Physikalisch-Technische Bundesanstalt

DKD

Guideline DKD-R 8-1	**Calibration of piston-operated pipettes with air cushions**

Edition 12/2011, Revision 1

https://doi.org/10.7795/550.20240415

Deutscher Kalibrierdienst (DKD) – German Calibration Service

Since its foundation in 1977, the German Calibration Service has brought together calibration laboratories of industrial enterprises, research institutes, technical authorities, inspection and testing institutes. On 3rd May 2011, the German Calibration Service was reestablished as a *technical body* of PTB and accredited laboratories.

This body is known as *Deutscher Kalibrierdienst* (DKD for short) and is under the direction of PTB. The guidelines and guides developed by DKD represent the state of the art in the respective areas of technical expertise and can be used by the *Deutsche Akkreditierungsstelle GmbH* (the German accreditation body – DAkkS) for the accreditation of calibration laboratories.

The accredited calibration laboratories are now accredited and supervised by DAkkS as legal successor to the DKD. They carry out calibrations of measuring instruments and measuring standards for the measurands and measuring ranges defined during accreditation. The calibration certificates issued by these laboratories prove the traceability to national standards as required by the family of standards DIN EN ISO 9000 and DIN EN ISO/IEC 17025.

Contact:
Physikalisch-Technische Bundesanstalt (PTB)
DKD Executive Office
Bundesallee 100 38116 Braunschweig
P.O. Box 33 45 38023 Braunschweig
GERMANY
Telephone: +49 531 592-8021
Internet: www.dkd.eu

Suggestion for the citation of sources:
Guideline DKD-R 8-1 Calibration of piston operated pipettes with air cushions, Edition 12/2011, Revision 1, Physikalisch-Technische Bundesanstalt, Braunschweig und Berlin. DOI: 10.7795/550.20240415

Authors:
Members of DKD’s Technical Subcommittee *Volume and Density*.

Published by Physikalisch-Technische Bundesanstalt (PTB) for the German Calibration Service (DKD) as result of the cooperation between PTB and DKD’s Technical Committee *Chemical Measurands and Material Properties* / Subcommittee *Volume and Density.*

Foreword

DKD guidelines are application documents regarding the EN ISO/IEC 17025 requirements. The guidelines contain a description of the technical, the process-related and the organizational procedures which accredited calibration laboratories use as a model for defining internal processes and regulations. DKD guidelines may become an essential component of the quality management manuals of calibration laboratories. By implementing the guidelines, it is ensured that the devices to be calibrated are all treated equally in the various calibration laboratories and that the continuity and comparability of the work of the calibration laboratories are improved.

The DKD guidelines should not impede the further development of calibration procedures and processes. Deviations from guidelines as well as new procedures are allowed in agreement with the accreditation body if there are technical reasons to support this action.

This guideline was prepared by the *Volume/Density* Technical Subcommittee in cooperation with PTB and accredited calibration laboratories. The guideline was adopted in the meeting of the *Mass/Balances/Volume/Density* Technical Committee on 27 September 2011.

As preparation for this guideline, a pilot study (report [4]) on the calibration of piston-operated pipettes was conducted with 13 participants (national/international). The aim of the 2010 pilot study (report [4]) was to improve the comparability of measurements for the calibration of piston-operated pipettes.

Furthermore, the results of additional investigations of the effect of environmental influences on the dispensing result were incorporated into the guideline [2, 3, 5].

In terms of technical content, the present version of DKD-R 8-1 (Revision 1) is identical to Revision 0 of the guideline. The list of applicable standards and regulations as well as some of the designations have been updated. The reference to EN ISO/IEC 17025 has been adjusted to be in line with the current edition of this standard.

Table of Contents

1 Purpose and scope of application

This guideline defines the minimum requirements for the calibration procedure. Furthermore, it takes into account the special influences and the measurement uncertainty contributions for the calibration of *piston-operated pipettes with air cushions**. This guideline does not refer to pipettes *without* air cushions (positive displacement pipettes).

This guideline applies to the calibration of:

- Single-channel piston-operated pipettes with a fixed volume
- Single-channel piston-operated pipettes with a variable volume
- Multi-channel piston-operated pipettes

* In this guideline, these will only be called *piston-operated pipettes*.

Applicable standards and regulations

EN ISO 8655:2022 Parts 1, 2, 6	Piston-operated volumetric apparatus
ISO/TR 20461	Determination of uncertainty for volume measurements made using the gravimetric method, February 2023
JCGM 100: 2008	Evaluation of measurement data – Guide to the expression of uncertainty in measurement, September 2008
DKD-R 7-2	Richtlinie zur Kalibrierung nichtselbsttätiger Waagen; Ausgabe 01/2018 (German translation of EURAMET cg-18 Version 4.0)
EURAMET cg-18	Guidelines on the Calibration of Non-Automatic Weighing Instruments, Version 4.0, November 2015, https://www.euramet.org/publications-media-centre/calibration-guidelines/
EURAMET cg-19	Guidelines on the Determination of Uncertainty in Gravimetric Volume Calibration, Version 3.0, September 2018, https://www.euramet.org/publications-media-centre/calibration-guidelines/
ISO 3696	Water for analytical laboratory use – Specification and test methods, 1987 [the German translation DIN ISO 3696 was published in June 1991]
EA-4/02 M: 2022	Evaluation of the Uncertainty of Measurement in calibration, April 2022, https://european-accreditation.org/

