

# Userguide

Liquid Handling 7 | No 025

## Calibration and adjustment of dispensing systems in the laboratory

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### Abstract

The calibration of pipettes and dispensers means that under specified ambient conditions a defined volume of water dispensed from a dispensing system should have a certain expected weight. To be more precise, this involves determining the correlation between the dispensed volume and the nominal volume or selected volume of a dispensing system. Calibration does not require intervention which modifies the dispensing system on a permanent basis.

### Introduction

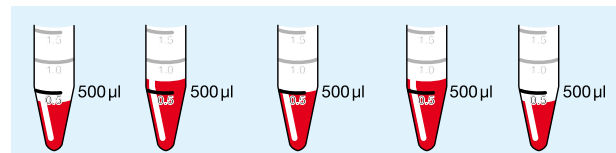
The test norm most commonly used for pipettes and dispensers and their calibration is the standard DIN EN ISO 8655. This standard lays down the maximum permissible random and systematic measurement deviations for the various liquid handling systems. The error limits specified in the standard always refer to the overall system, consisting of the dispensing device and accessories such as the pipette tip. Compliance with regard to error limits must be checked by the user under the control of inspection, measuring and test equipment or analytical quality assurance at least once a year. The user is thus free to specify shorter intervals. The frequency of usage, number of users of the dispensing system and the aggressiveness of the liquid to be dispensed play a key role in this decision.

### Random and systematic measurement deviation

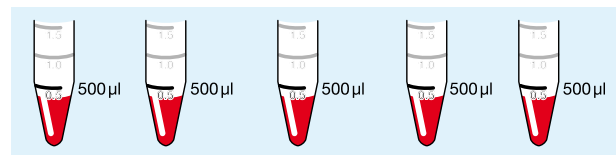
Random measurement deviation, also known as imprecision, is a measure for the variation of individual measured values. Slight imprecision occurs when there is only minor deviation between the repeat measurements. To achieve this a precise device is required as well as proper handling, cleanliness and practical experience (Fig. 1). A pipette may on the one hand be highly precise but on the other hand, it may also be precisely false, i.e. provide incorrect measurements. Systematic measurement deviation, also known as inaccuracy, is a measure of the

extent of the deviation between the mean value of the measurement results and the nominal value. Slight inaccuracy is seen when there is only deviation between the measured volume and the nominal volume (Fig. 1).

#### Imprecise



#### Precise, but inaccurate



#### Precise and accurate

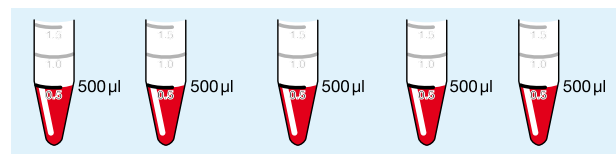


Fig. 1: Simple presentation of the random and systematic measurement deviation (imprecision and inaccuracy), nominal value 500 µl

Random and systematic measurement deviation

Figure 2 shows the distribution of measured values in a measurement series with different random and systematic errors. In Figure 3 the number of pipetting processes is plotted against the frequency of the measured volumes. The result is typically a Gaussian distribution curve. The greater the volume range spanned by the curve, the more imprecise the measurement. The accuracy of a dispensing system can be checked with a gravimetric, photometric or titrimetric test procedure.

Predominant type of error	a and b	a	b	—
a) random error				
b) systematic error				
random measurement deviation	bad	bad	very good	good
systematic measurement deviation	bad	good	bad	good

Fig. 2: Random and systematic measurement deviation of a pipette illustrated by coordinate axes

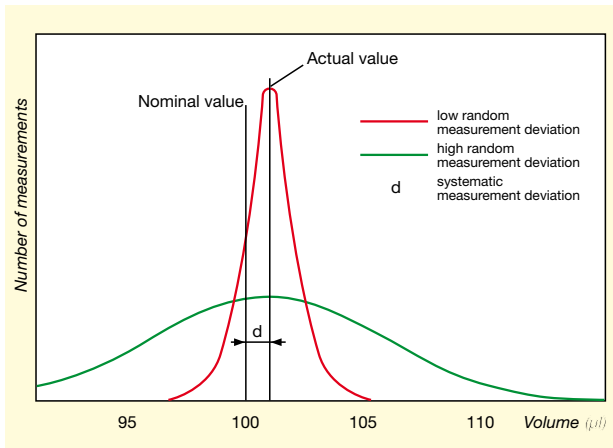


Fig. 3: Nominal value: set volume of the pipette (here: 100 µl); Actual value: the volume measured most frequently during all pipetting procedures; d: systematic measurement deviation, deviation of the actual value from the nominal value.

