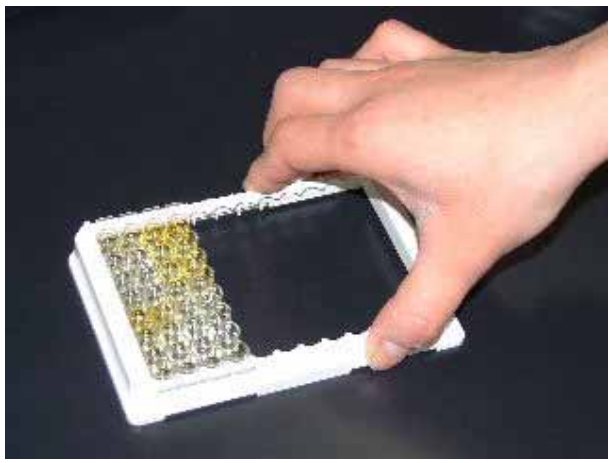
○ After delivery of the liquid to a well, perform “touch and go” to the wall of the well.

○Do not dip the top of the tip in the well.

8. Shaking of the well-plate for mixing

After addition of a reagent solution, the microplate should be shaken to mix the solutions. A short shaking is enough because of the small volume of the reaction mixture.

We recommend to use a microplate mixer (microplate shaker) as shown above. Set the plate on it, and shake at 800 rpm for approx. 10 seconds. Repeat 3 times.



If a microplate shaker is not available, shake the plate by hand as is described below.

Place the plate on the flat and smooth surface of a laboratory table, hold the plate and move the plate roundly to draw circles rapidly for approx. 10 seconds while lightly pressing the plate on the surface. Repeat 3 times.

9. Color generation

Bluish color generation will occur during incubation with TMB solution owing to enzyme action of HRP.

10. Careful addition of reaction stopping solution

As the reaction stopper is strongly acidic, please, be careful in treatment of the solution.
We recommend wearing glasses or goggles for protection of your eyes. After addition of the stopping solution the color of the reaction mixture in the well will change from blue to orange-yellow.

11. Measurement of absorption using a plate reader

After stopping the enzyme action, absorbance is read using a densitometer for microplates (microplate reader). Two types of microplate readers are available, one in which the wavelength is fixed using a filter, and the other in which wavelength is variable. Use the reader under good maintenance. In most plate readers, the concentration of the substance assayed automatically calculated. Because operation of the reader is different depending on the machine makers, read and follow the instruction of the reader you are going to use.

If possible measure absorbance at both 450nm (for Shibayagi's kits with TMB as a chromogenic substrate) and 620nm, and calculate the difference between absorbance at 450nm and 620nm. By doing this, we can compensate influences of some non-specific factors of wells like small scar on the bottom of well, and the variation in the structure of wells, e.g., thickness of the bottom. The difference is expressed as Abs.450(Δ620)nm.

12. Preparation of standard curve and calculation of assay values

As to the nature of ELISA standard curve, refer to Part III in page 12..

In manual calculation, prepare a standard curve using bi-logarithmic section paper by plotting Abs.450(Δ620)nm on Y-axis against standard concentration (ng/ml) on X-axis.

Because the coefficients of variation (CV) in absorbance do not change so much throughout all the assay range, it is proper to choose logarithmic expression in both concentration and absorbance. This expression allows easy reading of lower concentration area.

Read the concentrations of the substance to measure in samples from their Abs.450(Δ620)nm, and multiply the assay value by sample dilution rate if samples have been diluted.

In most microplate readers, some calculation programs have been installed, and the assay

results are automatically calculated by proper setting.

For calculation of assay results with EXCEL, refer to “VI. How to calculate ELISA assay value by Excel” in page 36.

13. Assay validation and validation tests for performance of an ELISA kit

Any report using an ELISA system should provide the validation data to show evidences that the assay system gives reliable data. So, the validation tests are necessary to establish and apply an ELISA system.

In immunoassay system there are 3 kinds of errors, namely accidental error (random error), systematic error, and gross error (mistake).

○Accidental or random error means, in other expression, variation of assay results. This is expressed by standard deviation, or relative standard error (RSD, in other words coefficient of variation (CV).

RSD = CV = SD/mean x 100 (%)

○Systematic error means difference from true value or bias which is related to accuracy.

○Gross error is, in other word, mistake. This kind of error derives from careless mistake in doing assay, troubles of equipments, denaturation of reagents, etc., which causes cancellation of assay.

ICH (International Conference of Harmonization, Japan, U.S. and EU) issued a guideline for immunoassay validation. Governments of above countries urged kit makers and users to follow this guideline.

Important items for ELISA are as follows. In this text the author will try to explain in later sections.

For standard curve

○Detection limit (relating assay sensitivity)

○Quantitation limit (relating assay sensitivity)

○Linearity (not requested for immunoassay)

○Range (= assay range covered by standard curve)

For random error

○Precision

Repeatability (= intra-assay precision)

Intermediate precision (= inter-assay precision in one laboratory)

Reproducibility (= Variation among different assay laboratories)

For systematic error

○Specificity

○Accuracy

For assay system

○Robustness (= stability of assay system under various conditions)

a. Standard curve and assay sensitivity

Assay sensitivity is defined as follows.

(From Note No. 338 by Ministry of Welfare and Labor, Japan)

Detection limit

(Definition by ICH based on proposal of ISO and IUPAC)

$DL = 3.3s/a$

s: SD of blank absorbance
a: Slope of calibration curve near DL
DL: Detection limit
0.004x3.3=0.0131
DL=0.0131/0.0289=0.453

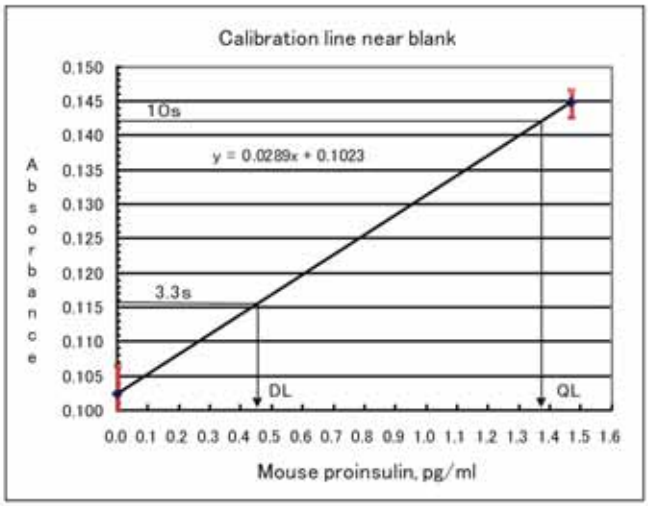
If the equation above is transformed
(s/a)/DL = 0.3
So, CV at DL is 30%

Quantitation limit

$QL = 10s/a$

0.004x10 = 0.04
QL=0.04/0.0289=1.38
CV at QL is 10%

Conc. pg/ml	Abs. 1	Abs. 2	Mean	SD	CV
2.94	0.177	0.168	0.172	0.006	3.57
1.47	0.146	0.143	0.145	0.002	1.36
0	0.105	0.099	0.102	0.004	4.22



We can think that the sensitivity is the standard concentration to give significantly higher absorbance than that of blank.

Another way to estimate the sensitivity is to show the concentration of the standard at the middle point of standard curve. From this value we can guess the practical assay range.

As for lower limit of assay range, we should start from QL rather than DL because at DL, assay precision (CV) will be 30% and is thought to be too big. At QL, CV will be 10% and would be permissible.

The highest limit of assay range has some problems. Some people define the upper limit of assay range to be the concentration which gives an absorbance of highest absorbance –3SD.

I myself think that absorbance should not be larger than 2.5. We cannot trust a colorimeter if the absorbance is more. The slope of ELISA standard curve, if standard concentration goes up, gradually becomes smaller and smaller until reaching its maximum. If slope becomes smaller, the CV of the calculated assay value increases, so, only a minor change of absorbance causes a big change of calculated concentration.

b. Assay precision (“Repeatability” according to ICH definition)

This is also called within assay variation or intra-assay variation. If the variation is small, the mean assay value will be trustworthy.

This is expressed by CV % (coefficient of variation) of a sample which is measured by in one

assay trial. One assay sample is measured using several wells, and the mean and standard deviation (SD) are calculated, then CV% is obtained as CV (%) = SD/mean x 100. Usually, several samples are measured and their average CV % is shown. An ELISA system is expected to give CV% less than 5% from our experience.

An example of within assay variation [Rat C-peptide ELISA KIT (U-type)]

Two samples, A and B were measured using 8 wells for each sample.

