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High precision weighing and uncertainty assessment in reagent preparation

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Abstract. The volume of the reagent in one tube was difficult to be measured in reagent preparation, as the volume of each portion was extremely small. Formerly, the measurement errors of direct weighing method were large. Therefore, the method of twice minusing method was used to weighing in order to obtain the reagent preparation result more accurately. The sources of uncertainty of this method were analyzed and the uncertainty was evaluated, the uncertainty of twice minusing method and direct weighing method was compared. The results showed that the expanded uncertainty of the reagents in one of the eight-connected tubes was 2.266×10^{-4} g (k = 2) when using twice minusing method, and the expanded uncertainty was 1.704×10^{-3} g (k = 2) when using direct weighing method, so the results of twice minusing method were more reliable.

Keywords: metrology, uncertainty, GUM method, error analysis.

1. Introduction

Polymerase Chain Reaction (PCR) is a widely used technique in molecular biology, the octal tube is an indispensable reagent in this technology. It is widely used in fields such as molecular biology, medicine , life sciences to preserve PCR reaction reagents and reactants. When preparing PCR reagents, the nucleic acid amplification reaction solution, primer probe mixture, and enzyme mixture need to be mixed in certain proportions. The volume of each reagent and the total volume after mixing may affect the accuracy of the amplification experiment. This indicates that a measuring method needs to be used to assist in verifying the results of PCR reagent preparation, ensuring that the volume of each reagent well is consistent and that the prepared volume is the same as the required volume. Therefore, a high-precision weighing measurement method is needed to verify the quality of the reagents, and the uncertainty of the measurement method's results needs to be assessed. Finally, based on the weighing results, the pipetting performance of the instrument is adjusted to ensure that the PCR experiment can proceed smoothly.

When configuring laboratory reagents, whether it is an electronic pipette or a manual pipette, its pipetting performance is difficult to evaluate because the volume required per person is very small, in the microliter range. Different reagents need to be mixed together, resulting in a high level of difficulty in measurement. This study used the GUM method to assess the uncertainty of PCR reagent quality

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measurement results and analyzed the main sources of uncertainty generated during the measurement process.

2. Verification of reagent preparation results based on high-precision weighing

When the eight-connected tubes leave the factory, the eight reaction tubes are connected together. When weighing, it is necessary to cut the connections between each reaction tube in order to separate them for dispensing and weighing, as shown in the schematic diagram in Figure 1.

Figure 1. Cut the eight-connected tube

The eight-connected holder shown in Figure 2 was used in this study. During the experiment, the tubes and their holder were weighed together. This method has two advantages: firstly, according to the principle of electronic balance weighing, there is a serious drifting phenomenon near the zero point of the balance. Weighing the tubes and their holder together can help stabilize the balance during weighing. Secondly, it is easy to fix the position of the tubes holder on the balance pan. Mark the position of the holder at the center of the balance pan, so that the position of the holder is the same every time the weighing experiment is carried out, thus reducing the influence of the balance's load error on the weighing results.

Figure 2. The bracket of eight-connected tube

The method of minusing weighing is a weighing method that determines the mass of a sample by taking the difference between two weighing result. The method of twice minusing weighing method is a method that calculates the difference obtained from two minusing method weighing results.

In this study, the high-precision weighing method uses twice minusing weighing method. In practical use of twice minusing weighing method, all cut-off octuplets are first placed in a rack and placed in the center position of the balance pan. A weighing is performed when they are initially placed, and the order of the eight tubes is recorded. Then, each reaction tube is taken out one by one, and a weighing is performed after each removal. The weighing is repeated ten times, and the arithmetic mean is calculated. This way, for each set of octuplets, nine arithmetic means are obtained. By subtracting the data after removal from the data before removal, the mass of each reaction tube can be obtained using the minusing weighing method. The reaction tubes are then placed on the rack in their original order for reagent preparation. Subsequently, each well of the octuplets contains prepared reagents. Following the same

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removal order, each reaction tube is taken out one by one, and the same weighing method is used to obtain the mass of the reagents, the test tube, and the rack. Nine arithmetic means are calculated for this set of data. By subtracting the data after removal from the data before removal, eight sets of data are obtained, representing the sum of the mass of each reaction tube and the mass of the reagents inside each tube. Finally, by subtracting the corresponding masses of the two sets of data for each tube, the mass of the reagents of every reaction tube can be determined using the twice minusing weighing method.

Uncertainty is a composite concept, and it arises from both random effects and systematic effects. These two factors are dialectically unified and can be transformed into each other under different circumstances. Therefore, uncertainty cannot be simply divided into random uncertainty and systematic uncertainty. Instead, uncertainty should be divided into "uncertainty component caused by random effects" and "uncertainty component caused by systematic effects".