2 Symbols

2.1 Abbreviations and symbols

Abbreviations/symbols	Explanation
a_0 to a_4	Constants (ITS-90 temperature scale) for calculating the water density
c	Sensitivity coefficient
CV	Random error as variation coefficient indicated in percent
e_S	Systematic error
g	Gravitational acceleration
h_W	Lifting height of the liquid column in the pipette tip
i	Continuous index
k_1 to k_3	Constants (ITS-90 temperature scale) for calculating the air density
m	Mass of the test liquid (corresponding to the difference of the balance readings)
m_E	Loss of mass due to evaporation
n	Number of individual measurements
p_L	Atmospheric pressure
s	Random error
t_W	Temperature of the test liquid
t_L	Air temperature during weighing
t_{L0}	273.15 K
t_M	Temperature of the piston-operated pipette during measurement
t_{M20}	Piston-operated pipette reference temperature of 20 °C
u	Standard measurement uncertainty
U	Expanded measurement uncertainty ($k = 2$)
V_0	Nominal volume
V_S	Selected volume
V_{20}	Volume at 20 °C reference temperature
V_T	Volume of the air cushion (dead volume)
Z	Correction factor describing the relationship between the mass which has been determined during weighing, and the volume
ρ_L	Air density
ρ_W	Density of the water used as a test liquid
ρ_G	Density of the standard weights used to calibrate the balance (equal to 8000 kg/m^3)
ϕ	Relative humidity
γ	Cubic coefficient of expansion of the material from which the pipette is made

2.2 Units of measurement

Units of measurement	Explanation
µL	Microlitre
mL	Millilitre
g	Gram
mg	Milligram
K	Kelvin
°C	Degrees Celsius
hPa	Hectopascal
%	relative humidity in percent
g/cm^3	Gram per cubic centimetres
µL/mg	Microlitre per milligram

3 Definitions

Calibration certificate:
Calibration certificates document the results of calibrations, including the measurement uncertainty. In this guideline, the term *calibration certificate* applies to the following documents (with restrictions):

- Calibration certificates from calibration laboratories whose accreditation bodies have signed the ILAC-MRA (see www.ilac.org)
- Calibration certificates from National Metrology Institutes with CMC entries (Appendix C of the CIPM MRA, see www.bipm.org)

The following terms have been taken from EN ISO 8655-1.

Piston-operated pipettes:
Piston-operated pipettes are volume measuring devices that are used to aspirate and dispense fixed or variable quantities of liquid. Single-channel piston-operated pipettes only have one piston/cylinder set. Multi-channel piston-operated pipettes have one piston/cylinder set for each channel; the same volume of liquid can be dispensed into several receptacles at the same time. A differentiation is made between piston-operated pipettes with or without air cushions (positive displacement pipettes).

Nominal volume:
The nominal volume (V_0) of a volume measuring device is the volume defined by the manufacturer to identify and specify the measuring range. For multi-channel piston-operated pipettes, the nominal volume is specified for an individual channel.

Useful volume range:
The useful volume range of a volume measuring device with a variable volume is a sub-range of the nominal volume; within this sub-range, dispensing can be completed under observance of the maximum permissible errors defined in the international standard ISO 8655. The upper

limit of the useful volume range is always the nominal volume. If not specified otherwise by the supplier, the lower limit is 10 % of the nominal volume.

Selected volume:
The selected volume V_S of a volume measuring device with a variable volume is the volume set by the user to dispense a selected volume from the useful volume range of a piston-operated device. In the case of volume measuring devices with a fixed volume, the selected volume is the nominal volume.

Volume of the air cushion (dead volume)
The volume of the air cushion (V_T) is the geometric space between the piston and the tip opening. The expansion of the air cushion volume is defined by the first stop of the piston in the pipette.

4 Aim of the calibration

The calibration of piston-operated pipettes with air cushions serves to define the deviation of the dispensed volume from the selected testing volume. The measurement trueness and the measurement uncertainty analysis of the determined measuring results are decisive for the implementation of quality-related metrological specifications in fields such as medicine and pharmacology. Thereby, metrological traceability to national or international standards must be ensured.

As stipulated in DIN EN ISO/IEC 17025:2018 (ISO/IEC 17025:2017), national and international intercomparisons / comparison measurements are required to ensure the comparability of the calibration results.

5 General requirements for the calibration capability of piston-operated pipettes with air cushions

The general requirements for the calibration capability of piston-operated pipettes with air cushions can be divided into three categories:

- Requirements of standard EN ISO 8655
- Requirements contained in the product information of the manufacturers
- Additional requirements of standard practice

5.1 Requirements of standard EN ISO 8655

Please refer to Part 1, Part 2 and Part 6 of EN ISO 8655 for information on these requirements.

5.2 Requirements contained in the product information of the manufacturers

These requirements differ according to the information and scope of the corresponding manufacturer's product information. Some of the most important requirements are:

- Information on the sterilizability of the piston-operated pipettes and spare parts
- Notes on operation, application exclusions, maintenance, cleaning and servicing

- Indication of a manufacturer's specification with the permitted tolerances of the random and systematic errors with reference to the adjustment (in/ex and reference temperature)
- Indication of the approved pipette tips to be used

5.3 Requirements arising from observing the calibration process

The requirements arising from standard practice primarily apply to the immediate usability of the piston-operated pipette. This includes:

- Labelling the piston-operated pipette with
    - the serial number or another unique identification number,
    - the nominal volume (piston-operated pipette with a fixed volume),
    - the volume range (piston-operated pipette with a variable volume),
    - the unit of measurement, for example: "µL" or "mL", or with
    - information on the type and manufacturer.

- No internal or external damage, e.g.
    - cracks, fissures
    - in the case of piston-operated pipettes with a variable volume: no unintentional adjustment of the digital indicator
    - deformed, scratched or heavily contaminated pipette shaft

- No residual liquids and dirt particles in the piston-operated pipette
- Complete and secure sealing of the piston

This also includes the requirements for the pipette tips or piston-operated pipette/pipette tip system:

- Use of original tips from the manufacturer or tips approved by the manufacturer
- Sufficiently firm positioning of the tip on the pipette cone
- Tight and secure sealing on the pipette cone
- The tips must ensure a continuous discharge of liquid
- The tips must have a uniform discharge opening
- The test liquid *water* must be dispensed without residue

In certain cases, it may make sense to conduct a calibration in the "as found" condition, i.e., in a condition where not all of the calibration capability criteria are met.