Well/Sample	A	B
1	1015	214
2	1027	222
3	1038	211
4	1043	219
5	1029	232
6	1034	209
7	1039	231
8	1041	224
mean	1033	220
SD	9.60	8.51
CV (%)	0.93	3.86

Unit: pg/ml

Comment on "3 dilution and duplicates assay"

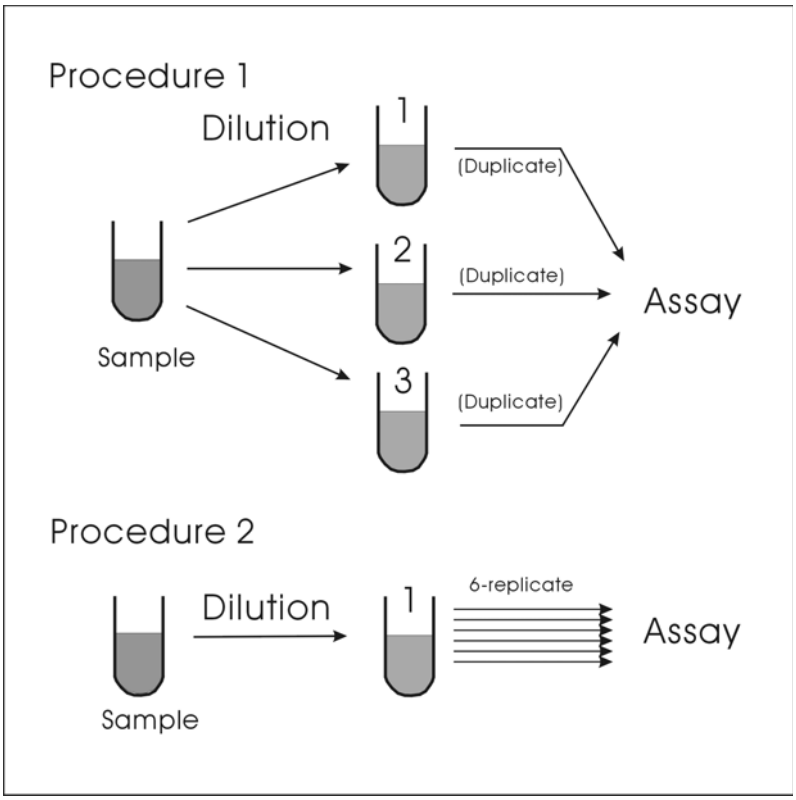
A test for repeatability " 3 dilution-duplicates assay" is often used.

This testing procedure is to take 3 aliquots are taken from a sample, and are diluted separately. Then each diluted samples are assayed in duplicates, and their assay values are compared (means and CVs) as shown in the figure below as "procedure 1". However this is not suitable for testing precision of an assay kit. The results of procedure 1 contain variation composed of pipetting variation and dilution variation and assay kit variation. If the volume for dilution is very small, influence of pipetting technique is large.

On the other hand, variation of 6-replicate assay is consisted mostly of assay kit variation and influence of assay technician's skill is less. Six replicates assay has high reliability of CV.

If both procedures are used together, proficiency of technician would be clarified.

Rf: According to ISO 8655-5, maximum permissible random error for single-stroke dispenser with nominal volume of 10µl is 0.1µl. If a pipette of a larger nominal volume like 50µl is used, the maximum permissible random error is 0.2µl. Pipettes are so made. However, practically, proficiency of pipetting technique is requested more seriously in precise delivering if the sample is serum or plasma. They are viscous and should be pipetted very carefully. (Please, refer to the pipette's sections in p.25 and 52.)



c. Reproducibility (“Intermediate precision” according to ICH definition)

Reproducibility means that, if one sample is measured repeatedly in different assay trials, the assay values obtained in those trials are always constant or not.

This is also called between assay variation or inter-assay variation, and is also expressed by CV%. One sample is measured in several assay trials, and from assay values of trials mean assay value and SD, CV (%) is calculated. If the number of wells in each trial is larger, the more trustable reproducibility will be given. We expect CV% less than 10%, hopefully less than 5%.

An example of between assay variation [Rat C-peptide ELISA KIT (U type)]

Three samples, C, D, and E were measured using 4 wells/sample, and assays were repeated 4 times on different days.

Sample/day	Day 1	Day 2	Day 3	Day 4	mean	SD	CV (%)
C	1499	1435	1491	1458	1471	29.68	2.02
D	599	605	569	559	583	22.55	3.87
E	63.9	58.2	59.9	64.2	61.6	2.98	4.83

Unit: pg/ml

Reproducibility of ICH definition is difficult to estimate without any comparative project, especially for single assay laboratory.

d. Specificity

It is necessary to be confident that the ELISA system in question measures only the substance aimed, and no other substances positively measured due to cross-reaction. The test

on this problem is carried out using several candidate substances suspected to cross-react to the system from similarity of their chemical structure to the aimed substance. Varied amounts of candidate substances are measured, and the assay values are examined. If those candidates give no detectable assay values, specificity of the assay system will be confirmed.

If any candidate gives a significant assay value, its cross-reactivity is expressed as the percentage of the assay value to the amount of the candidate.

Sometimes, the apparent cross-reactivity is due to the contamination of the aimed substance in the candidate preparation

An example of cross-reactivity test [Rat C-peptide ELISA KIT (U type)]]

Species	Substance	Cross reactivity (%) *
Rat	C-peptide	100
	Insulin	Less than sensitivity
	Pro-Insulin	Less than sensitivity
Mouse	C-peptide	75
	Insulin	Less than sensitivity
Human	C-peptide	85
	Insulin	Less than sensitivity
	Pro-Insulin	Less than sensitivity
Pig	Insulin	Less than sensitivity
Cow	Insulin	Less than sensitivity

***Cross-reactivity was estimated with 15,000 pg/ml of the substance**

e. Accuracy

Accuracy of assay system, i.e. whether the assay value obtained really reflects the amount of the aimed substance or not, should be confirmed through several tests, like recovery test, linearity of dilution test, and comparison with other assay system.

○ **Spike recovery test**

Sample serum/plasma is divided into two portions. And a certain amount of the standard preparation is added to one portion (sometimes called “spike”), while the other is left intact. Both portions are assayed, and from these two assay values, recovery of the standard preparation added (spike-recovery) was estimated by subtraction. Recovery of the added standard should be around 100% within the range of assay precision. In the recovery tests shown below, three different amounts of the standard are added

Examples of recovery tests [Rat C-peptide ELISA KIT (U type)]

Duplicate assay Unit：pg/ml

Added	Found	Recovered	Recovery %
0.00	180	-	-

110	291	111	101
161	332	152	94.5
209	379	199	95.1

Added	Found	Recovered	Recovery %
0.00	360	-	-
263	616	256	97.1
526	909	549	104
658	1040	680	103

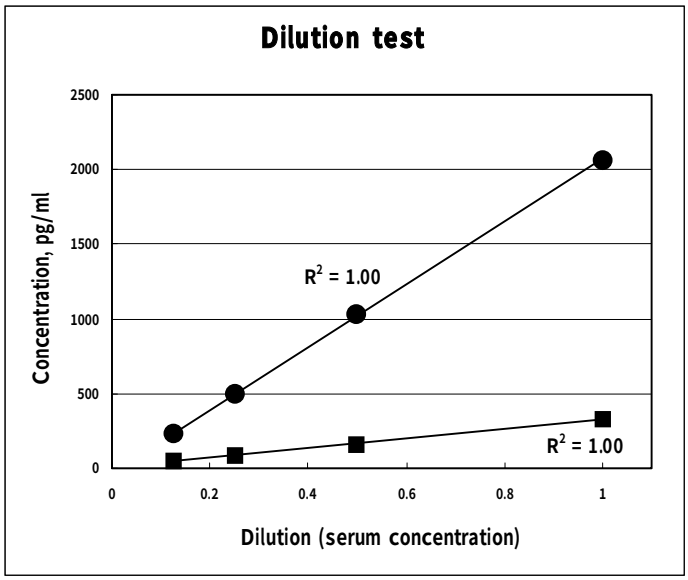
○ **Dilution test**

Dilution test is performed to see whether or not the assay system is influenced by constituents of serum or plasma. The blood sample is diluted with assay buffer by 2, 4, and 8 times for example, then those diluted samples and not diluted sample are assayed. All the assay values should be nearly the same after multiplied by dilution factor, in other words, if their assay values are plotted against concentration of the blood samples (undiluted =1.0), they must be on a linear line.

An example of dilution test (Rat C-peptide ELISA KIT) is shown below.

The test was carried out using two assay sample sera containing different amounts of the target substance.

As shown in the figure, plotted assay values were on linear lines, and each regression lines pass closely to the origin of the coordinates. This means the dilution curves are almost parallel to the standard curve, and assay values give the constant value if multiplied by dilution factors.