Assuming the eight reaction tubes in the eight-connected tube, along with the total mass of the reagents inside each tube, are represented by M_{a1} to M_{a8}, and the mass of the eight reaction tubes are represented by M_{b1} to M_{b8}. Let m_{a0} to m_{a8} represent the nine weighing measurements for the total mass of the reaction tube and reagents after preparation, taken in the order of removing each reaction tube, and let m_{b0} to mm_{b8} represent the nine weighing measurements for the mass of the reaction tube before reagent preparation, also taken in the same order. Here, m_{a0} and m_{a8} represent the total weight of the eight-channel tube and the rack when no reaction tube has been removed, and both m_{a8} and m_{b8} represent the mass of the same rack left after all reaction tubes have been removed. Therefore, the values of m_{a8} and m_{b8} can be used to verify the status of the electronic balance. If the difference between the two values is too large, the experimental results cannot be trusted, and the electronic balance needs to be calibrated and reset before conducting the experiment again.

Taking the measurement and calculation of the total mass of the first reaction tube in the eight-connected tube after adding the reagent, taking the measurement of M_{a1} as an example, the mathematical model for the total mass of the first reaction tube and the reagent is as follows:

$$M_{a1} = m_{a0} - m_{a1} \tag{1}$$

In equation (1), the expression:

$$m_{a0} = m_{a0r} + \Delta m_{a0} \tag{2}$$

$$m_{a1} = m_{a1r} + \Delta m_{a1} \tag{3}$$

The m_{a0r}in formula includes the random error during measurement, Δm_{a0}represents the systematic error when measuring m_{a0}.This is the same when measuring m_{a1}.

$$u(m_{a0}) = \sqrt{u^2(m_{a0r}) + u^2(\Delta m_{a0})} = \sqrt{u_{ran}{}^2(m_{a0}) + u_{sys}{}^2(m_{a0})} \tag{4}$$

$$u(m_{a1}) = \sqrt{u^2(m_{a1r}) + u^2(\Delta m_{a1})} = \sqrt{u_{ran}{}^2(m_{a1}) + u_{sys}{}^2(m_{a1})} \tag{5}$$

$u(m_{a0r})$ represents the uncertainty component caused by random effects when measuring m_{a0r}, $u_{sys}(m_{a0})$ represents the uncertainty component caused by systematic effects when measuring m_{a0r}, This is the same when measuring.

The errors in the balance caused by systematic effects usually include bias error, maximum permissible error, and indication error. Measures can be consciously taken during measurement to avoid the influence of bias error, for example, by specifying the position where the weighing is placed each time and marking the position. It is possible to simplify the calculation by disregarding the consideration of the balance's bias error. During the same period of weighing, the maximum permissible error and indication error of the electronic balance are generally considered invariant. Therefore, under the condition of unchanged influencing factors during the same period of weighing, the systematic error of the electronic balance can be considered approximately equal. Other factors include environmental factors and personnel factors. In this experiment, with other influencing factors unchanged and using the same electronic balance for weighing during the same period, it can be considered:

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$$u_{sys}(m_{a0}) = u_{sys}(m_{a1}) \tag{6}$$

The total mass of the reaction tube and reagent in the first position of the eight-connected tube:

$$M_{a1} = m_{a0} - m_{a1} = m_{a0r} + \Delta m_{a0} - (m_{a1r} + \Delta m_{a1}) = (m_{a0r} - m_{a1r}) + (\Delta m_{a0} - \Delta m_{a1}) \tag{7}$$

According to the introduction of reference [1], it is stated that "measurement uncertainty can be derived from the uncertainty components that affect the measurement results, regardless of how these components are grouped or further decomposed into lower-level components."

$$y_1 = m_{a0r} - m_{a1r} \tag{8}$$

$$y_2 = \Delta m_{a0} - \Delta m_{a1} \tag{9}$$

Then the uncertainty of measuring M_{a1} is $(y_1 + y_2)$.

Systematic effects contribute to uncertainty mainly due to the instrument's inherent flaws, imperfect experimental methods, and the influence of environmental conditions on the instrument. The characteristic of systematic effects is that the measured values consistently deviate in one direction. Within the same time period, the impact of systematic effects on weighing using a balance remains relatively constant. It can be assumed that the direction and magnitude of the changes in Δm_{a0} and Δm_{a1} are the same, indicating that Δm_{a0} and Δm_{a1} are positively correlated with a correlation coefficient $r = +1$. The uncertainty caused by random effects primarily originates from incidental factors that interfere with the experiment, leading to deviations in the measurement results. Its characteristics are non-directional and discrete. The main method to eliminate this effect is to conduct multiple repeated measurements of the same quantity. The impact of random effects on weighing using a balance result in random errors that vary in an unpredictable manner. The magnitude and direction of these errors cannot be determined. Therefore, m_{a0r} and m_{a1r} are uncorrelated with each other, and the correlation coefficient $r = 0$.