6 Ambient conditions

The calibration must be carried out at stable ambient temperatures.
The ambient conditions

- air temperature
- relative humidity
- atmospheric pressure*

have an influence on the

- weighing technology
- calibration object/piston-operated pipette
- test liquid

and therefore have a significant effect on the calibration result of the piston-operated pipettes and the corresponding measurement uncertainty budget.

An important requirement for a metrologically accurate calibration is that the specified ambient conditions are ensured by the use of air conditioning.
The calibration must be conducted after the temperature between the calibration object and the environment has been equalized. An equalization time of at least 2 hours must be observed for the calibration object.

The ambient conditions must be recorded.

During the calibration, also the fluctuations in the ambient conditions must be monitored. If deviations occur, the air temperature, the atmospheric pressure and the relative humidity have to be corrected.

* The current atmospheric pressure enters into the air density calculation and will be taken into account. In addition, the dependence of the atmospheric pressure on the altitude needs to be taken into account (report [2]).

7 Calibration procedure

Piston-operated pipettes are calibrated using the gravimetric method in accordance with EN ISO 8655-6.
With this procedure, the mass of the liquid volume is determined from the indication of the weighing instrument taking the air buoyancy into account, and is converted into the volume via the water density. In this way, the metrological traceability of the volume is realized by the physical quantity mass as the reference standard.
A full calibration includes the metrological recording of 10 measured values per testing volume and per pipette channel.

The measuring systems are analytical balances with the corresponding accessories (e.g., weighing vessel, evaporation trap, draught protection), which are supplied together with calibration software and which have been specially modified for pipette calibration.

Water (quality 3, ISO 3696) shall be used as a test liquid for the gravimetric testing method according to EN ISO 8655-6.

7.1 Calibration objects

The calibration objects are classified into single-channel and multi-channel piston-operated pipettes with air cushions. Single-channel piston-operated pipettes are available as volume measuring devices with a fixed or with a variable volume.
Multi-channel piston-operated pipettes are available with a variable volume.
Single-channel and multi-channel piston-operated pipettes are available with manual or electronic operation.
The piston-operated pipettes are available with various measuring ranges.
Here are some typical examples:

Single-channel piston-operated pipettes with a fixed volume	Single-channel piston-operated pipettes with a variable volume	Multi-channel piston-operated pipettes
10 µL	0.1 µL to 2.5 µL	0.5 µL to 10 µL
20 µL	0.5 µL to 10 µL	5 µL to 50 µL
50 µL	2 µL to 20 µL	10 µL to 100 µL
100 µL	10 µL to 100 µL	25 µL to 250 µL
200 µL	20 µL to 200 µL	30 µL to 300 µL
250 µL	50 µL to 200 µL	50 µL to 300 µL
500 µL	100 µL to 1000 µL	100 µL to 1200 µL
1000 µL	500 µL to 2500 µL	
2500 µL	500 µL to 5000 µL	
	1000 µL to 10000 µL	

Other volume ranges or measuring ranges are also available and used in practice.

7.2 Pipette tips – Accessories for dispensing

Pipette tips which are attached to the pipette shaft are used for volume dispensing with piston-operated pipettes. Only unused pipette tips approved by the manufacturer may be used. Like the piston-operated pipettes, the pipette tips have to be stored in the measuring room for at least two hours before starting calibration.
According to EN ISO 8655-2, a tip replacement is recommended after each individual measurement. However, deviations from this rule are allowed. According to this guideline, a pipette can be calibrated with one pipette tip per channel. However, the air cushion still needs to be pre-wet five times at the start of the calibration. Pre-wetting should also be conducted when the volume is changed (setting of a new test volume).
If residues remain in the tip, the tip has to be replaced categorically.

If a tip is to be replaced, the new pipette tip must be pre-wet five times as well.

If tip replacement according to the recommendation in EN ISO 8655 is applied, this must be documented in the calibration certificate.

7.3 Additional notes for the calibration process (as a supplement to EN ISO 8655-6)

After the water has been aspirated and the waiting period has been observed, the pipette must be lifted vertically from the water with a smooth and initially slow movement without touching the tube inner wall.

When the piston slides back after dispensing, the pipette tip must not be too close to the top of the weighing vessel or the supply vessel to ensure that no residual liquid or moist air from the wall of the weighing vessel is aspirated into the tip again.

The calibration should be started with the smallest partial volume (approx. 10 % of the nominal volume).

The following immersion depths and waiting periods should be observed:

Volume range	Immersion depth in mm	Waiting period in s
0.1 µL - 1 µL	1 – 2	1
> 1 µL - 100 µL	2 – 3	1
> 100 µL - 1000 µL	2 – 4	1
> 1000 µL	3 – 6	3

8 Measurement uncertainty

The measurement uncertainty is a value which is indicated together with the measuring result. The measurement uncertainty is determined by the measuring procedure and is assigned to the measuring result. The measurement uncertainty characterizes a range of values which, due to the measurement, can reasonably be attributed to the measurand. As a matter of principle, the measurement uncertainty is calculated according to the international guideline JCGM 100 “Evaluation of measurement data – Guide to the expression of uncertainty in measurement” or according to EA-4/02 M: 2022.

The dependence of the measurement uncertainty within one volume range has to be adequately described. Hereby, the nominal volume has to be taken as a basis and the volume ranges must be selected in relation to the different measuring ranges in a technically meaningful way (see 7.1).

An indication in absolute volume units, in a very narrow classification of the volume ranges, is not precise enough and not practical. This means that an assignment between nominal volume and measurement uncertainty is not given over the entire range.
The measurement uncertainty should be specified in percent.
The measurement uncertainty of the partial volumes must be specified (see 9).