Gravimetric test procedure

The prerequisite for a gravimetric test procedure is an analytical balance or equivalent weighing device. The scale graduation value must correspond to the selected volume of the dispensing system to be examined (Tab. 1). In order to minimize the evaporation loss during weighing, particularly with small volumes (< 50 µl), a weighing vessel suitable for the volume should be used and if possibly an evaporation trap (Fig. 4).

Random measurement deviation (Imprecision)	Scale graduation value	Type of balance
up to 0.01 µl	0.001 mg	Micro balance
up to 0.1 µl	0.01 mg	Semi-micro balance
up to 1 µl	0.1 mg	Analytical balance

Tab. 1: Indicative figures for the gravimetric testing of pipettes



Fig. 4: Pipette calibration using an analytical balance with moisture trap

In addition, the following items must be taken into account during gravimetric calibration:

- Testing must take place in a room free of drafts.
- In the test room there must be a relative humidity in excess of 50 % and a constant temperature ( $\pm 0.5$  °C) between 15 and 30 °C.
- Prior to testing the dispensing system and the test liquid should have stood for sufficient time in the test room (min. two hours) so that they are equalized with the room conditions.
- The test cycles must be as uniform as possible. It is important for the measuring time to be as identical as possible within each cycle as well as from cycle to cycle in order to reliably compensate for evaporation effects during a measurement series by way of calculation.
- Degassed and distilled, or ion-free, water must be used as the test liquid.

### Gravimetric test procedure

With variable pipettes three different volumes are tested:

- Nominal volume (greatest volume that can be adjusted by the user and set by the manufacturer)
- 50 % of the nominal volume
- The lower limit of the useful volume or 10 % of the nominal volume.

Generally ten measurements per test volume are performed. However, the user can alter the number of test volumes, the number of measurements per test volume and, with multichannel pipettes, the number of channels tested in a suitable manner so that testing satisfies his requirements in terms of accuracy.

According to initial adjustment by the manufacturer, pipettes and dispensers must be tested for the dispensing of test liquid into a weighing vessel. The test procedure is as follows:

- The weighing vessel is filled with test liquid up to a level of min. 3 mm.
- The temperature of the liquid, the room temperature and the air pressure (Tab. 2) should be measured (the temperatures and the air pressure are necessary for selection of the correction factor Z; see statistical analysis of measurement results).
- The pipette is equipped with the appropriate pipette tip, and the pipette tip pre-wetted five times with test liquid to produce a humidity balance in the “dead” air volume.
- The tip is then changed and pre-wetted one more time.
- The balance is calibrated.
- The pipette is held vertically and the pipette tip is immersed a few millimeters in the test liquid.
- The volume to be tested should be aspirated slowly and evenly. Here a waiting period of 1 s to 3 s should be taken into account.
- The pipette tip is then removed slowly from the liquid, wiping it against the wall of the vessel.
- The filled tip is slanted against the wall of the weighing vessel, and the test liquid is slowly dispensed to the first stroke (measuring stroke).
- The residual liquid is dispensed by pressing the pushbutton to the second stop (blow-out).
- The pushbutton is held down, and the tip drawn up against the wall of the vessel.
- The weighing value, i.e. the mass of liquid pipetted, is then calculated.

All measurements in a measurement series are performed as described.

Conversion		Multiply by
from	into	
bar	Pascal (Pa)	10 <sup>5</sup>
bar	Hectopascal (hPa)	10 <sup>3</sup>
bar	Kilopascal (kPa)	10 <sup>2</sup>
millibar (mbar)	Pascal (Pa)	10 <sup>2</sup>
millibar (mbar)	Hectopascal (hPa)	1
millibar (mbar)	Kilopascal (kPa)	0.1

Tab. 2: Conversion of different pressure units

### Statistical analysis of measurement results

This section describes the most important statistical variables for the control of a volume measurement and demonstrates a calculation using a straightforward example. The aim is to determine pipette's dispensing accuracy with a nominal volume of 1000 µl – nominal value ( $V_0$ ) –. Distilled water (20 °C) is aspirated five times, and the corresponding amounts of water are weighed using an analytical balance.