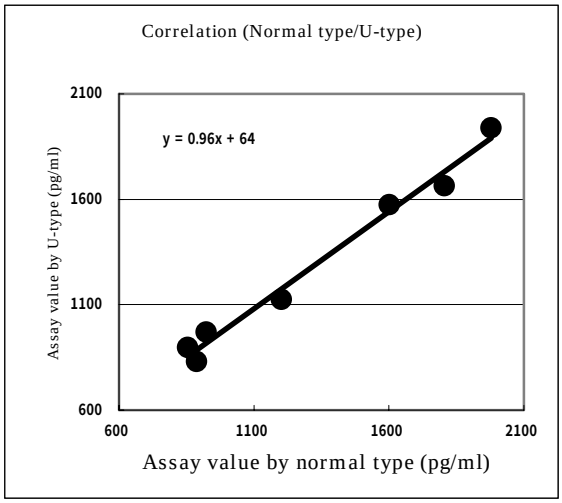


○ **Comparison with other assay system**

Comparison with other assay system is also an important test of the ELISA system in

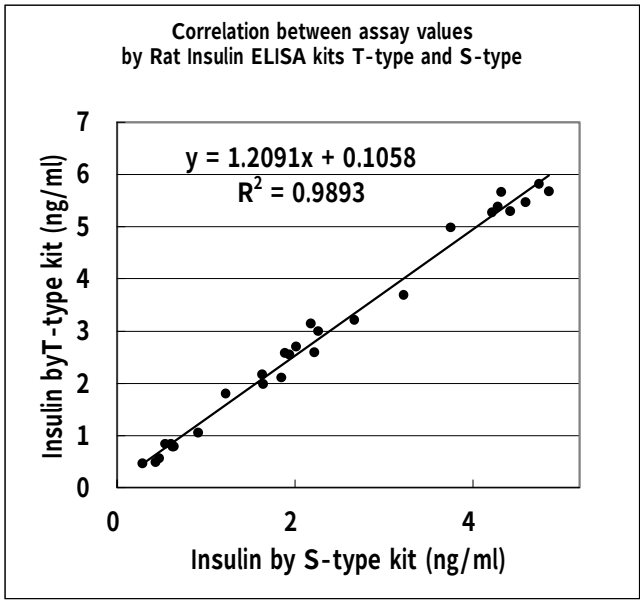
question. In trying this test, we have to collect many samples containing various amounts of substance to be measured in a wide range. Those samples are assayed by two assay systems for comparison. From assay values, their correlation coefficient and an equation of first order regression (y = ax+ b) are calculated. If the standard preparation is commonly used in two assay systems, the slope, a, must be nearly 1.0, and b must be small enough compared with assay values. If those two systems use different preparations of the standard, the slope, a, is not necessarily 1.0, depending on the difference of purity of the preparations. The correlation coefficient should be nearly 1.0.

An example of comparison between Shibayagi's two rat insulin assay kits is shown.



Another example is the comparison of rat insulin ELISA KIT (T-type) with S-type

T-type kit cross-reacts proinsulin, while S-type is more specific to insulin, and this difference possibly makes the slope value 1.2.



14. Evaluation of an ELISA kit from its diagnostic usefulness.

An ELISA system is evaluated also from its usefulness in diagnosis of any disease. If one

disease (D) is known to show significant increase or decrease of the blood level of a substance, and an ELISA system is established for this substance, this ELISA system is tested in clinical sections as follows.

Blood samples are collected from normal subjects, D-patients, patients of other D-like diseases to be distinguished from D, and measured for the substance in question. From the assay data, two indices, sensitivity and specificity (though names are same to the indices for assay performance) are calculated.

Sensitivity: percentage of significant change of the substance to disease D patients

Specificity: percentage of significant change of the substance in disease D to all signigicant change of the substance.

If these the percentages of these two indices are high, diagnostic usefulness of the ELISA is thought to be high.

VI. How to calculate ELISA assay value by EXCEL

In usual step for calculate the assay value of ELISA is to draw a standard curve, absorbance on Y-axis against concentration on X-axis, then to estimate assay value from the absorbance of the sample.

EXCEL is really an excellent tool, however, it does not give X value from Y. so, the usual standard curve by EXCEL is not useful for assay value calculation.

I suggest a method to calculate assay value by using a reverse standard curve where absorbance on X and concentration on Y. The procedure will be shown step by step.

In ELISA, the standard curve is nearly linear and excellent fitness is easily obtained by logarithmic transformation of both absorbance and concentration, the method starts from logarithmic transformation of the data.

Procedure of calculation step by step with an example of our insulin assay data

Input of data in EXCEL spread sheet.

Standard points of rat insulin: 0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 ng/ml

First make up a table for standard concentration and absorbance as shown below. The example shown here is a duplicate assay, and as TMB is used as chromogenic substrate, we measured absorbance at 450nm. If possible, as absorbance, difference of absorbance at 450nm and 620nm is preferable. Subtract the mean of blank absorption from each mean absorbance to make ΔBlank.

	A	B	C	D	E
1	Insulin	Abs.450(Δ620)nm		Mean	ΔBlank
2	10	2.316	2.214	2.265	2.233
3	5	1.312	1.227	1.270	1.238
4	2.5	0.614	0.641	0.628	0.596

5	1	0.217	0.209	0.213	0.181
6	0.5	0.112	0.108	0.110	0.078
7	0.25	0.064	0.061	0.063	0.031
8	0.1	0.045	0.044	0.045	0.013
9	0	0.031	0.032	0.032	

Add two columns for logarithmic transformation.

	A	B	C	D	E	F	G
1	Insulin	Ln(conc)	Abs.450(△620)nm		Mean	△Blank	Ln(△Blk)
2	10		2.316	2.214	2.265	2.233	
3	5		1.312	1.227	1.270	1.238	
4	2.5		0.614	0.641	0.628	0.582	
5	1		0.217	0.209	0.213	0.181	
6	0.5		0.112	0.108	0.110	0.078	
7	0.25		0.064	0.061	0.063	0.031	
8	0.1		0.045	0.044	0.045	0.013	
9	0		0.031	0.032	0.032		

For transformation, natural logarithm is more convenient

To B2 cell, write" =LN(A2) ", the logarithmic value will appear in B2, and using fill-handle, transform other cells in B column(B3-B8, except B9)

Then transform ΔBlk (F column) in the same way.

The result will be:

	A	B	C	D	E	F	G
1	Insulin	Ln(conc)	Abs.450(⊿620)nm		mean.	⊿Blank	Ln(⊿Blk)
2	10	2.302585	2.316	2.214	2.265	2.233	0.803346
3	5	1.609438	1.312	1.227	1.270	1.238	0.213093
4	2.5	0.916291	0.614	0.641	0.628	0.596	-0.51835
5	1	0	0.217	0.209	0.213	0.181	-1.70926
6	0.5	-0.69315	0.112	0.108	0.110	0.078	-2.55105
7	0.25	-1.38629	0.064	0.061	0.063	0.031	-3.49003
8	0.1	-2.30259	0.045	0.044	0.045	0.013	-4.38203
9	0		0.031	0.032	0.032		

By using this table , prepare "a standard curve for calculation" with following steps.

Preparation of reversed graph and regression equation

1. Click "Graph wizard" in tool bar.
2. Choose "scatter diagram"
3. Choose default, i.e." plotting only" then next.
4. Indicate the column of "Ln(conc)" (in our example, B2-B8 as date area.

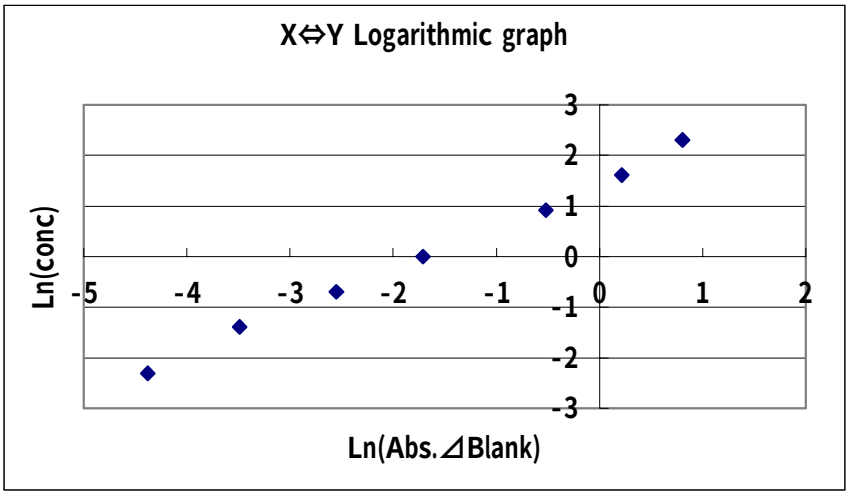
Click" column" as date series

5. Click "series" and choose data area of X.

6. Indicate the column of Ln(⊿Blank) (in our example G2-G8) as date area of X, then "next."

7. Click "Title and label" tag, and write the graph title, names of X and Y axis. Click "legend" tag and uncheck "show legend"

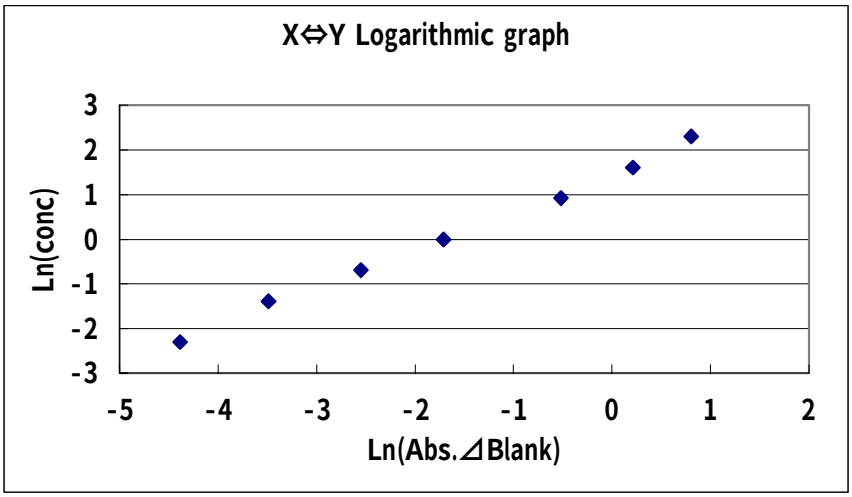
8. Click "finish to show a graph on the data sheet.