ISO/TR 20461, for the measurement uncertainty determination according to the gravimetric method, will be taken into account when establishing the measurement uncertainty budget. In accordance with ISO/TR 20461, the volume for the reference temperature of 20 °C will be calculated as follows:

$$V_{20} = \frac{m}{\rho_G} \cdot \frac{\rho_G - \rho_L}{\rho_W - \rho_L} \cdot [1 - \gamma(t_M - t_{M20})] \quad (1)$$

Furthermore, the calculation formulae for the water density and the air density must be taken into account. For the standard measurement uncertainty, the following equation is yielded:

$$u^2(V_{20}) = \left(\frac{\partial V_{20}}{\partial m}\right)^2 \cdot u^2(m) + \left(\frac{\partial V_{20}}{\partial t_W}\right)^2 \cdot u^2(t_W) + \left(\frac{\partial V_{20}}{\partial t_L}\right)^2 \cdot u^2(t_L) + \left(\frac{\partial V_{20}}{\partial p_L}\right)^2 \cdot u^2(p_L) + \ldots \quad (2)$$

The following fault tree is a comprehensive representation of all the factors which influence the measurement uncertainty and are taken into consideration by this guideline.

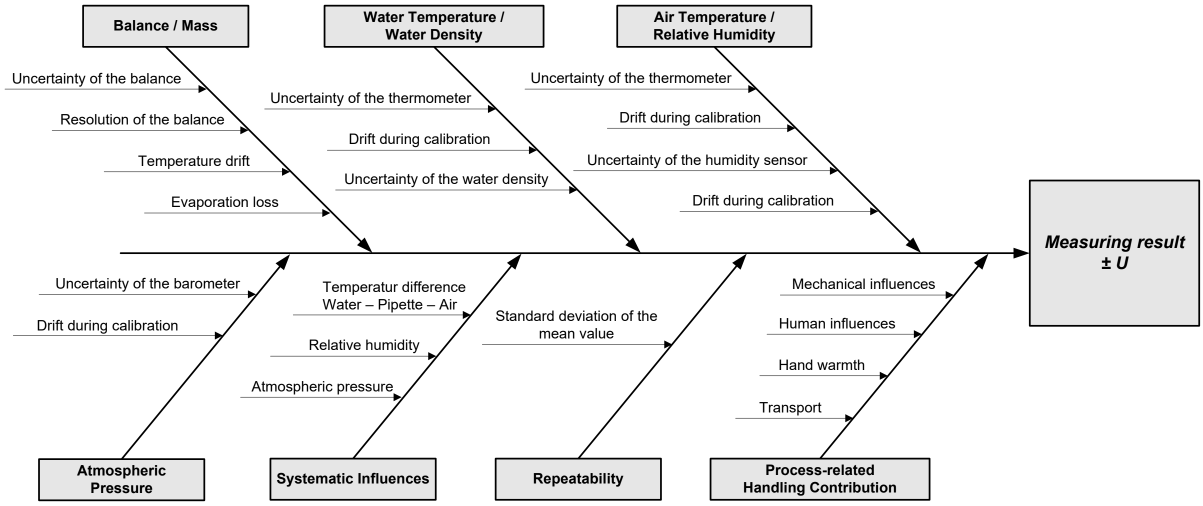

Figure 1: Influences on the measurement uncertainty during the calibration of piston-operated pipettes

8.1 General

The accredited measurement uncertainty budgets for the measurands and the calibration procedures are a precondition for ensuring the comparability of measuring results also across international borders.

Setting up the measurement uncertainty budgets for the gravimetric calibration procedure for piston-operated pipettes includes:

- Optimization and definition of the calibration procedure
- Definition of concrete ambient conditions
- Metrological evaluation of the various calibration objects from different device manufacturers
- Taking into account the process-related handling contribution

The process-related handling contribution is dependent on the user and on the design of the piston-operated pipettes, e.g. whether they are single-channel or multi-channel ones. This measurement uncertainty contribution is composed of random and systematic components.

If individual influences on the calibration result and on its measurement uncertainty cannot be determined precisely, their maximum possible contribution to the uncertainty must be estimated and taken into account. The basis/source of this estimation must be indicated.

The measurement conditions of the calibration must be described as completely as possible because the measurement uncertainties are also dependent on the conditions of use.
In the case of comparison measurements, the calibration conditions should be defined in order to ensure the comparability of the measuring results.

In future, the measurement uncertainties described below will be used in the appendix of the accreditation certificate as the basis of the “smallest assignable measurement uncertainty”. These uncertainties are part of the calibration and measurement capabilities (CMC). The concept of the CMC is defined in EA-4/02 M: 2022.

The “smallest assignable measurement uncertainty” can be achieved if all of the following conditions are strictly observed. If this cannot be accomplished, the measurement uncertainty must be adjusted accordingly and taken into account using additional contributions to the measurement uncertainty budget (see also [2]). As a result, the actually achieved measurement uncertainty may be larger.

8.2 Uncertainty contributions from the balance

As a rule, one can assume that the ambient conditions during the calibration of the balance and during the calibration of the pipette are nearly identical.

The calibration task, the measuring range, the resolution of the balance and the corresponding measurement uncertainty must be adapted to each other in order to ensure a user-specific usage according to EURAMET cg-18.
The balance should be calibrated user-specifically, i.e., the measuring range of the calibration task (pipette) should correspond to the calibrated weighing range.
Please note that “zero” is not a real measuring point. If no minimum load is specified by the manufacturer, the calibration should start at ≥ 1 mg.

Prior to a calibration of piston-operated pipettes, it must be ensured that the balance is calibrated according to EURAMET cg-18. This ensures that the contributions *resolution of the balance*, *repeatability*, *eccentricity* and *non-linearity* which are assigned to the weighing are included in the current calibration certificate.

The calibration certificate of the balance used is the basis of the further measurement uncertainty analysis of the gravimetric method.
The uncertainty contribution *reading*, or respectively, *resolution of the balance*, has to be considered in the measurement uncertainty budget twice (tare weighing and gross weighing). The result of the weighing is the difference between the indicated values.

Another contribution to be taken into account is the influence of the ambient temperature according to the specifications of the manufacturer; this contribution can be found in the manufacturer’s specifications.

An additional contribution comes from the drifting of the balance due to aging and wear. This influence can be determined by means of intermediate checks or by re-calibration. Hence, this contribution should be taken into account as a result of long-term observation and conclusions can be drawn.

