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Short communication

# An expression of within-plate uncertainty in sandwich ELISA

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**Abstract**

This paper puts forward a method to describe an equation of the within-plate uncertainty (relative standard deviation (R.S.D.) of measurements) as a function of analyte concentration in sandwich enzyme-linked immunosorbent assay (ELISA). A kit for thyroid stimulating hormone is taken as an example. The pipetting procedures of analyte solution and chromogen–substrate solution and absorbance inherent to the wells of a microplate are identified as major error sources and their variability is included as parameters in the uncertainty equation. These parameters can be determined by the experiments with distilled water. The theoretical R.S.D. is shown to be in good agreement with the results of the repeated experiments using the real samples. Since the theory gives a continuous plot of R.S.D. against concentration, the uncertainty structure of the ELISA kit can be recognized over a wide concentration range and the detection limit and quantitation range can easily be determined on the plot.


*Keywords:* ELISA; Thyroid stimulating hormone; Within-plate uncertainty

## 1. Introduction

The uncertainty of measurement is the major unifying principle for data quality [1]. The definition by ISO is that the uncertainty is the parameter, associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand [2]. The measurand means a particular quantity subject to measurement, e.g., concentration, and not an actual measurement result. The ISO definition can be put into other words: the uncertainty is the interval within which the true value lies with a specified probability [1].

The uncertainty is generally expressed by standard deviation (S.D.). Evaluation methods for the uncertainty are specified by *Guide to the Expression of Uncertainty in Measurement* (GUM) [2]. Type A evaluation of uncertainty is performed by the statistical analysis of a series of observations. The well-known equation for S.D., $\sqrt{1/(n-1)\sum_{k=1}^{n}(q_k-\bar{q})^2}$, is used for the type A evaluation. Type B evaluation is a means other than the repeated observations (type A). It may include previous measurement data, knowledge of the behavior and properties of relevant materials and instruments, manufacturer's specifications and so forth.

The rule of error propagation [3] is also taken into account by GUM [2]. A measurand, $Y$, if not estimated directly, can be related to $N$ other quantities, $X_1, X_2, \ldots, X_N$: $Y = f(X_1, X_2, \ldots, X_N)$. The S.D. of $Y$, called combined standard deviation [2], $u_C$ is expressed as $\sqrt{\sum_{i=1}^{N}(\partial f/\partial X_i)^2(\mathrm{d}X_i)^2}$ where $\mathrm{d}X_i$ denotes the S.D. of $X_i$.

The profound influence of the concept, uncertainty, on many aspects of analytical chemistry is no need to say [1]. Neither is the widespread utility of enzyme-linked immunosorbent assay (ELISA). The purpose of this paper is to propose a general method for drawing the continuous plot of relative standard deviation (R.S.D.) against analyte concentration, called precision profile [4], in a sandwich ELISA kit. The type A evaluation and combined standard deviation are referred to. The within-plate uncertainty of a kit itself is

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focused on and the matrix effects of samples (e.g., blood or food) are not considered.

The importance of the precision in ELISA has often been stressed [4–16]. To the authors' knowledge, however, attempts to elucidate the uncertainty structure of sandwich ELISA could not been found in literature.

## 2. Materials and methods

The ELISA kit (Cretin TSH ELISA II) for thyroid stimulating hormone (TSH) was donated from Eiken Chemical Co. Ltd., Tokyo. It included the microplate coated with the mouse anti-TSH monoclonal antibody, mouse anti-TSH monoclonal antibody labelled with horseradish peroxidase, washing solution, chromogen–substrate (*o*-phenylenediamine) and stop solution (sulfuric acid). A Reference 4910 pipette (Eppendorf) was used for sample preparation. Absorbance was measured by a $V_{max}$ kinetic microplate reader (Molecular Devices).

All the procedures for the ELISA analysis are shown in Fig. 1. Fifty microliters of a standard TSH solution were taken into a well of a microplate and 100 μL of the labeled antibody solution were added to the well. The mixture was incubated for 4 h at room temperature. After washing and tapping, 100 μL of the chromogen–substrate solution were added to the well, shaken and incubated for 30 min at a room temperature. Then, 100 μL of the stop solution was added and the absorbance was measured at 490 nm.