As the graph appears as above is not good looking, so we should move X and Y axis.

To move X axis, double click the figure on X-axisto show X-axis setting window, and at "scale" tag uncheck the automatic checking of "intersection with Y, and write the intersection wanted (in our example, -5", then OK. Y-axis is move in the similar way to X-axis. Wite "-3" in our case.

Then the graph appears as shown below.

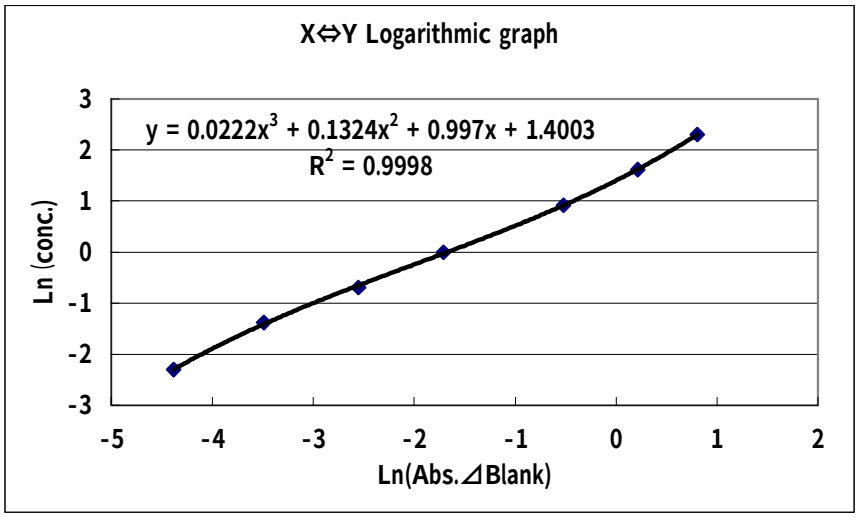


Now let's get regression equation

9. Click one data point on the graph to make color change, and click "graph" in the task bar, then choose "Add regression curve"

10. Choose "multimember regression", and set the order "3". Click "option" tag, and check "show equation" and "show R-2"., then "OK".

Now, we get the reverse standard curve with equation and R^2. From R2, we can estimate fitness of the curve.



The R^2 value, 0.9998, obtained indicates that the fitness of the third order regression curve in our example seems to be excellent.

Let's check fitness of the equation by calculation the assay value from standard data.

Using the equation we can calculate the assay value of samples and also fitness of the regression equation to the standard curve. Before calculation of sample assay values, I recommend to check the fitness.

In order to examine fitness, we add two columns next to the column Ln(ΔBlk) (columns H and I). For column H, we will fill calculated logarithmic concentrations, i.e. Cal.Ln(conc), then we tranform them to normal value, and write them to the column I as Calc.conc. We will explain the step using our example.

To the cell H2, copy a part of the third order equation shown in graph, and change x to G2 like;

=0.02＊G2^3－0.1156＊G2^2+0.9754＊G2+1.3934

and push Enter key. Then 2.298192 will be written into H2 cell. The again point H2 to activate H2, and drag the fill handle of the cell until H8 to calculate all the standard data. The next step is to write "＝EXP(H2)" to the I2, and push Enter key. Then the value **9.956164** will be written in I2. After activation of the cell I2, drag the fill handle until I8. Then all the logarithmic concentration will be transformed into normal value.

You can compare those value in the column I with those in column A, you can examine the fitness.

The results of our example calculation are shown in the table below.

	A	B	C	D	E	F	G	H	I
1	Insulin	Ln(conc)	Abs.450(⊿620)nm		mean.	⊿Blank	Ln(⊿Blk)	Cal. Ln(conc)	Cal.Conc. Fitness
2	10	2.302585	2.316	2.214	2.265	2.233	0.803346	2.298192	9.956164
3	5	1.609438	1.312	1.227	1.270	1.238	0.213093	1.618981	5.047943
4	2.5	0.916291	0.614	0.641	0.628	0.596	-0.51835	0.915984	2.499233
5	1	0	0.217	0.209	0.213	0.181	-1.70926	-0.02788	0.972509
6	0.5	-0.69315	0.112	0.108	0.110	0.078	-2.55105	-0.65002	0.522038
7	0.25	-1.38629	0.064	0.061	0.063	0.031	-3.49003	-1.4103	0.24407
8	0.1	-2.30259	0.045	0.044	0.045	0.013	-4.38203	-2.29422	0.10084
9	0		0.031	0.032	0.032				

Calculation of sample assay values

Prepare a table of [sample absorbance-blank absorbance] for each well, and transform them into logarithmic values. Then fill the logarithmic assay values in the columns “LN(AV)1” and “LN(AV)2,” using the equation as in the fitness examination. Then fill the columns “AV1” and “AV2” with the assay values transformed from the logarithmic assay values. I would be convenient to calculate mean assay values, SD, and CV as shown in the example table below. (AV: assay value)

No.	Abs.1	Abs.2	LN (Abs1)	LN(Abs2)	LN(AV)1	LN(AV)2	AV1	AV2	Mean	SD	CV
1	0.125	0.127	-2.07944	-2.06357	-0.30001	-0.28836	0.740811	0.749495	0.745153	0.006141	0.824131
2	0.138	0.136	-1.9805	-1.9951	-0.22739	-0.2381	0.796608	0.78812	0.792364	0.006002	0.757479
3	0.075	0.077	-2.59027	-2.56395	-0.67968	-0.65976	0.506778	0.516974	0.511876	0.00721	1.408496
4	0.096	0.093	-2.34341	-2.37516	-0.49469	-0.51827	0.609763	0.595548	0.602655	0.010052	1.667886
5	0.186	0.191	-1.68201	-1.65548	-0.00772	0.01192	0.992305	1.011992	1.002148	0.013921	1.389083
6	0.156	0.162	-1.8579	-1.82016	-0.13738	-0.10963	0.87164	0.896166	0.883903	0.017343	1.962064
7	0.256	0.251	-1.36258	-1.3823	0.231465	0.216493	1.260445	1.241715	1.25108	0.013244	1.058621
8	0.897	0.889	-0.1087	-0.11766	1.293463	1.284792	3.645387	3.613915	3.629651	0.022254	0.613122
9	1.254	1.238	0.226338	0.213497	1.633	1.619408	5.119207	5.050098	5.084653	0.048868	0.961079
10	2.213	2.254	0.794349	0.812706	2.286936	2.309934	9.844731	10.07376	9.959246	0.161948	1.626109

In our example, results with 10 samples are shown. More samples can be treated.

Preparation of a template for calculation

It would be convenient to prepare a template for ELISA calculation and store the file. What you should do is only to take out the file and fill the table with absorbance of standard and samples, and store the results table with a new file name. Duplicate assay is intended. The figures 1and 2 indicate well1 and well2, respectively.

Preparation of a template for standard curve.

Procedure

First, prepare a table in EXCEL. Let us call the cells A-1~H-8.

Cells in line 1 are used only for identification. Just write as are shown in the model table.

In the cell C2, write "=LN(A2)"

In the cell E2 write "=(C2+D2)/2

Leave F2 untouched. To the cell F2 write "=E2-average absorbance of blank" after assay.

In the cell G2 write "=LN(F2)"

Leave the H2 untouched. (After assay write the equation to H2)

In the cell I2 write "=EXP(H2)"

Then you will get a table as shown below.

	A	B	C	D	E	F	G	H	I
1	Conc.	Ln(conc)	Ab 1	Ab 2	Mean	ΔBlank	Ln(ΔBlank)	CalLn(conc)	Cal conc
2		#NUM!			0		#NUM!		1

Store the template and use a copy.

How to use the template for the standard curve

Using the data for standard solutions, first finish calculation until the column G.
Before starting calculate the average of blank absorbance.

a. In the column A write concentrations of standard solutions starting from the highest. Then B2 cell will be filled with the logarithmic transformation of the highest standard concentration.

b. Activate B2 cell and drag the fill handle until B8 to complete transformation.

c. Write pairs of absorbance of wells in columns C and D.

d. Point E2 to activate, and drag fill handle until E8 to obtain means.

e. Write "=E2-blank absorbance(calculated above)" in F2, then drag fill handle to F8 for ΔBlank.

f. Activate G2, then drag the fill handle until G8 to obtain logarithm of ΔBlank.

g. Get the reverse regression curve as stated above (p.32)

h. Write the equation of 3rd order regression curve in H2 as described above (p.34), then click.

i. Activate H2, and drag the fill handle until H8.

j. Point I2, then drag I2 fill handle until I8.

k. Compare the assay values in the column I with the concentrations in A for fitness.

The equation written in H2 can be used for sample calculation, by copying.

Preparation of a template for sample calcualtion

First, prepare a table in EXCEL. Let us call the cells A-1~N11 (The template uses only

A-1~N2).

Cells in line 1 are used only for identification. Just write as are shown in the model table.