As piston-operated pipettes are calibrated according to the gravimetric method, it is of extreme importance that the balance is regularly observed. For this reason, intermediate checks with suitable, calibrated weights (calibration certificate) have to be carried out within the calibration period.

During the dispensing of the piston-operated pipettes, free liquid surfaces occur, so that loss by evaporation should be taken into account as a contribution.
Loss by evaporation can either be determined, or it can be estimated – on the basis of one's own previous experience – with respect to the pipette volume.
To minimize the influence of evaporation, modern weighing equipment for calibrating piston-operated pipettes is equipped with evaporation traps.

8.3 The measurement uncertainty contributions "water temperature/water density"

Distilled or deionized water is used as the test liquid for calibrating piston-operated pipettes. The water must be at least quality level 3 according to ISO 3696 (electrolytic conductivity < 5 µS/cm).
Prior to and during the calibration, one must ensure that the water used is free of bubbles and that it is adjusted to the air temperature (deviation of < 0.5 K).
In comparison measurements/intercomparisons, or when specifying the smallest assignable measurement uncertainty, the limit should be narrower, i.e. 0.2 K.
In all other cases, the measurement uncertainty must be adjusted accordingly.
The air temperature must be selected within the range of 20 °C to 25 °C. In countries with a reference temperature of 27 °C, the temperature range may be adjusted accordingly. Measures must be taken to prevent evaporative cooling, e.g. by covering the recipient or by using a thermostat.

Compliance with the specified ambient conditions *air temperature/relative humidity* during the calibration is ensured by a suitable air conditioning system. Due to this, also the stability of the water temperature is positively influenced.

The temperature of the test liquid used (water) is determined by using a calibrated thermometer. In the measurement uncertainty budget, the uncertainty of the thermometer and the fluctuation of the water temperature during the calibration are taken into account.

The influence of the temperature in the weighing vessel may be neglected.

The uncertainty for calculating the water density according to [6] is estimated to be $10 \cdot 10^{-6}$ as the precise isotope ratio and the gas content are not known.
The water density is needed to calculate the volume of the test liquid.

8.4 The measurement uncertainty contributions “air temperature” and "relative humidity"

During the calibration of piston-operated pipettes, the ambient conditions *air temperature* and *relative humidity* are realized in specified parameters by air conditioning. The measurement data of the ambient conditions are measured, recorded and documented by means of suitable, calibrated thermometers and humidity sensors.

The air density may be calculated according to the EURAMET cg-19 equation (4).

An experimental study [4] has proved that during the calibration, the fluctuations in the air temperature should be < 0.5 K. The water temperature should be adjusted to the air temperature (see Section 8.3).

The relative humidity should be 45 % to 60 %. Low air humidities lead to a reduction of the measured volumes.

Calibrating piston-operated pipettes with the smallest assignable measurement uncertainties requires the ambient conditions to remain within small tolerance limits.

The air temperature and the relative humidity also have an influence on the volume dispensed by the pipette (see Section 8.7).
During the calibration, the drift behaviour of the air temperature should therefore not exceed 0.5 K and the drift behaviour of the relative humidity should not exceed 5 %.

8.5 The measurement uncertainty contribution “atmospheric pressure”

The atmospheric pressure should be measured and documented using a calibrated precision barometer. The atmospheric pressure is a measurand which is needed to calculate the air density and, thus, the volume. The precision barometer used should have a minimum resolution of 1 hPa.

The atmospheric pressure also has an influence on the dispensed volume in the pipette (see Section 8.7).

8.6 Thermal expansion of the volume measuring device

Due to the different types and designs of the individual pipettes, the cubic coefficient of expansion cannot be universally determined.
The cubic coefficient of expansion of a piston-operated pipette is made up of the linear expansion coefficient of the components which connect the piston stroke stops and the coefficient of expansion of the piston cross section.
Material properties, material pairings, and a variety of geometries and designs, all have an influence on the cubic coefficient of expansion. However, these influences cannot be represented in mathematical terms and, therefore, cannot be defined by any of the manufacturers.
For that reason, the cubic coefficient of expansion is taken into account in the measurement uncertainty budget with the value “zero”.
The measurand, however, then refers to the temperature taken during the measurement.

The reference temperature for calibrating piston-operated pipettes is 20 °C according to EN ISO 8655-1.
If the calibration is carried out at another temperature, this deviation must be taken into account (e.g., increase in the measurement uncertainty). For this purpose, data on the entire temperature behaviour of the calibration object must be available, e.g., from the manufacturer.

It is indispensable to ensure that the piston-operated pipettes are stored in the measuring room for a sufficiently long period of time (at least for 2 hours). As it is not possible to measure the temperature of the pipette directly, it will be equated with the ambient temperature. The measurement uncertainty contribution must be estimated.

8.7 Observation of the systematic influences of the piston-operated pipettes with air cushions during the calibration

Volume dispensing in a piston-operated pipette with air cushion is a thermodynamic process which begins when the pipette tip is immersed into the water and ends when it is removed (separation of the liquid column).

Comprehensive descriptions can be found in the reports [2] and [5].

The influences depend, in particular, on the size of the air cushion and on the lifting height in the pipette tip. The following influences will be examined in the sections below:

- Temperature differences between the water, the pipette and the air
- Relative humidity
- Atmospheric pressure

8.7.1 Temperature differences between the water, the pipette and the air

Temperature differences between the water, the pipette and the air in the pipette system/pipette tip lead to significant influences on the dispensed volume. These influences are decisive components of the measurement uncertainty budget.

During the calibration, the difference between the air temperature and the water temperature should be less than 0.5 K [4].
To achieve the smallest assignable measurement uncertainty, or for comparison measurements and intercomparisons, the temperature difference should, however, be 0.2 K at maximum. (The attached measurement uncertainty budget is based on this value.)