## 3. Theory

An uncertainty equation for sandwich ELISA is derived based on the possible error sources involved in the experimental procedures shown in Fig. 1. The uncertainty refers to the measurement variability among the different wells within a microplate.

The errors can be grouped into two origins: sample preparation (till the stop solution in Fig. 1) and absorbance measurement. They are considered to be probabilistically independent of each other. By taking into account the rule of error propagation [3,17], we can describe the variance, $\sigma_T^2$, for the total analysis:

$$\sigma_T^2 = \sigma_P^2 + \sigma_M^2 \quad (1)$$

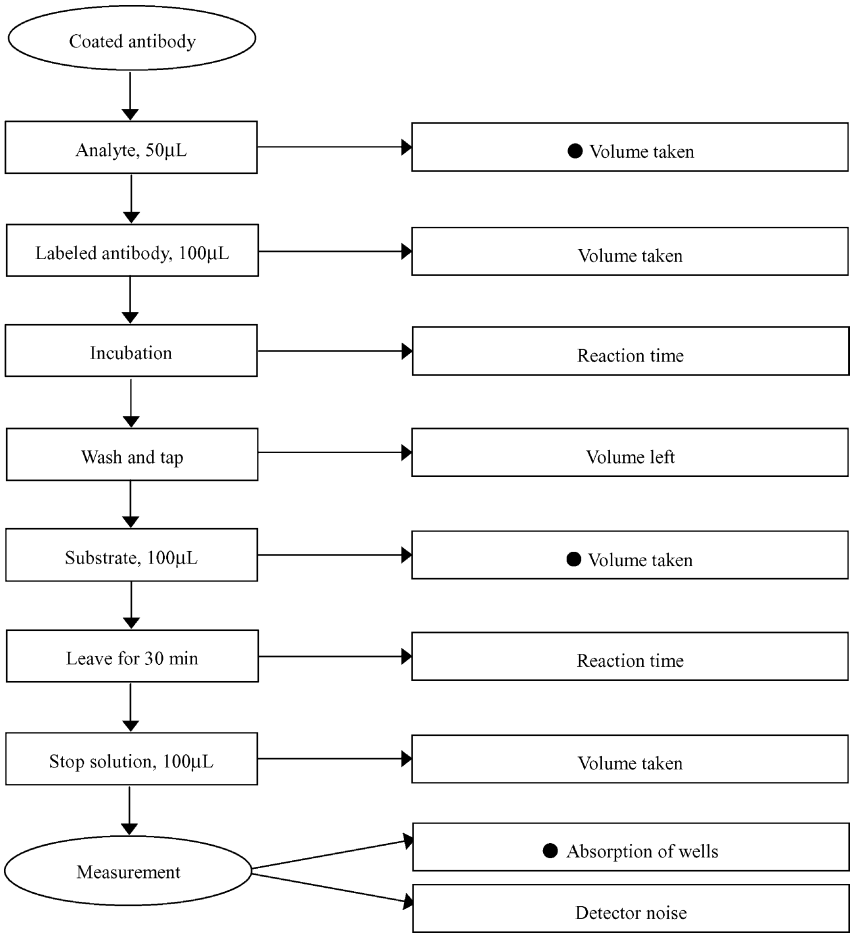


Fig. 1. Scheme of analytical procedures and errors. (●) means the error sources included in the uncertainty equation (Eq. (5)).

where $\sigma_P$ denotes the S.D. for the preparation error and $\sigma_M$ is the S.D. for the measurement error. Here, the dimensions of $\sigma_T$, $\sigma_P$ and $\sigma_M$ are assume to be absorbance. Dividing the both sides of Eq. (1) by the square of the averaged absorbance measurements, we can obtain the R.S.D., $\rho_T$, of total errors:

$$\rho_T^2 = \rho_P^2 + \rho_M^2 \quad (2)$$

where $\rho_P$ denotes the R.S.D. of preparation and $\rho_M$ is the R.S.D. of measurement.

### 3.1. Preparation error

Among all the possibilities, the following errors are considered to contribute overwhelmingly to the R.S.D., $\rho_P$, for the sample preparation (see the following section):

$$\rho_P^2 = \rho_X^2 + \rho_S^2 \quad (3)$$

where $\rho_X$ denotes the R.S.D. of the pipetted volumes of the analyte solution and $\rho_S$ is two thirds times the R.S.D., $\rho_C$, of the pipetted volumes of the chromogen–substrate solution ($\rho_S = 2/3\rho_C$, see Ref. [6]).