$u(m_{a0r})$ and $u(m_{a1r})$ are components of the standard uncertainty y_1, and m_{a0r} and m_{a1r} are uncorrelated with each other. Therefore, the combined standard uncertainty of y_1, denoted as $u(y_1)$, is:

$$u(y_1) = \sqrt{u^2(m_{a0r}) + u^2(m_{a1r})} = \sqrt{u^2{}_{ran}(m_{a0}) + u^2{}_{ran}(m_{a1})} \tag{10}$$

$u(\Delta m_{a0})$ and $u(\Delta m_{a1})$ are components of the standard uncertainty y_2,and Δm_{a0} and Δm_{a1} are positively correlated with a correlation coefficient $r = +1$. According to the synthesis standard uncertainty propagation law in reference [1], when the input quantities are strongly positively correlated and the correlation coefficient is 1, the synthesis standard uncertainty should be calculated according to equation (11):

$$u_c(y_2) = \left| \sum_{i=1}^{N} \frac{\partial f}{\partial m_i} u(m_i) \right| \tag{11}$$

The error transfer coefficient of each component in y_2 is:

$$C_1 = \frac{\partial y_2}{\partial(\Delta m_{a0})} = 1 \tag{12}$$

$$C_2 = \frac{\partial y_2}{\partial(\Delta m_{a1})} = -1 \tag{13}$$

According to the formula (11) substitution, the combined standard uncertainty $u(y_2)$ of y_2 is:

$$\begin{aligned} u(y_2) &= |C_1 u(\Delta m_{a0}) + C_2 u(\Delta m_{a1})| \\ &= \left|C_1 u_{sys}(m_{a0}) + C_2 u_{sys}(m_{a1})\right| \\ &= \left|u_{sys}(m_{a0}) - u_{sys}(m_{a1})\right| = 0 \end{aligned} \tag{14}$$

In summary:

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$$u(M_{a1}) = u(y_1) + u(y_2) = \sqrt{u^2{}_{ran}(m_{a0}) + u^2{}_{ran}(m_{a1})} \quad (15)$$

Based on the above reasoning, it can be concluded that the uncertainty in weighing other well positions can be obtained in a similar manner. It is known from the formula (15) that the uncertainty in weighing by subtraction method due to systematic effects can cancel each other out. For example, in [2], the uncertainty in weighing by subtraction method is only related to random effects, and it is determined by the standard uncertainty caused by random effects before and after the subtraction. In general, the repeatability of measurements is considered to be caused by random effects, and the standard uncertainty introduced by repeatability is usually evaluated as Class A.

According to the introduction mentioned in reference: "When a measurement result is used for the next measurement, its uncertainty can be considered as a component of the uncertainty of the next measurement result". Therefore, the mass of the reagent can be obtained by subtracting the total mass of the reaction tube obtained from the subtraction method from the mass of the empty reaction tube. The uncertainties obtained from the twice minusing method can be treated as components to further calculate the uncertainty in the second subtraction method, which represents the uncertainty in measuring the mass of the reagent in this study. Hence, the uncertainty in subtracting corresponding well positions between the twice minusing method data can be calculated by synthesizing the uncertainties in measurement and subtraction.

The mathematical model for the mass of the reagent in the first reaction tube of the eight-connected tube obtained from twice minusing weighing method is:

$$M = M_{a1} - M_{b1} = (m_{a0} - m_{a1}) - (m_{b0} - m_{b1}) \quad (16)$$

The uncertainty in measuring M_{b1} is the same as that in measuring M_{a1}, as obtained from formula(15):

$$u(M_{b1}) = \sqrt{u^2{}_{ran}(m_{b0}) + u^2{}_{ran}(m_{b1})} \quad (17)$$

The components of the standard uncertainty of $u(M)$ are $u(M_{a1})$ and $u(M_{b1})$.When M_{a1} and M_{b1} are measured using the minusing weighing method, their uncertainties are only related to the components of uncertainty caused by random effects before and after each measurement. The variations in their respective random errors are unpredictable, therefore $u(M_{a1})$ and M_{b1} are uncorrelated, and the correlation coefficient $r = 0$. This situation satisfies the conditions given in equation (24) in reference [1]. Therefore, the combined standard uncertainty of the quadratic difference method can be obtained as:

$$u_c(M) = \sqrt{u^2(M_{a1}) + u^2(M_{b1})} = \sqrt{u^2{}_{ran}(m_{a0}) + u^2{}_{ran}(m_{a1}) + u^2{}_{ran}(m_{b0}) + u^2{}_{ran}(m_{b1})} \quad (18)$$