8.7.2 Relative humidity

The influence of the relative humidity is an important criterion for defining the ambient conditions (see Section 8.4) and must be documented in the calibration procedure/QM manual. The evaporation of the calibration liquid is directly dependent on the relative humidity of the environment as the liquid evaporates during the aspiration process and as even the smallest evaporated quantities of liquid can lead to a large volume displacement in the air cushion (factor: approx. 1250). This influence is reduced by pre-wetting five times.

To achieve the smallest assignable measurement uncertainty, or for comparison measurements and intercomparisons, a relative humidity of (50 ± 5) % should, however, be achieved. (The attached measurement uncertainty budget is based on this value.)

8.7.3 Atmospheric pressure

Calibrations of piston-operated pipettes with air cushions at various altitudes have a significant influence on the measuring results. The influence of the altitude on the calibration result is discussed in the report [3] and confirmed in an experimental study.
See also the paper [2] that demonstrates by means of a theoretical model that the dispensed volume is significantly reduced at larger altitudes due to the low air density.

To achieve the comparability of the calibration results, corrections of the altitude must be made. The current atmospheric pressure during the measurement must be indicated in the calibration certificate.

The change in volume which results from the calibration at a location $X2$ (with the atmospheric pressure $p_{\mathrm{L},X2}$) compared to a location $X1$ (with the atmospheric pressure $p_{\mathrm{L},X1}$) is determined by applying the following formula:

$$\Delta V = -V_{\mathrm{T}} \cdot \rho_{\mathrm{W}} \cdot g \cdot h_{\mathrm{W}} \cdot \left(\frac{1}{p_{\mathrm{L},X2} - \rho_{\mathrm{W}} \cdot g \cdot h_{\mathrm{W}}} - \frac{1}{p_{\mathrm{L},X1} - \rho_{\mathrm{W}} \cdot g \cdot h_{\mathrm{W}}} \right) \quad (3)$$

Note: The lifting height h_{W} may, in good approximation, be considered equal at both locations.

For general meteorological atmospheric pressure fluctuations, a contribution of ± 20 hPa (triangular distribution) is taken into account in the measurement uncertainty budget. A correction will not be made.

8.8 Repeatability

The empirical standard deviation of the mean value of a series of 10 individual measurements is applied as the *repeatability*. The empirical standard deviation characterizes the scattering of the measured values under the same measurement conditions as during the calibration of piston-operated pipettes and is calculated by applying the following formula:

$$s = \sqrt{\frac{\sum_{i=1}^{n} (V_i - \bar{V})^2}{n-1}} \quad (4)$$

The standard measurement uncertainty (confidence interval of the mean value) of the repeatability is determined according to the *Type A evaluation of uncertainty* (GUM) and calculated by applying the following formula (see also [2]):

$$u(s) = \frac{s}{\sqrt{n}} \quad (5)$$

Empirically, the experimental standard deviation lies at approx. one third of the tolerance specified by the manufacturer for the repeatability (max. random error).
For multi-channel piston-operated pipettes, this usually requires a higher value than for single-channel piston-operated pipettes.

8.9 Process-related handling contribution

The *process-related handling contribution* is a minimum value which cannot be fallen short of. This value encompasses the influences on the dispensed volume which occur due to the handling of the devices during the calibration of piston-operated pipettes, and it should be taken into account in the budget as follows:

- for single-channel piston-operated pipettes with a fixed volume: at least 0.07 % of the nominal volume, and for single-channel piston-operated pipettes with a variable volume and for multi-channel pipettes: 0.1 % of the nominal volume (empirical values for the standard uncertainty based on experience).

Various influences contribute to the process-related handling contribution; the most important influences are:

- Mechanical influences
- Operator-based influences
- Hand warmth
- Transport

8.9.1 Mechanical influences

The term *mechanical influences* comprises, among other things, the following influences:

- Hysteresis of the digital indicator (in the case of variable pipettes, not in the case of electronic pipettes)
- Reproducibility of the piston stroke

8.9.2 Operator-based influences

The *operator-based influences* during the calibration of piston-operated pipettes strongly depend on the experience of the operator, which has to be developed and maintained by regular training.

- Waiting period after the aspiration
- Steady pace of the pipetting rhythm
- Pipette’s angle of inclination during aspiration/dispensing

- Operating force (not in the case of electronic pipettes)
- Immersion depth

8.9.3 Hand warmth

An additional influence to be taken into account is *hand warmth*. Hand warmth is an individual influence which depends on the operator and on the pipette type. Due to the position of the lifting piston in the piston-operated pipette, hand warmth has the strongest effect on the nominal volume.

To minimize the influence of *hand warmth*, direct contact with the calibration object should be kept to a minimum.

Hand warmth leads to a reduction of the volume (temporal drift). If the influence of hand warmth occurs during the calibration, this contribution has to be estimated and taken into account in the measurement uncertainty.

8.9.4 Transport

The influence of transport on the measurement uncertainty only refers to the proper shipment of piston-operated pipettes, e.g., temperature fluctuations or mechanical vibrations that occur during shipment.

9 Measurement uncertainty budgets

Note: Three sample budgets are attached as an Appendix.

Appendix A	Sample measurement uncertainty budget for calibrating piston-operated pipettes with a fixed volume, nominal volume 1000 µL
Appendix B	Sample measurement uncertainty budget for calibrating piston-operated pipettes with a variable volume, nominal volume 100 µL
Appendix C	Sample measurement uncertainty budget for calibrating multi-channel piston-operated pipettes, nominal volume 10 µL

The attached measurement uncertainty budgets are valid for the corresponding nominal volumes. The expanded measurement uncertainty for the mean, or respectively, the lower testing volume is calculated by 75 %, or respectively, 50 % of the expanded measurement uncertainty of the nominal volume.