The results of the five weighing steps are:

$$\begin{aligned} m_1 &= 0.998 \text{ g} \\ m_2 &= 0.999 \text{ g} \\ m_3 &= 0.995 \text{ g} \\ m_4 &= 0.996 \text{ g} \\ m_5 &= 0.975 \text{ g} \end{aligned}$$

To calculate the associated volumes  $x_i$ , the density of the water has to be taken into account. Tables show that 1000 µl distilled water weighs exactly 0.9983 g at 20 °C. Using the following formula, the actual volumes  $x_i$  can be calculated:

$$(x_i) = \frac{m_i}{0.9983 \text{ g}} \cdot 1000 \text{ µl}$$

$$\begin{aligned} x_1 &= 1000 \text{ µl} \\ x_2 &= 1001 \text{ µl} \\ x_3 &= 997 \text{ µl} \\ x_4 &= 998 \text{ µl} \\ x_5 &= 977 \text{ µl} \end{aligned}$$

To simplify matters, the weighing values can also be converted to the associated actual volume values with the correction factors Z for distilled water specified in DIN EN ISO 8655:

$$x_i = m_i \cdot Z$$

The fifth weighing step shows marked deviation from the other four. It is apparently erroneous. One possible cause is that the drop on the pipette tip was not wiped off. Such obvious errors are known as gross errors. This result can be disregarded for further statistical analysis. As the other four results also vary, the "true value" must lie somewhere between them.

Approximation to the true value is obtained from the arithmetical mean value  $\bar{x}$ .

This is the sum ( $\sum$ ) of the usable results  $x_i$ , divided by the number  $n$  of results:

$$\bar{x} = \frac{\sum_{i=1}^n x_i}{n}$$

$$\bar{x} = \frac{x_1 + x_2 + x_3 + x_4}{4}$$

$$\bar{x} = \frac{1000 \mu\text{l} + 1001 \mu\text{l} + 997 \mu\text{l} + 998 \mu\text{l}}{4}$$

$$\bar{x} = 999 \mu\text{l}$$

To assess whether the measurement accuracy of a dispensing device lies within the specified error limits, it is necessary to calculate the systematic and random measurement deviation.

#### Calculation of systematic measurement deviation

The systematic measurement deviation or inaccuracy  $d$  is defined as the percentage-based deviation of the mean value  $\bar{x}$  from the nominal value  $V_0$ , referred to the nominal value.

$$d = \frac{\bar{x} - V_0}{V_0} \cdot 100 \%$$

In the above example it is:

$$d = \frac{999 \mu\text{l} - 1000 \mu\text{l}}{1000 \mu\text{l}} \cdot 100 \%$$

$$d = -0.1\%$$

Besides inaccuracy a second variable is required to describe measurement results. To demonstrate this, we assume that the following values were calculated from a different series of weighing results:

$$x_{1'} = 1011 \mu\text{l}$$

$$x_{2'} = 1010 \mu\text{l}$$

$$x_{3'} = 986 \mu\text{l}$$

$$x_{4'} = 989 \mu\text{l}$$

With these values calculation of the arithmetical mean value would also be  $\bar{x}' = 999 \mu\text{l}$ . But the deviation between the individual results is much greater: the greatest and smallest value vary by 25  $\mu\text{l}$ . In the above example the difference was only 4  $\mu\text{l}$ . To what extent the individual results vary in relation to the arithmetical mean value is given by the standard deviation  $s$ .

$$s = \pm \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n-1}}$$

For the first measurement series this is:

$$s = \pm \sqrt{\frac{(1000-999)^2 + (1001-999)^2 + (997-999)^2 + (998-999)^2}{4-1}} \mu\text{l}$$

$$s = \pm \sqrt{\frac{10}{3}} \mu\text{l}$$

$$s = \pm 1.83 \mu\text{l}$$

In the case of the second measurement series this would be with a similar calculation:

$$s' = \pm 13.34 \mu\text{l}$$

#### Calculation of random measurement deviation

The random measurement deviation (imprecision), also called the coefficient of variation CV, is defined as the ratio of the standard deviation  $s$  to the arithmetical mean value  $\bar{x}$  expressed as a percentage:

$$VK = \pm \frac{s}{\bar{x}} \cdot 100 \%$$

For the first measurement series this is:

$$VK = \pm \frac{1.83 \mu\text{l}}{999 \mu\text{l}} \cdot 100 \%$$

$$VK = \pm 0.183 \%$$

The second measurement series, however, results in:

$$VK' = \pm 1.34 \%$$

### Pipette calibration software

Laboratories working in the field of diagnostics and research make extremely high demands on the accuracy and precision of dispensing devices. The guidelines for Good Laboratory Practice (GLP) stipulate regular control of the specified error limits. Pipettes can be used for lengthy periods without requiring maintenance as a basic rule. However, leakage may occur, e.g. through etching of piston grease, if aggressive liquids are frequently pipetted. This must be detected in good time. Leading manufacturers of pipettes and dispensing systems thus offer calibration software that can be used to control and document systematic and random measurement deviation of a pipette over its entire lifetime. Pipette calibration software is a comprehensive program for the automatic acquisition and processing of measurement data from gravimetric and photometric pipette testing. It can be used to test all pipette types from various manufacturers as well as different dispensing systems, e.g. bottle-top dispensers.