The uncertainty introduced by the measurement repeatability during each weighing in this experiment is assessed as Type A standard uncertainty, in the [3], the uncertainty of each weighing is calculated using the Bevès formula, as given in equation (19). Under the condition of repeatability, the Type A standard uncertainty introduced by the measurement repeatability of the electronic balance can be obtained by equation (20) when the same object is independently weighed ten times using the same balance.

$$s(m_k) = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}[m_i - \overline{m}]^2} \quad (19)$$

$$u_{ran}(\overline{m}) = s(\overline{m}) = \frac{s(m_k)}{\sqrt{n}} \quad (20)$$

Taking the example of calculating the mass of reagent in the first well of the octal tube, the same electronic balance was used to perform ten consecutive independent weighings (n = 10) of the substance in each well. The measurement results were within the range of 50~60 g, and the specific data is shown in Table 1.

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Table 1. Weighing experimental data of twice minusing method

m_{a0}/g	m_{a1}/g	m_{b0}/g	m_{b1}/g
56.6273	56.4763	56.4561	56.3262
56.6273	56.4767	56.4562	56.3264
56.6272	56.4764	56.4562	56.3263
56.6271	56.4762	56.4563	56.3265
56.6271	56.4762	56.4563	56.3267
56.6270	56.4761	56.4561	56.3267
56.6269	56.4762	56.4563	56.3269
56.6269	56.4762	56.4561	56.3267
56.6271	56.4761	56.4561	56.3269
56.6268	56.4762	56.4562	56.3264

By substituting the experimental data into equation(20), the components of uncertainty for the twice minusing method when measuring the mass of the reagent in the first well of the octal tube can be obtained as follows: $u_{ran}(m_{a0})$ = 5.385×10^{-5} g; $u_{ran}(m_{a1})$ = 5.616×10^{-5} g; $u_{ran}(m_{b0})$ = 2.769×10^{-5} g; $u_{ran}(m_{b1})$ = 7.754×10^{-5} g.

By substituting the data into equation (18), the standard uncertainty $u_c(M)$ for weighing the reagent mass in the first well using the twice minusing method is calculated to be 1.133×10^{-4} g.

According to the components of uncertainty for weighing using the twice minusing method, the combined uncertainty is approximately normally distributed. Taking a confidence factor k = 2, therefore, the confidence probability P = 0.95, the expanded uncertainty is calculated based on equation(21).

$$U = 2u_c(M) \quad (21)$$

By substituting the data, the expanded uncertainty U for weighing using the twice minusing method is calculated to be 2.266×10^{-4} g.

3. Comparisons Results

When weighing the mass of reagents using the direct method, the procedure involves placing an empty octal tube rack at the center position of the balance pan. Subsequently, each empty reaction tube from the cut octal tube is sequentially placed onto the rack on the balance pan for weighing. After each weighing, the empty reaction tube is removed and replaced with the next one, ensuring that only the rack and the specific reaction tube being weighed are present on the balance pan at a time. This process is repeated for all eight empty reaction tubes and the rack, and the sum of their masses is recorded. This weighing process is repeated ten times for each position, and the arithmetic mean is calculated.

Following this, the reagents are prepared in all eight reaction tubes of the octal tube, with the empty rack placed at the center position of the balance pan. The prepared reaction tubes are sequentially placed onto the rack on the balance pan for weighing, obtaining the data for the reagents, tubes, and rack for each corresponding position. Finally, the mass of each reagent in the reaction tube is obtained by subtracting the mass of the corresponding empty reaction tube from the mass of the reaction tube with the reagent for each position.

The standard uncertainty of weighing reagents using the direct method is similar to that of the twice minusing method.

$$u_c(M) = \sqrt{u^2(M_{a1}) + u^2(M_{b1})} = \sqrt{u^2{}_{ran}(M_{a1}) + u^2{}_{sys}(M_{a1}) + u^2{}_{ran}(M_{b1}) + u^2{}_{sys}(M_{b1})} \quad (22)$$

In the direct weighing method, the systematic errors caused by the instrumental effects of the balance primarily include the standard uncertainty $u_{mpe}(m)$ introduced by the maximum permissible error and the standard uncertainty $u_w(m)$ introduced by the standard weights.