10 List of references

[1] H. WOLF: „Volumenbestimmung im Mikroliterbereich“, Physikalisch-Technische Bundesanstalt, PTB-Bericht *PTB-Th Ex-16*, Braunschweig, 2001

[2] K.-H. LOCHNER, R. FELDMANN, J. PFOHL: „Analyse der Einflussgrößen auf die Kalibrierung von Kolbenhubpipetten mit Luftpolster“, Expert Report DKD-E 8-2, 2013

[3] CHR. SPÄLTI, M. POLIN, S. FINKBEINER: „Einfluss der Höhenlage auf das Volumenergebnis einer Kolbenhubpipette mit Luftpolster“, Expert Report DKD-E 8-3, 2013

[4] B. WERNER, U. BREUEL, N. SCHIERING, O.SCHNELLE-WERNER, L. HALLBAUER: „Experimentelle Studie zur Kalibrierung von Kolbenhubpipetten mit Luftpolster“, Expert Report DKD-E 8-1, 2013

[5] K.-H. LOCHNER: „Untersuchung der Messgenauigkeit von Kolbenhubpipetten mit Luftpolster“ (Forschungsbericht AIF Nr. 9152), Forschungsgemeinschaft für Technisches Glas e.V., 1995

[6] M. TANAKA, G. GIRARD, R. DAVIS, A. PEUTO, N. BIGNELL: Recommended table for the density of water between 0 °C and 40 °C based on recent experimental reports; Metrologia 2001, 38, 301-309

Appendix A

Sample measurement uncertainty budget for calibrating piston-operated pipettes with a fixed volume

Nominal volume 1000 µL

Quantity X_i	Best estimate x_i	Half width of distribution a	Probability distribution $P(x_i)$	Divisor k	Standard measurement uncertainty $u(x_i)$	Sensitivity coefficient c_i	Uncertainty contribution $u_i(y)$
Balance / Mass							
Measurement error of the balance	0 mg	30 µg	normal	2	15.000 µg	0.001 µL/µg	0.015 µL
Resolution of balance (with load)	999.60 mg	5 µg	rectangular	$\sqrt{3}$	2.887 µg	0.001 µL/µg	0.003 µL
Resolution of balance (without load)	0.00 mg	5 µg	rectangular	$\sqrt{3}$	2.887 µg	0.001 µL/µg	0.003 µL
Temperature drift	0 mg	0.2 K	rectangular	$\sqrt{3}$	0.115 K	0.001 µL/K	1.2E-04 µL
Evaporation loss	0 mg	20 µg	rectangular	$\sqrt{3}$	11.547 µg	0.001 µL/µg	0.012 µL
Water temperature / Water density							
Indication of the thermometer	21.60 °C	0.012 K	normal	2	0.006 K	0.21 µL/K	0.001 µL
Drift during calibration	0 K	0.2 K	rectangular	$\sqrt{3}$	0.115 K	0.21 µL/K	0.024 µL
Water density	997.86 kg/m³	10 ppm	rectangular	$\sqrt{3}$	0.00001 mg/µL	-1000 µL²/mg	-0.006 µL
Air temperature							
Indication of the thermometer	21.8 °C	0.13 K	normal	2	0.065 K	0.0045 µL/K	2.9E-04 µL
Drift during calibration	0 K	0.2 K	rectangular	$\sqrt{3}$	0.115 K	0.0045 µL/K	5.2E-04 µL
Air pressure							
Indication of the barometer	1008.0 hPa	0.05 hPa	normal	2	0.025 hPa	0.0012 µL/hPa	3.0E-05 µL
Drift during calibration	0 hPa	1 hPa	rectangular	$\sqrt{3}$	0.577 hPa	0.0012 µL/hPa	6.9E-04 µL
Relative humidity							
Indication of the humidity sensor	53 % RH	0.6 % RH	normal	2	0.300 % RH	0.0001 µL/% RH	3.0E-05 µL
Drift during calibration	0 % RH	5 % RH	rectangular	$\sqrt{3}$	2.887 % RH	0.0001 µL/% RH	2.9E-04 µL
Temp. difference water-pipette-air	0.0 K	0.2 K	rectangular	$\sqrt{3}$	0.115 K	2.2 µL/K	0.254 µL
Relative humidity	53 % RH	5 % RH	rectangular	$\sqrt{3}$	2.887 % RH	0.07 µL/% RH	0.202 µL
Atmospheric pressure / Altitude	1008.0 hPa	20 hPa	triangular	$\sqrt{6}$	8.165 hPa	0.014 µL/hPa	0.114 µL
Repeatability	0 mg	0.67 µL	normal	$\sqrt{10}$	0.211 µL	1	0.211 µL
Process-related handling contribution	0 mg	0.70 µL	rectangular	$\sqrt{3}$	0.404 µL	1	0.404 µL
Y (Volume)	1002.9 µL						

$u(y)$ =	0.57 µL
$U(y)$ =	**1.2 µL**
$w(y)$ =	0.06 %
$W(y)$ =	**0.12 %**

Appendix B

Sample measurement uncertainty budget for calibrating piston-operated pipettes with a variable volume