Ab: absorbance of sample before subtraction of blank absorbance

ΔBk: absorbance of samples subtracted blank absorbance

LN( ): natural logarithm of ΔBk

Cal:natural logarithm of calculated sample assay value

Av: Assay value of sample, transformed to normal number

Mean: acerage of well 1 and well 2, SD: standard deviation, CV: Coefficient of variation

Procedure

In the cells in the column A, write sample number.

The cell D2 is left empty until use. After assay write "=B2-Blank"

Blank: mean blank absorbance value

The cell E2 is left empty until use.

In the cell F2, write "=LN(D2)"

Leave the cell H2 untouched. (After assay write the regression equation)

The cell I2 is left untouched

In the cell J2, write "=EXP(H2)"

In the cell L2 write "=(J2+K2)/2

After assay fill M2 with SD using function STDEV

In the cell N2, write "=M2/L2*100"

(Do not include quotation marks in writing.)

Then you will get a template table as shown below.

Store the template until use, and use it after making a copy.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	No.	Ab1	Ab2	⊿Bk1	⊿Bk2	LN(1)	LN(2)	Cal.1	Cal.2	Av.1	Av.2	Mean	SD	CV
2	1					#NUM!	#NUM!			1	1	1	0	0
3	2													
4	3													
5	4													
6	5													
7	6													
8	7													
9	8													
10	9													
11	10													

How to use the template for sample calculation

After preparation of this template table, store the file. After an assay, take out the file and once store with proper naming. then input the first pair of absorbance in the cells B2 and C2, and "=D2-average blank absorbance" in D2, and regression equation in H2, as shown in the previous page. In this case, By dragging of the fill handle of D2 to E2, the function will be copied to E2, and the cell numbers are changed automatically. The situation is the same with G2, I2, and K2. The results of calculation appear in those cell of the line 2. By dragging each fill handle down to the last cell, results of calculation will appear when each pair of sample absorbance are input. The calculation will be completed after the input of absorbance of the last sample.

Number of samples is not limited.

You can download the template ready to use from Shibayagi's web site.

http://www.shibayagi.co.jp/

When using downloaded template, prepare a copy first to avoid erasing.

VII. Important points in performing ELISA and improvement of assay performance

1. Sampling and treatments of samples

Serum or plasma?

In general, we recommend using serum samples.

In getting plasma, heparin is most often used as an anti-coagulant at a final concentration of 1.2-12U/ml (10-100μg/ml), however, in some kits, such as rat/mouse TSH assay kit, heparin interferes with the reaction, causing concentration-dependent poor assay values. In such case, we recommend to use EDTA-2Na (at a maximum final concentration of 1mg/ml). Use of fluoride must be avoided because fluoride ion is a potent inhibitor of peroxidase. Even if wells are washed after the binding reaction to capture antibody a trace amount of remaining fluoride ion may interfere with the enzyme activity.

An important phenomenon with frozen plasma is that an insoluble substance (fibrin) will be formed when thawed. In this case, the sample must be vortexed and centrifuged, then the insoluble cluster flowing in the plasma should be taken out by a thin wire needle sharply bent at an end. If such fibrin remains in the sample, it may clog the tip of a pipette and influences assay variability.

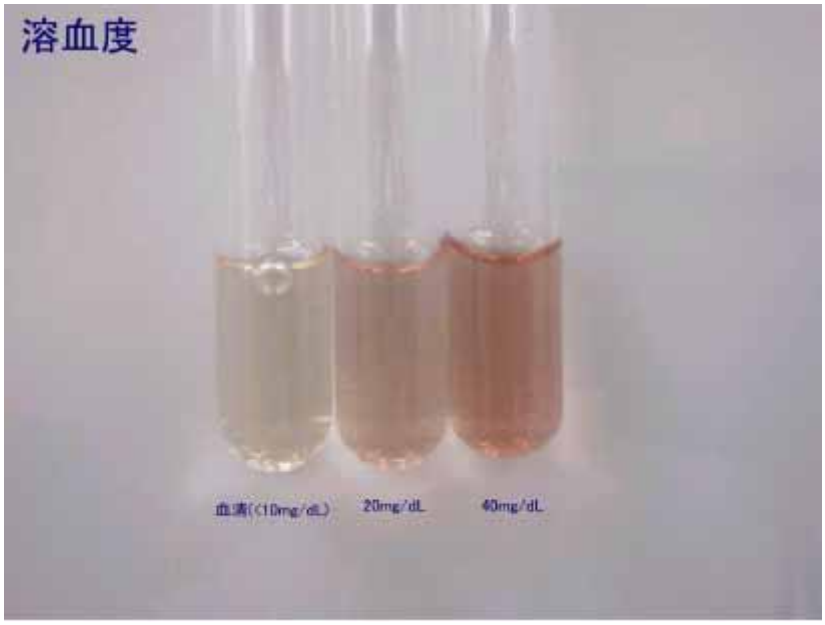
Influence of hemolysis

As an example, the results of a test with rat insulin assay kit are shown below. The test was carried out with n=5.

With hemoglobin concentration above 40mg/dl, the assay values are lowered significantly.

Samples		Hemoglobin concentration (mg/dl)			
		0	10	20	40
1	Assay value, ng/ml %	0.521 100%	0.533 102%	0.500 96.0%	0.456 87.5%
2	Assay value, ng/ml %	1.04 100%	0.998 96%	0.989 95.1%	0.925 88.9%
3	Assay value, ng/ml %	3.56 100%	3.35 94.1%	3.39 95.2%	2.56 71.9%

Hemoglobin concentration and sample color



Be careful! Mouse blood is easily hemolysed.

Preservatives

Antiseptics

Sodium azide (NaN_3) is very often used as an antiseptic. But better to avoid this substance because it is a potent inhibitor of peroxidase from the similar reason to fluoride ion.

In ELISA, NaN_3 in samples is washed out after antigen-antibody reaction, but we are not sure about complete elimination. We are afraid of influence of peroxidase inhibiting action of NaN_3 remaining in wells after washing.

ProClin 300 (at final concentration 0.02-0.01%) may be allowed.

Protease or peptidase inhibitors

In some cases, a preservative such as aprotinin/trasylol, which is a kallikrein (a serine protease) inhibitor, is necessary to protect the substance to be assayed. Aprotinin is an alkaline polypeptide of 6.5kDa, and also inhibits trypsin, chymotrypsin, and plasmin. Aprotinin binds these protease at 1:1 ratio. Aprotinin should be added to serum (plasma) at a final concentration of 100-500KIU/ml.

An example of using aprotinin

Aprotinin (50,000KIU) (Wako Pure ChemicalsCat. No. 010-11834) is dissolved in 5ml

of physiological saline to make 10,000KIU/ml. The this solution is added to the blood sample at 1/100.(v/v) (final concentration is 100KIU/ml). Then serum or plasma is separated.

Amount of aprotinin is expressed as KIU（kallikrein inhibitor unit）, TIU (trypsin inhitor unit),

BAPNA unit (N α -benzoyl DL-arginine-p-nitroanilide unit), BAEE unit (Benzoyl-L-arginine ethylester unit), or UIP (Peptidase inhibitor unit).

1 TIU is the amount to reduce 2 trypsin units by 50%.

1trypsin unit is the amount of trypsin to hydrolyses 1 μmole of BAPNA per minute at 25°C, pH 7.8.

1 KIU is the amount to inhibit 32.5FIP unit trypsin.

FIP unit: amount of enzyme to hydrolyse 1 μ mole of BAEE per minute,

1 TIU 900KIU (J. Gen. Physiol. 19, 991, 1996) 229,100 KIU = 254 TIU

1 KIU=8UIP

In GLP-1(glucagon-like peptide-1) assay, it is necessary to use DPP-1(dipeptidyl peptidase IV) inhibitor because the active forms of GLP-1, GLP-1(7-36)amide and GLP-1(7-37), are hydrolyzed by DPP IV to GLP-1(9-36)amide, and GLP-1(9-37), respectively, and cannot be measured.

pH of samples

Serum or plasma, when fresh, shows pH near neutral, however, it very quickly goes to alkaline more than pH 8 by losing CO_2. Our observations on the change of pH of mouse serum and plasma are shown below.

Mouse: BALB/cA, 6 weeks of age, ♂, free access to food and water.

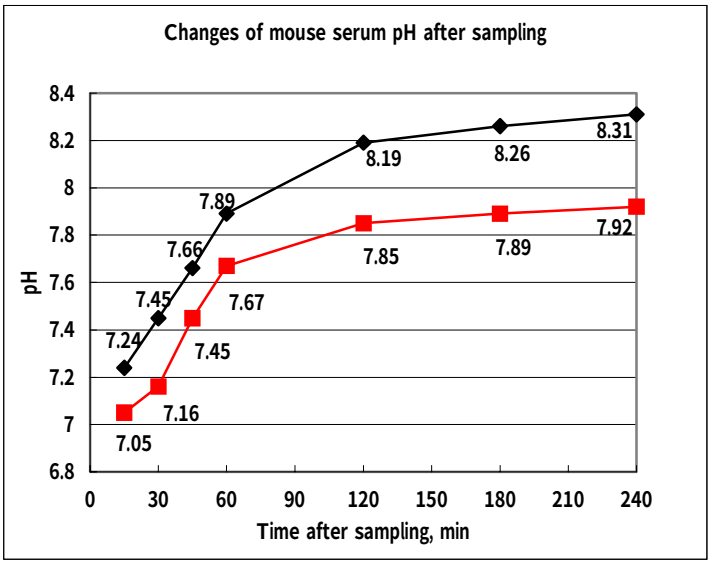
Samples sera from 2 mice were obtained by heart puncture, and kept standing at 2~8°C in PP vials after preparation,

Sample plasma from 2 mice were obtained by heart puncture using EDTA-2Na, and kept standing at 2~8°C in PP vials after preparation,

pH meter: Twin PH (HORIBA) Model: B-111

a. Serum

Immediately after preparation sera showed physiologically neutral pH, however, within 1 hour after preparation, pH went up rapidly by losing CO_2, and reach around 8 after 2 hours.