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The standard uncertainty introduced by the maximum permissible error of the balance is evaluated using Type B uncertainty assessment. Referring to[4], this information can be obtained., for a Class I electronic balance with an actual scale interval of 0.1 mg, the maximum permissible error (MPE) within the range of 50~200 g is ±1 mg. Therefore, the half-width of the interval is 1, and the distribution within this interval follows a uniform distribution, so the coverage factor $k = \sqrt{3}$, The uncertainty introduced by the maximum permissible error of the electronic balance is denoted as $u_{mpe}(m)$:

$$u_{mpe}(m) = \frac{0.001}{\sqrt{3}} = 5.774 \times 10^{-4}\text{g} \tag{23}$$

The standard uncertainty introduced by the standard weights of the electronic balance is typically evaluated as Type B. For a Class I electronic balance with an actual scale interval of 0.1 mg, the maximum permissible error (MPE) within the range of 50~200 g is 1 mg. According to the provisions of [4], the expanded uncertainty of the mass of standard weights (k=2) should not exceed the maximum permissible error $1/3$ of the balance.

$$U_W \leq \frac{|MPE|}{3} \tag{24}$$

Assuming that:

$$U_W = \frac{|MPE|}{3} = 3.333 \times 10^{-4}\text{ g} \tag{25}$$

It can be derived that:

$$u_w(m) = \frac{U_W}{k} = 1.667 \times 10^{-4}\ g \tag{26}$$

Therefore, the uncertainty introduced by the systematic effects in the direct weighing method is:

$$u_{sys}(m) = \sqrt{{u_{mpe}}^2(m) + {u_w}^2(m)} = 6.010 \times 10^{-4}\text{ g} \tag{27}$$

When using the direct weighing method to weigh the object, it was repeatedly weighed independently for ten consecutive times. The measured results were all within the range of 50~60 g, as shown in Table 2.

Table 2. Weighing experimental data of direct method

m_{a1}/g	m_{b1}/g
55.5583	55.5381
55.5585	55.5382
55.5583	55.5385
55.5582	55.5383
55.5583	55.5384
55.5584	55.5383
55.5583	55.5384
55.5584	55.5385
55.5582	55.5386
55.5583	55.5384

The formula (20) was applied using the experimental data, and the result obtained is $u_{ran}(M_{a1})$ = 2.906×10^{-5} g; $u_{ran}(M_{b1})$ = 4.724×10^{-5} g.

Assuming that the uncertainties caused by systematic effects in two consecutive direct weighing measurements are equal, substituting them into equation (22) yields the combined standard uncertainty $u_c(M)$ for measuring the mass of the reagent in the first well of the eight-connected tube as 8.518×10^{-4} g.

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Considering that the combined uncertainty in direct weighing can be approximated as a normal distribution, with a confidence factor $k = 2$, the confidence probability $P = 0.95$. The expanded uncertainty is calculated using equation (28), resulting in an expanded uncertainty:

$$U = 2u_c(M) \tag{28}$$

Substituting the given data, the expanded uncertainty U for the direct weighing method is calculated to be 1.704×10^{-3} g.

By comparing the formulas for combined standard uncertainties, we can conclude that the uncertainty in the twice minusing method of weighing is only related to random errors, while the standard uncertainty in the direct weighing method is not only influenced by random errors but also by the systematic errors of the electronic balance. According to [5], due to the stochastic nature of random effects, the uncertainty caused by them varies each time during weighing. On the other hand, the uncertainty introduced by systematic effects is usually larger in magnitude than that caused by random effects. Therefore, in order to determine which method provides more reliable measurement results, it is necessary to evaluate whether the uncertainty caused by random effects or that caused by systematic effects is greater.

Since the uncertainty introduced by systematic effects in the weighing process is much larger than the uncertainty caused by random effects, and the twice minusing method cancels out the systematic errors of the balance, using the twice minusing method significantly reduces the uncertainty and improves the reliability of the measurement results.

4. Conclusions

This study investigated and analyzed the uncertainties in measuring the quality and volume of PCR reagents. It compared two weighing methods: the twice minusing method and the direct weighing method. By analyzing the sources of uncertainties for each method, the number of the uncertainties were calculated, providing evidence for the feasibility of the twice minusing method. The results showed that when using the twice minusing method to weigh the reagents in the tube, the expanded uncertainty was determined to be 2.266×10^{-4} g ($k = 2$). On the other hand, when using the direct weighing method, the expanded uncertainty was found to be 1.704×10^{-3} g ($k = 2$). Therefore, the results obtained from the twice minusing method were considered more reliable. It provides a reliable verification method for the preparation of reagents.

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