Nominal volume 100 µL

Quantity X_i	Best estimate x_i	Half width of distribution a	Probability distribution $P(x_i)$	Divisor k	Standard measurement uncertainty $u(x_i)$	Sensitivity coefficient c_i	Uncertainty contribution $u_i(y)$
Balance / Mass							
Measurement error of the balance	0 mg	15 µg	normal	2	7.500 µg	0.001 µL/µg	0.0075 µL
Resolution of balance (with load)	100.059 mg	0.5 µg	rectangular	$\sqrt{3}$	0.289 µg	0.001 µL/µg	0.0003 µL
Resolution of balance (without load)	0.000 mg	0.5 µg	rectangular	$\sqrt{3}$	0.289 µg	0.001 µL/µg	0.0003 µL
Temperature drift	0 mg	0.2 K	rectangular	$\sqrt{3}$	0.115 K	0.0001 µL/K	1.2E-05 µL
Evaporation loss	0 mg	15 µg	rectangular	$\sqrt{3}$	8.660 µg	0.001 µL/µg	0.0087 µL
Water temperature / Water density							
Indication of the thermometer	21.70 °C	0.012 K	normal	2	0.006 K	0.021 µL/K	0.0001 µL
Drift during calibration	0 K	0.2 K	rectangular	$\sqrt{3}$	0.115 K	0.021 µL/K	0.0024 µL
Water density	997.84 kg/m³	10 ppm	rectangular	$\sqrt{3}$	0.00001 mg/µL	-100 µL²/mg	-0.0006 µL
Air temperature							
Indication of the thermometer	21.8 °C	0.13 K	normal	2	0.065 K	0.00045 µL/K	2.9E-05 µL
Drift during calibration	0 K	0.2 K	rectangular	$\sqrt{3}$	0.115 K	0.00045 µL/K	5.2E-05 µL
Air pressure							
Indication of the barometer	1009.0 hPa	0.05 hPa	normal	2	0.025 hPa	0.00012 µL/hPa	3.0E-06 µL
Drift during calibration	0 hPa	1 hPa	rectangular	$\sqrt{3}$	0.577 hPa	0.00012 µL/hPa	6.9E-05 µL
Relative humidity							
Indication of the humidity sensor	53 % RH	0.6 % RH	normal	2	0.300 % RH	0.00001 µL/% RH	3.0E-06 µL
Drift during calibration	0 % RH	5 % RH	rectangular	$\sqrt{3}$	2.887 % RH	0.00001 µL/% RH	2.9E-05 µL
Temp. difference water-pipette-air	0.0 K	0.2 K	rectangular	$\sqrt{3}$	0.115 K	0.22 µL/K	0.0254 µL
Relative humidity	53 % RH	5 % RH	rectangular	$\sqrt{3}$	2.887 % RH	0.007 µL/% RH	0.0202 µL
Atmospheric pressure / Altitude	1009.0 hPa	20 hPa	triangular	$\sqrt{6}$	8.165 hPa	0.0012 µL/hPa	0.0098 µL
Repeatability	0 mg	0.067 µL	normal	$\sqrt{10}$	0.021 µL	1	0.0211 µL
Process-related handling contribution	0 mg	0.10 µL	rectangular	$\sqrt{3}$	0.058 µL	1	0.0577 µL
Y (Volume)	100.38 µL					$u(y)$ =	0.071 µL
						$U(y)$ =	**0.15 µL**
						$w(y)$ =	0.07 %
						$W(y)$ =	**0.15 %**

Appendix C

Sample measurement uncertainty budget for calibrating multi-channel piston-operated pipettes

Nominal volume 10 µL

Quantity X_i	Best estimate x_i	Half width of distribution a	Probability distribution $P(x_i)$	Divisor k	Standard measurement uncertainty $u(x_i)$	Sensitivity coefficient c_i	Uncertainty contribution $u_i(y)$
Balance / Mass							
Measurement error of the balance	0 mg	15 µg	normal	2	7.500 µg	0.001 µL/µg	0.0075 µL
Resolution of balance (with load)	10.010 mg	0.5 µg	rectangular	$\sqrt{3}$	0.289 µg	0.001 µL/µg	0.0003 µL
Resolution of balance (without load)	0.000 mg	0.5 µg	rectangular	$\sqrt{3}$	0.289 µg	0.001 µL/µg	0.0003 µL
Temperature drift	0 mg	0.2 K	rectangular	$\sqrt{3}$	0.115 K	1.0E-05 µL/K	1.2E-06 µL
Evaporation loss	0 mg	10 µg	rectangular	$\sqrt{3}$	5.774 µg	0.001 µL/µg	0.0058 µL
Water temperature / Water density							
Indication of the thermometer	22.20 °C	0.012 K	normal	2	0.006 K	0.0021 µL/K	1.3E-05 µL
Drift during calibration	0 K	0.2 K	rectangular	$\sqrt{3}$	0.115 K	0.0021 µL/K	0.0002 µL
Water density	997.72 kg/m³	10 ppm	rectangular	$\sqrt{3}$	0.00001 mg/µL	-10 µL²/mg	-0.0001 µL
Air temperature							
Indication of the thermometer	22.4 °C	0.13 K	normal	2	0.065 K	0.000045 µL/K	2.9E-06 µL
Drift during calibration	0 K	0.2 K	rectangular	$\sqrt{3}$	0.115 K	0.000045 µL/K	5.2E-06 µL
Air pressure							
Indication of the barometer	1009.0 hPa	0.05 hPa	normal	2	0.025 hPa	0.000012 µL/hPa	3.0E-07 µL
Drift during calibration	0 hPa	1 hPa	rectangular	$\sqrt{3}$	0.577 hPa	0.000012 µL/hPa	6.9E-06 µL
Relative humidity							
Indication of the humidity sensor	53 % RH	0.6 % RH	normal	2	0.300 % RH	0.000001 µL/% RH	3.0E-07 µL
Drift during calibration	0 % RH	5 % RH	rectangular	$\sqrt{3}$	2.887 % RH	0.000001 µL/% RH	2.9E-06 µL
Temp. difference water-pipette-air	0.0 K	0.2 K	rectangular	$\sqrt{3}$	0.115 K	0.019 µL/K	0.0022 µL
Relative humidity	53 % RH	5 % RH	rectangular	$\sqrt{3}$	2.887 % RH	0.001 µL/% RH	0.0029 µL
Atmospheric pressure / Altitude	1009.0 hPa	20 hPa	triangular	$\sqrt{6}$	8.165 hPa	0.0003 µL/hPa	0.0024 µL
Repeatability	0 mg	0.033 µL	normal	$\sqrt{10}$	0.011 µL	1	0.0105 µL
Process-related handling contribution	0 mg	0.010 µL	rectangular	$\sqrt{3}$	0.006 µL	1	0.0058 µL
***Y* (Volume)**	10.040 µL					*u(y)* =	0.016 µL
						***U(y)* =**	**0.032 µL**
						w(y) =	0.16 %
						***W(y)* =**	**0.32 %**

Published by:

Physikalisch-Technische Bundesanstalt
Deutscher Kalibrierdienst
Bundesallee 100
38116 Braunschweig

www.dkd.eu
www.ptb.de