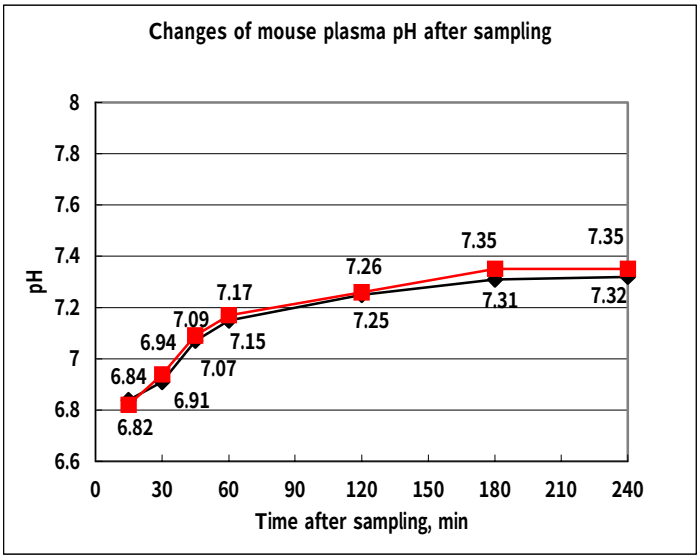
Individual difference was also observed.

In the present observation, sera were kept at 2~8°C. If the temperature goes up, pH will change more quickly.

When frozen the solubility of CO2 is reduced, and shows alkaline pH very quickly after thawing.

b. Plasma

The figure shows the observation with plasma obtained using EDTA-2Na.



EDTA itself is rather strong acid, but its sodium salt is weakly acidic, and used as anticoagulant. So, the plasma showed slightly acidic pH at 15 min. after preparation. pH also went up, but not so markedly, to 7.2 after 1 hour, and 7.3 after 4 hours.

EDTA-2Nais generally used at final concentration of 1mg/ml, namely 0.1%. It reduced plasma pH to 6.8 at first, and showed some buffering action to resist pH upraising due to CO_2 loss.

If we use heparin sodium as anticoagulant, the situation may be different. Heparin is a sulfated polysaccharide. According to Merck Index, pH of 1% aqueous solution of heparin sodium is 6.8~7.5. Heparin sodium is used with final concentration of 10μg/ml (1.2U/ml), i.e. 0.001%. So, its effect on plasma pH will be negligible, and change of pH after preparation may be similar to that of serum.

In alkaline pH, the antigen-antibody reaction is interfered. In ELISA, if serum or heparinized plasma samples are directory added to the empty antibody-coated wells and incubated at room temperature for several hours, pH would be upraised, resulting in cancellation of the assay or giving inaccurate assay values. So, in such case, beforehand dilution of serum or heparinized plasma with assay buffer will be helpful.

When in cell- or tissue culture media or in vitro incubation media are assayed, pH if those samples may be also upraised if buffered with bicarbonate. They have to be treated with phosphate buffer before assay.

Storage temperature and freezing-thawing

Sample storage temperature is better to be lower than -35°C. Ultra-low temperature such as -80°C is recommended for a long-term storage.

A long storage in a home freezer is not recommended because the sample is frozen very slowly and the solute may separate from solvent, and is concentrated at to the bottom of the storage tube. This may sometimes cause inactivation.

When samples are frozen and stored, first snap-freeze the sample using dry ice-acetone mixture or dry ice-ethanol, then transfer them into a freezer.

Repeated freezing and thawing is also harmful to the protein, and may cause inactivation.

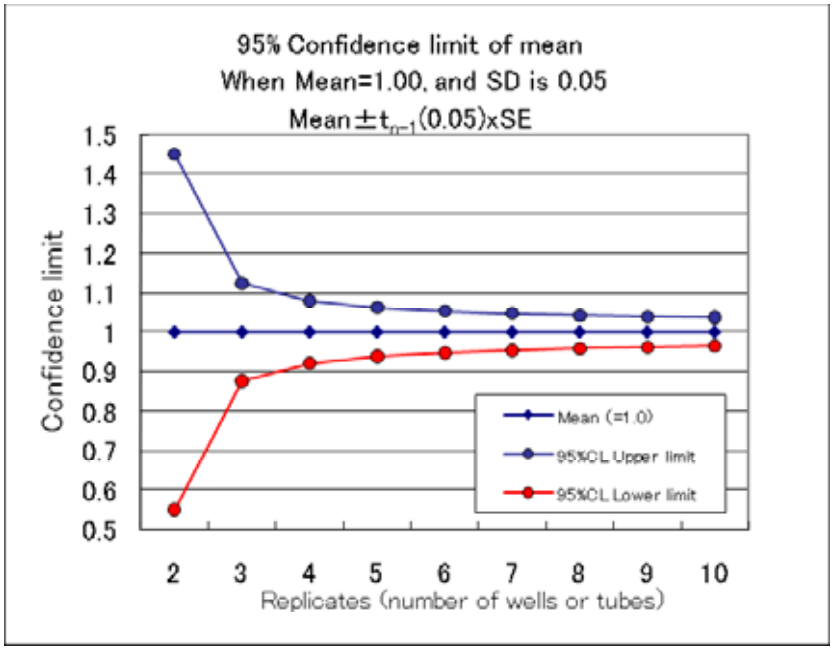
When samples are taken out from the freezer and thawed, never forget to vortex these

samples because the solution after thawing is not homogeneous, and the bottom area contains more solute.

How many wells should we run per one sample?

– Number of replicate and confidence limit of mean

In ELISA someone assay samples in duplicates, and others in triplicates. The graph shown below indicates how 95% confidence limits change according to number of replicate.



If number of replicate is n, 95% confidence limit of the mean is calculated as;

Mean ± $t_{n-1}(0.05)$ x SE

SE: standard error

$t_{n-1}(0.05)$: value of t found in t-table at p=0.05 and degree of freedom= n-1

If a sample is measured in duplicate, and mean assay value is 1.00 with SD is 0.05 (i.e. CV =5%), 95% confidence limit will be from 0.55 (lower limit) to 1.45(upper limit). This means that the reliability of the obtained mean value is very low.

Then, how about in the case of triplicates assay? With same mean assay value of 1.00 and SD of 0.05, the confidence limit will be from 0.88 to 1.12. Addition of only one well more changes the reliability nearly 4x. The influence of replicates> 4 on reliability is not so big.

So, the author recommends triplicates assay from the point of efficiency in reliability and economy.

2. Stability of assay samples

In assay, the problem of sample stability, i.e. how long the substance to be measured can keep its immunoreactivity, in serum or plasma, is very important. Blood samples also contain enzymes to destroy peptides or proteins, and stability against those enzymes differs from substance to substance.

In establishment of any assay system, the stability of the target substance has to be examined by changing storage conditions and periods. As test samples quality control sera (QC) are suitable. QC are prepared in a larger volume to contain low, (middle), and high levels of target substance, and stored at ultra-low temperature in many aliquots, and are measured together with samples in every assay to make sure of the assay reproducibility and robustness.

Examples of guideline for stability test using LQC (low content QC) and HQC ,by triplicates assay.

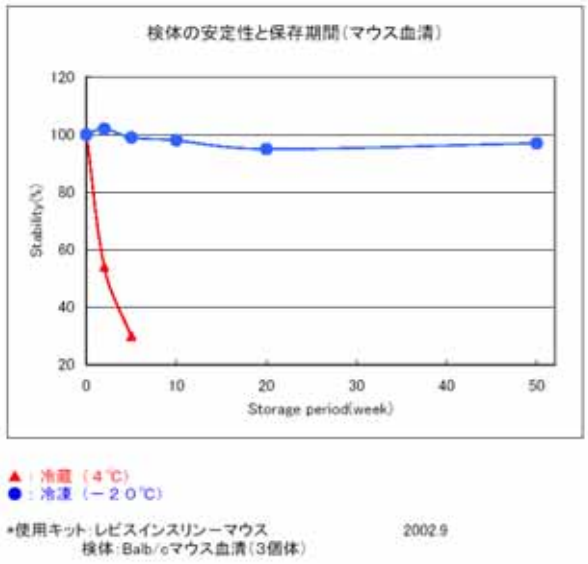
Stability at room temperature: 2 hours

Stability at refrigerator temperature: 24 hours

Stability against freezing-thawing: 3 times

Stability for long period: at –20℃ or－80℃

We tested mouse insulin stability in serum. Serum samples were stored in a refrigerator (4°C) and in a freezer (-20°C). As shown in the right figure, insulin was stable for 50 weeks at –20°C, while it lost reactivity was lost quickly at 4°C.