### 3.2. Measurement error

The squared R.S.D., $\sigma_M^2$, of measurement error depends on the variance, $\sigma_W^2$, of the absorbances inherent to the wells of a microplate:

$$\sigma_M^2 = \left(\frac{\sigma_W}{Y}\right)^2 \quad (4)$$

where $Y$ denotes the absorbance measurement and a calibration line, $Y = aX + b$, can be substituted for $Y$. The other error sources (detector noise, etc.) are discussed in the following section.

### 3.3. R.S.D. of total analysis

Substituting Eqs. (3) and (4) for Eq. (2), we cab obtain the total error of analysis:

$$\rho_T^2 = \rho_X^2 + \rho_S^2 + \left(\frac{\sigma_W}{Y}\right)^2 \quad (5)$$

The uncertainties, $\rho_X$, $\rho_S$ and $\sigma_W$, are all constant. A similar equation to Eq. (5) has been derived for competitive ELISA [6], but it takes a different form because of the different experimental procedures.

## 4. Results and discussion

It is possible that every procedure of the sandwich ELISA is a source of uncertainty, but only a few procedures are considered influential (see (●) in Fig. 1). The error sources neglected from Eq. (5) are discussed below.

In the sandwich ELISA, 100% of the antigen analyte are supposed to react with the antibody. Therefore, as long as the anti-TSH antibodies, coated and in solution, exceed the analyte in amount, the variability in the antibody amount between the wells of the microplate is of little concern. The following procedures are irrelevant: the scattering of the incubation time and change in temperature during the incubation period; the volume of liquid left after the wash and tap; the timing to add the stop solution. The volume of the stop solution exerts no influence on the absorbance measurements. This is because the absorbance is measured in the vertical direction of the wells and the product of concentration and light path length is kept constant irrespective of the added volume of the stop solution. The detector noise can be cancelled out by repeating the absorbance measurements for a sample with an A/D converter and averaging the results (here, $n = 10$).

Fig. 2 shows the calibration line (A), precision profiles (B and D) and contribution of the individual terms of the uncertainty equation (Eq. (5)). The calibration line is straight on the natural scales, but looks like curvilinear in the semi-logarithmic plot (A).

In the precision profile (B), the R.S.D. of absorbance measurements decreases with increasing analyte concentration. The R.S.D. estimated from the repetition of the real samples (●; $n = 8$) well corresponds to that from the uncertainty theory (—; Eq. (5)). At concentrations less than 0.01 μIU/assay, the difference between theory and practice can be spotted. However, we should note that the difference is enhanced by the semi-logarithmic plot and that the theoretical line is not a least-squares fit to the experimental results (●).

The values of $\rho_X$ and $\rho_S$ used for the theory are 0.97% and 0.33%, respectively. They were all determined by the weight measurements for the repeated pipetting of distilled water, since the viscosity of the analyte and substrate solutions seemed to be almost comparable to that of water. The S.D., $\sigma_W$ (=0.002 Abs), of well absorbances was obtained among the empty wells of a microplate. The actual equation used for the uncertainty estimation is:

$$\rho_T^2 = (0.97)^2 + (0.33)^2 + \left(\frac{0.002}{Y}\right)^2 \quad (6)$$

For the precision profiles, the blank absorbance including the average of the well absorbances is subtracted from the sample measurements (actually, $Y$ in Eq. (6) $= Y - b$). No arbitrary constants are involved in the uncertainty equation.

Fig. 2C shows the relative contribution of the error sources included in Eq. (6). The $Y$-axis denotes (individual term of the right side of Eq. (6))/(left side of Eq. (6)). At low concentrations, the absorbance of the wells (▲) is the most influential factor on the ELISA uncertainty, but at high concentrations, the variability of the pipetted sample volumes (◆) affects the entire uncertainty most. The pipetting of the chromogen–substrate solution (□) is not significant in the range examined.