### Possible sources of error when pipetting with air-cushion pipettes

Over time, even experienced users may produce erroneous measurements; reference is made to their localization and elimination in Table 3.

### Adjustment of air-cushion pipettes

On manufacture piston stroke pipettes are calibrated to distilled water under the specified measurement conditions. The air-cushion pipettes Eppendorf Reference® and Research® bear a so-called calibration seal identifying their calibration. If there is any doubt about the accuracy of the pipetting volume, readjustment of the pipette should be the very last possibility. A volume that is too low is often an infallible sign of leakage. If a pipette without any leakage whatsoever nevertheless shows a positive or negative deviation, all measurement conditions should be checked once again and if necessary, taken into account in the calculations:

- Appropriate tips (system)
- Temperature of sample
- Temperature of pipette
- Temperature of air
- Conversion from mg to µl.

Only when these checks have eliminated all doubt can it then be assumed the adjustment of the pipette has been altered by certain influences (e.g. replacement of several key components).

Influencing parameter	Effect*	Can be influenced by	Can be recognized by
Difference in density of the liquid to be pipetted versus that of the water used for adjustment	up to 1.0 %	Readjusting the pipette (observe user information)	Comparing the density of the liquid to be pipetted with that of water
Difference in vapor pressure of the liquid to be pipetted versus that of the water used for adjustment	up to 2.0 %	Sufficient pre-wetting of the pipette tip; observe EN ISO 8655-6	Dripping tip
Uneven piston movement	up to 0.5 %	Smooth operation of piston; cleaning and lubricating of the piston	Monitoring one's own pipetting technique
Uneven rhythm and timing during pipetting	up to 1.5 %	Even pipetting technique	Maximum permitted errors are exceeded
Depth of plunging of the pipette tip and handling angle during pipetting	up to 1.0 %	Holding pipette in vertical position. Observe user information or EN ISO 8655-6	Visual control of plunging depth and handling angle
Failure to pre-wet pipette tip	up to 2 %	Pre-wetting of pipette tip	Maximum permitted errors are exceeded
Failure to wipe pipette tip on the vessel wall	up to 3.0 %	Wiping of the pipette tip on the vessel wall. Observe EN ISO 8655-6	Maximum permitted errors are exceeded
Leaky pipette tips	0.5 % up to 50 %	Using original or recommended pipette tips	Dripping tip or maximum permitted errors are exceeded

\*: The possible measurement deviations are reference values and are quoted in percent from the nominal value.

**Tab. 3:** Possible sources of error for pipetting with air-cushion pipette

### Re-adjustment in case of error

For re-adjustment, the pipette, its appropriate pipette tip and the water must have the same temperature (15 °C to 30 °C,  $\pm 0.5$  °C constant). The pipette display is set to the nominal volume required, and the volume pipetted and weighed ten times. The mean value of these weighing steps, corrected by the density of water at the given temperature, is the actual volume of the pipette. Adjustment is carried out according to the manufacturer's instructions (refer to the operating manual of respective pipette) using the special tool provided. After adjustment the pipette display is set to the nominal volume in the usual

manner. If the nominal volume still does not correspond to the result of the measurements, the above procedure is repeated. As adjustment affects the entire measuring range, in the case of pipettes with a variable volume all volumes specified in the technical data of the pipette must also be tested without fail (generally 10 %, 50 % and 100 % of the nominal volume).

Pipettes whose factory settings have been altered by re-adjustment must have clear visible identification according to DIN EN ISO 8655.

### Re-adjustment for liquids with a density other than water

It is possible to re-adjust piston-stroke pipettes with an air cushion for a specific volume of liquid that has a density different than water that the volume displayed corresponds to the pipetted volume. In the case of adjustable-volume pipettes, all other values are then deleted, which essentially turns them into fixed-volume pipettes. A pipette adjusted as such only displays a pipetting value that corresponds to

the type of liquid used and the tested volume! For this reason the re-adjusted pipette must be marked as a "fixed-volume pipette for solution X". If errors occur in the case of liquids with an increased vapor pressure (e.g. organic solvents), this cannot be compensated by re-adjustment. The use of a positive displacement pipette is recommended for applications involving such liquids [1].

### Literature

[1] Eppendorf Userguide No. 019: Liquid Handling 1



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