Freezer of –20°C is not trustable for the constancy of temperature, and freezing at –20°C may cause heterogeneous concentration of protein solution, use of a freezer of –35°C or lower temperature is recommended. Snap freezing of samples in dry ice-acetone mixture or in liquid nitrogen before placing them in a freezer is also recommended.

3. Influence of humidity and air stream

As ELISA is carried out in small volume of reaction mixture in shallow wells, the ratio of surface area to the liquid volume is large. This means that evaporation of water from the surface during incubation cannot be ignored. During all the incubation process, the well-plate should be covered using the attached plate cover. Plate cover is effective only under the most suitable condition, i.e. room temperature, humidity more than 50%, and air stream of less than 0.2m/sec. If the humidity is less than 30%, and the air is moving at more than 0.4m/sec, even with plate cover, good calibration curve cannot be obtained. Absorbance of all the wells including blank will become high, and differences of absorbance between lower standard points become smaller and assay sensitivity becomes worse. Such trouble may be due to abnormal adsorption by water evaporation at the edge of the solution in the well. The best way is to control of humidity and air stream by using a humidifier and turn down the air flow of the air conditioner. But if the suitable condition cannot be attained, we recommend to get a small semi-transparent plastic box, and put moistened paper towel on the bottom. Place the well-plate with plate cover in the box and close during incubation. Maybe you can seal the well-plate with Parafilm, however, it would be troublesome to use Parafilm in every incubation process.

4. Pipettes

Selection of pipettes

Pipettes for samples and standard solutions are especially important because their precision directly influence the assay precision. When you add samples or standards at a volume of 5 or 10 μl, for example, use a pipette with the maximal setting volume of 5 or 10μl, and never use with the maximum setting volume of 100μl or more. In other words, use volume-matched pipette. If you measure 100μl with maximum 100μl pipette, the precision of the delivery volume is about 1%, however, when you measure 5μl with the same pipette, the precision will be nearly 30%.

With a widely used tip-type pipette, the important factor is the temperature of the solution to be delivered. If the solution temperature is higher than room temperature, it may cause an expansion of the air in the tip and the pipette, thus delivers smaller volume of the solution.

On the other hand, colder solution than room temperature causes shrinkage of the air, and delivered volume will be more than setting. If the sample solution were highly viscous, the liquid attached to the inner wall of the tip would be more than that of thinner solution, so the first delivery volume would be less. The amount of solution attached to the outside of the tip would be also important, and in order to remove this, “touch and go” procedure is necessary.

We do not recommend a multi-channel pipette. We are afraid that it possibly scratches the bottom of wells because of difficulty of holding it in parallel with wells, resulting in removal of antibody coating the surface of the bottom and big variation.

In pipetting reagent solution to be added to all the wells, we recommend a repeating dispenser such as ”Eppendorf multipette plus”. Using this type of pipette, we can dispense rapidly and are able to save time without scratching the bottom.

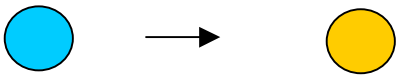
Whenever you start using a new type of pipette, please, read the instruction paper from the pipette maker carefully to avoid misuse of the pipette. Please, refer to Part V.

VIII. Trouble shooting in ELISA

Contents
Coloration in general
Standard curve
Samples
Assay precision (variation)
Plate seal
Reagents

Coloration in general

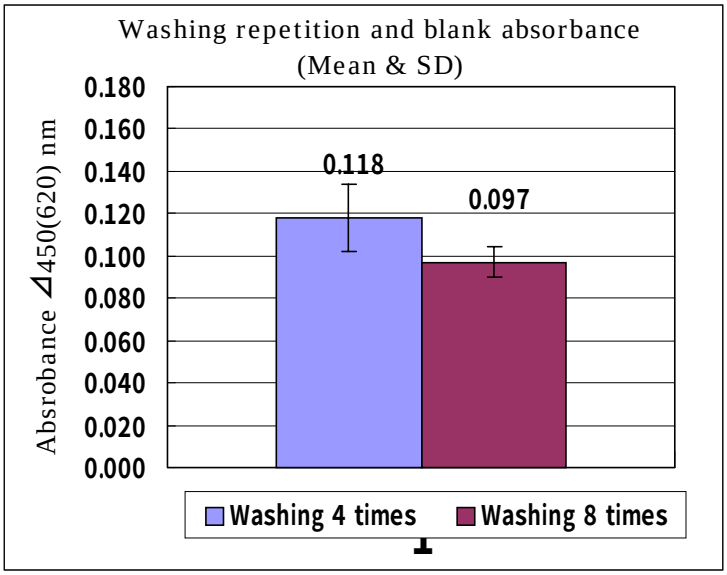
Troubles	Possible Causes and Measures
Poor or no coloration after the last step.	1) The standard or samples might not be added. 2) Reagents necessary for coloration shown below might not be added. ①Biotin-labeled antibody ②HRP-conjugated avidin ③TMB or OPD 3) Wrong reagents related to coloration might have been added. Wrong dilution of biotin-labeled antibody or HRP-avidin conjugate. 4) Contamination of enzyme inhibitor(s). Bottles or vessels used for dilution of HRP-avidin conjugate might have been contaminated with sodium azide (NaN_3) or fluoride, and inactivates HRP.. Especially, NaN_3 is very often added to various buffer solutions and contaminates vessels. So, please, be careful. 5) Influence of the temperature under which the kits had been stored. Freezing and thawing might cause denaturation of HRP-avidin conjugate. 6) Excessive hard washing of the well plate. Especially, in using an automatic plate washer, if you set the washing power to give a strong water pressure, it may result in washing off of the antibody coated on the well. The machine should be adjusted to give the best coloration. Also, in manual washing using a jet bottle, be careful not to press the bottle strongly. Pour the washing buffer gently onto the well. We recommend you to increase frequency of the gentle washing rather than strong flashing for better washing. 7) Addition of TMB solution soon after taking out from a refrigerator might cause poor coloration owing to low temperature. All the reagent solutions for ELISA should be used after fully warmed up to room temperature (20-25°C)
Satisfactory absorbance was not obtained by the densitometer though the coloration is apparent to the eye.	The plate-reader might not be adjusted to the correct wavelength (450nm for TMB). ⇒ Check the reader and adjust the wavelength. In the case of a filter-type plate-reader, check the wavelength of the filter, and replace for right one when wrong.
The wells showed coloration before the addition of reaction stopper.	The chromogenic substrate TMB used in our assay kits shows bluish color when oxidized by HRP at the neutral pH, and turns to yellow-orange color under an acidic pH caused by addition of sulfuric acid (the reaction stopper). This color absorbs the light of 450nm wave length. So, the coloration before the addition of the reaction stopper is natural.

	Coloration After enzyme action After reaction-stopper 
All the wells showed high coloration.	TMB solution might have been oxidized by some agents..

Standard curve

Troubles	Possible Causes and Measures
Flat standard curve. All the wells for the standard curve showed very low coloration, though sample wells gave good coloration.	Standard solutions are not added. In the dilution of original standard solution, other solution than the right original standard solution might be used, or simply the original standard solution was forgotten.
Flat standard curve. All the wells for both the standard curve and samples showed very low coloration.	Please, read the section of coloration in general.
Absorbance of the blank (0-conc.) was higher than that of the lowest standard.	1) Possibility of edge effect. Wells locating at the edge (columns 1 and 12, and lines A and H) of a well-plate are liable to be influenced by heat from outside circumstances, and the reactions in those wells may proceed more rapidly or more slowly than in other inside wells. We call this phenomenon “edge effect”. Sometimes, a well-plate, kit reagents, and samples which have been stored in a refrigerator are used soon after taken out while they are still cooler than room temperature, wells at the plate edge would be warmed up than other inside wells, causing reactions to proceed rapidly. In winter those wells may be heated by warm air derived from a heater and a radiant heat from a stove or a steam heater. As a result, the absorbance wells along the edge may be higher than those of low standards, or in duplicated standard wells those wells located along the edge of the plate always give higher absorbance than corresponding inside wells. To minimize the edge effect, 1) get back well-plates, reagents, and sample fully to the room temperature, 2) avoid working near heat source and away from the outlet of air conditioner, and 3) cover the well-plate with a transparent or semi-transparent plastic box twice bigger than the plate during incubation process. The wells used for blank are in most cases located at A1-A2 or H1-H2 (in duplicated standard wells). And are most liable

	to be influenced by the edge effect. One possible cause of the edge effect on such blank wells may be heat from the fingers which keep holding the well-plate during the addition of TMB solution. Please, do not hold the well-plate. To avoid the holding, we recommend the use of “non-slip sheet”…A very cheap and thin rubber sheet generally used under a doormat. In our laboratory, we cut such sheet to a size a little bigger than a well-plate, then put a plate on it. By doing this the plate will never move while pipetting without holding. If you do not like to use non-slip sheet, just hold the plate at a side with a fingertip to minimize the heat transfer. 2) Possibility of dried-up well The long interval between the end of washing buffer removal and addition of the reagent solution of the next step may cause the dryness of the wells owing to the air stream from the air conditioner. Please, check such possibility and try to make the interval as short as possible. 3) Wrong dilution of reagent supplied as concentrated solution. Please, check the dilution procedure. 4) Incomplete washing and carry-over. If you use an automatic plate washer, check the aspiration and washing volumes, and the amount of solution left in the well. Do not use pipette in washing. In order to avoid carry-over, every addition of the reagent solution and washing buffer (including standard solutions) should start from the blank wells to higher standard wells. Especially in the first addition of washing buffer after the reaction between the coated antibody and standard/sample solution, we recommend you to use a repeating dispenser set at 250μl to add washing buffer. In the second washing downward, a flashing bottle with a nozzle will do. 5) Improvement of blank absorbance and its variation by increase repetition of washing. As shown in the graph below, in our trial, increase of washing repetition from 4 times to 8 times lowers blank absorbance as well as standard deviation. This is especially effective in the step of washing after incubation with HRP-conjugated antibody or biotin-labeled antibody and HRP-conjugated avidin.