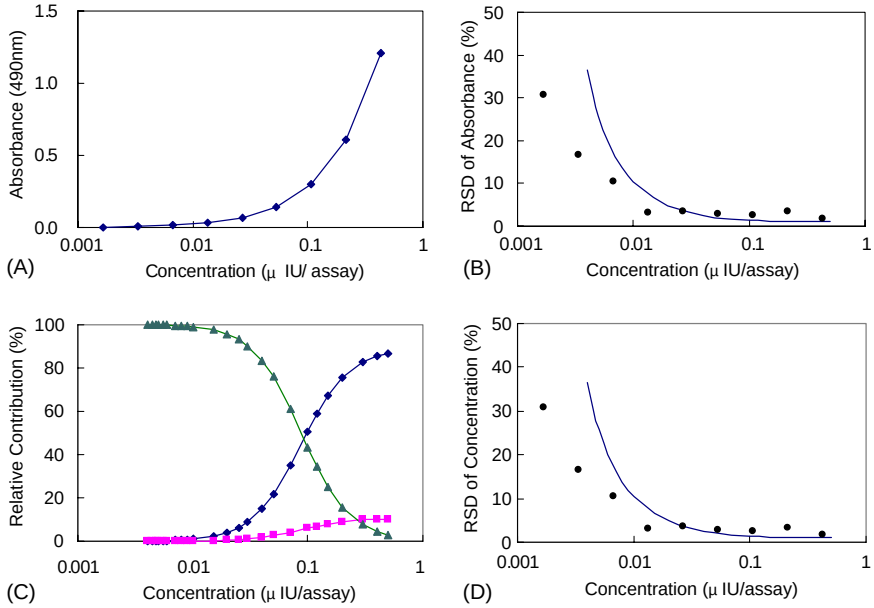


Fig. 2. Calibration line (A), contribution of errors (C) and precision profiles for measurements (B) and concentration estimates (D). (A) $Y = 2.84X - 0.0045$. (B and D) (●) the experimental R.S.D. of absorbance measurements ($n = 8$); (—) the theoretical R.S.D. from Eq. (5). (C) $Y$-axis is the relative contribution of error (the individual term of the right side of Eq. (5))/(the left side of Eq. (5)); (◆) first term ($\rho_X^2$); (□) second term ($\rho_S^2$); (▲) third term $(\sigma_W/Y)^2$.

As long as the calibration line is straight and passes through the origin ($Y = aX$), the precision profile for the R.S.D. of measurements is exactly the same as that for the R.S.D. of concentration estimates as shown in Fig. 2B and D. Let $s$ be the measurement S.D. and $Y$ be the measurement. In general, the measurement R.S.D., $s/Y$, can be converted to the concentration R.S.D., $\rho_C$, through the calibration curve, $Y = f(X)$, as follows: $\rho_C = (s/(dY/dX))/f^{-1}(Y)$. If $Y = aX$, then $s/(dY/dX) = s/a$, $f^{-1}(Y) = Y/a$ and $\rho_C = (s/a)/(Y/a) = s/Y$. However, unless the calibration curve is linear, this relationship does not necessarily hold true ($\rho_C \neq s/Y$). In competitive immunoassays, the calibration lines are non-linear and the precision profiles for measurement and concentration are different [6].

The detection limit, $L_D$, has been defined by international organizations such as ISO, IUPAC and ICH as follows: [18] $L_D = 3.3s_Y/a$ where $s_Y$ denotes the S.D. of blank responses and $a$ the slope of a linear calibration line. The range of quantitation, $R_Q$ (upper and lower limits of quantitation), is also defined as $R_Q = 10s_Y/a$. In general, the values of $L_D$ and $R_Q$ can be determined by the uncertainty equation (Eq. (5)).

In the homoscedastic situations where $s_Y$ is constant, the simple relationship is derived: $(s_Y/a)/L_D = 1/3.3 = 30\%$ and $(s_Y/a)/R_Q = 1/10 = 10\%$. This implies that $L_D$ and $R_Q$ are the concentrations corresponding to 30% and 10% R.S.D., respectively. Therefore, $L_D$ and $R_Q$ can easily be spotted in the precision profile (Fig. 2B or D).

The methodology proposed in this paper will underlie the theoretical estimation of the within-plate uncertainty in a variety of sandwich ELISA.

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