	 5) Possibility of excessive reaction Incubation of the well-plate must be carried out at the right temperature for the right period indicated in the instruction paper.
Absorbance of the standard decreased when standard concentration increased.	Order of the standard solutions might be reversed.
The standard curve obtained was not smooth.	There might be some mistake in the serial dilution of the original standard solution. Pay attention to volumes of standard and buffer solutions and enough mixing. For beginners, we recommend to use serial dilution with the interval of 1/2. This will be helpful in avoiding dilution mistake.
The standard curve obtained moved toward right than that shown in the instruction paper.	1) The standard preparation might have been denatured. 2) The wrong initial dilution of the original standard solution. 3) The vortex mixing in the dilution of original standard solution might not be enough, and the mixture might not be homogeneous. 4) The reaction period might not be enough by any mistake.
In duplicated standards, those wells located along the edge of the plate always gave higher absorbance than inner wells.	This may due to “edge effect”. Please, read above section describing the edge effect.

Samples

Troubles	Possible Causes and Measures
Assay values were not obtained because samples gave lower absorbance than that of blank though the standard curve seemed to be normal.	1) In case NaF-coated blood-sampling tubes are used, or NaN_3 is added to assay sample as a preservative, they may influence the enzyme action of HRP though wells are washed before the addition of HRP-conjugate. The best way is not to use NaF and NaN_3. The preservative NaN3 can be replaced with ProClin 300 (final concentration, 0.02-0.01%). In some kits, heparin is harmful for antigen-antibody reaction. We rather recommend serum than plasma. 2) Inactivation of the substance to be measured. Inactivation may happen during storage of samples. Blood samples should be snap-frozen and stored at lower than –35°C, preferably ultra-low temperature like –60°C. Addition of a kallicrein inhibitor like aprotinin (100~500KIU/ml) is recommended. We suggest examining the best procedure to avoid inactivation for your laboratory. DPP-IV inhibitor is necessary in measurement of GLP-1. 3) In order to check the presence of any interfering substances in blood samples, please, try a dilution test where sample is assayed after serial dilution, and see if the “assay value x dilution factor” gives always same value. 4) Low assay values owing to hemolysis. A slight hemolysis will not influence on assay value, however, too much hemolysis (hemoglobin concentration more than 40mg/ml) may interfere significantly with the assay reaction. 5) Low assay values due to pH of serum/plasma samples. Blood samples like serum or plasma easily lose carbon dioxide soon after preparation, and pH will become alkaline (> pH 8) which may interfere with antigen-antibody binding. 6) Blood samples obtained from animals by heavy ether anesthesia contain ether which may influence on assay reaction. 7) Deep ether anesthesia may reduce the blood levels of some hormones like TSH and prolactin. 8) Response of blood levels of anterior pituitary hormones may be influenced by many anesthetics via hypothalamic control. For example, effect of cold exposure on TSH level is blocked or retarded by many anesthetics.
Lower assay values with normal animal samples compared with those obtained with other maker’s kit, or those appeared in previous reports.	This may be due to the difference in purity of the standard preparations. If the standard preparation is of lower purity, assay values will be high, and highly pure standard preparation will give lower assay values, when the assay values are expressed by the weight of the standard preparation employed. If the values are expressed as the international standard unit (or weight) the assay values will remain similar range. This phenomenon very often happened if you check various reports published in the past.

Lower assay values with normal animal samples compared with those obtained in the previous assays using the same kit.	1) The substance in the sample might have been denatured during the storage. 2) There might be some seasonal or diurnal changes in the blood level of the substance to be measured. 3) We recommend the use of positive control samples which have been prepared and divided into many aliquots, and stored at ultra-low temperature. One aliquot is measured as a sample in every assay. This will be helpful in quality control of assays.
Higher assay values with normal animal samples compared with those obtained with other maker's kit, or those appeared in previous reports.	1) This might be due to the same reason stated above, i.e. difference in the purity of the standard preparation used. 2) The standard preparation in the present assay might be inactivated by denaturation. Please, check the standard curve if the curve has not shifted to the right. 3) There might be some seasonal or diurnal changes in the blood level of the substance to be measured. You can check it by using control samples in each assay.
Assay values were not obtained with samples from the tissue or cellar extraction, chromatographic, isoelectric focusing, or electrophoresis.	1) pH of the sample might be out of the permitted range of the assay (pH 6.5 –8.0). Please, check pH and, if necessary, neutralize by dilution with buffer, or by small volume of acidic or alkaline solution. 2) Contamination with some organic solvents. Organic solvents may interfere with the assay reaction. Please, dilute them with buffer, or remove solvents with nitrogen stream or placing under reduced pressure for same period. Validity of a treatment method should be checked by a recovery test.

Assay precision (variation)

Trouble	Possible Causes and Measures
Big variation between two wells in duplicated assay was observed.	The major factors making assay variation bigger are heterogeneous proceeding of the reaction, unsuitable pipetting, and heterogeneity of samples. Factors causing heterogeneous proceeding of assay reactions. 1) Scratching the bottom of the well by aspirator tip during aspiration of washing buffer. 2) Scratching the bottom of the well by pipette tip during addition of standards, samples, or reagents. Addition of the reagent solution using a pipette with 8 or 12 tips is not recommended because holding of such pipette in parallel with the well-plate is very difficult and may easily cause

	scratching. The antibody coated on the bottom of well is not so stable, and may be removed by scratching or hard water jet. The loss of antibody makes heterogeneity among wells. 3) Assay might be started while the well-plate was still cooler than room temperature, and this made the heterogeneity in the temperature among wells, and the edge effect happened. The well-plate and all reagents as well as assay samples which have been stored in a refrigerator, should be set back to room temperature by placing them outside at least for 1.5 hour before starting assay. 4) Air stream, warmer or cooler than room temperature from air conditioner, cooling system of various apparatus including personal computer might influence well-plate temperature. Heat radiation from a stove or instrument also might give the same effect (edge effect). 5) Air stream from air conditioner or other instruments might dry wells. 6) Insufficient removal of washing buffer from the wells might dilute reagent solution added in the following step of the procedure. The washing buffer should be completely removed by striking well plate upside down on folded several sheets of paper towel. Assay variation due to pipetting 1) Variation of pipetting of standard solutions and samples directly caused assay variation. To use proper pipette in proper way is one of the most important points in ELISA. The upper limit of the pipette must be coordinated to the volume of standard or sample. Never use 100µl pipette, for instance, for delivering 5 - 10µl standard or sample solution. Also, it is necessary to check the variation of every pipette with proper interval. 2) There are two methods for the tip-type pipette do not mix up “Pre-wetting method” and “Co-washing method”. These methods were described in the text (Part V). 3) Temperature of solution to be delivered might influence variation in assay. If solution to be delivered is cooler or warmer than room temperature, the volume of the solution may be smaller or larger than setting, respectively. All the solutions used in assay should have been stayed at room temperature for enough period to get to the room temperature. 4) Choking up of the pipette tip might influence the variation by decreasing the volume to be delivered. If plasma samples are frozen-stored and thawed, there formed fibrin particles which chokes up a pipette tip, interfering with pipetting. Please, check carefully the state of the samples after thawing, and if you find fibrin particles, remove them by any proper way.

	5) Big variation would be obtained if the sample is not homogeneous. When serum/plasma samples are stored frozen, please be careful about the freezing method and temperature. Slow freezing causes concentration gradient of protein components, leaving high concentration in the bottom. Also, when thawing thinner part will remain as ice at the top. So, samples should be snap-frozen in a very low temperature obtained by using dry ice-acetone mixture or dry ice-ethanol mixture, and stored under ultralow temperature if possible. After thawing, please, stir the sample well on a vortex mixer.

Plate seal

Trouble	Possible Cause and Measure
Plate seal could not be removed completely, and some parts remained on the plate.	The seal might be removed soon after the plate was taken out from a refrigerator. The plate seal has been attached to the plate with heat (without any glue). If you try to remove seal from the plate soon after taking out from a refrigerator, it is not easy to remove completely. Please, wait until the plate gets back to the room temperature. At room temperature, the seal is easily peeled off.

Reagents

Trouble	Possible Cause and Measure
Some crystals were found in the reagent solution.	From the solubility of the components, some crystals might be formed in reagent solution. In such case, please, warm up the solution by placing the bottle in warm water (lower than 37^{o}C) until those crystals are dissolved, then stir the bottle and place it in water of room temperature for some period.

Revised 2010/03/25