

Calibrating a Micropipette

Martin de Groot

Kelvin Training

This paper discusses the calibration of pipette, with particular attention to the type that is used and calibrated most frequently. This is the so-called air-displacement pipette, single channel, adjustable volume pipet. The new ISO/IEC 17025, published November 2017, demands more attention to the agreement of the calibration procedure with the customer. The paper describes points that need to be agreed with the customer and calibration by the gravimetric method (weighing). The alternative calorimetric method (ISO 8655-7) is not described in this paper.

The Use of Micropipettes

A good explanation of the do's and don'ts when using the pipette is shown in a 10 minute YouTube film of the University of Leicester [1] (see Figure 1).

There is a significant difference in use of these pipettes by either the forward or reverse method. Figure 2 shows the difference between the two approaches.

It is generally recommended that tips of the original supplier should be used with the pipette. Alternative tips can only be used after sufficient experience to ensure the tip fits the particular pipette type. The tip shape, surface roughness, and wettability (adhesion) are of influence on the amount of water taken in by the pipette. Take appropriate care in attaching the tip on the pipette. There must be a close seal between pipette and tip that can easily be misfit by rough treatment. Tips are for single use only.

Make sure that you are not contaminating the pipette body with the liquids that you take in. Immerse the pipette tip 2 mm to 3 mm in the liquid to be taken in. Operate the pipette with care and (for variable volume pipettes) always set the volume from higher to lower volume to avoid that the measurement reproducibility is influenced by the hysteresis of the pipette mechanism.



Figure 1. This YouTube film is recommended as a good short introduction to air displacement pipettes and their use [1].

The calibration procedure must reproduce the method of use as much as possible. For the gravimetric approach, a volume of water is added to a vessel on a balance. This volume is weighed and the weight is converted to volume through the known density of the reference weight(s) used to calibrate the balance and the equations of the densities of water and air. This correction factor is called the Z-factor as defined in ISO 8655-6 [2].

Method and Points of Attention

The new ISO/IEC 17025:2017 is even more particular than the 2005 version about the prior arrangements to be fixed with the customer. Calibrations need to meet the customer requirements whenever possible. There are a few items to observe when calibrating pipettes.

Calibrate the pipette in the same way as it is used: if the customer uses forward (Figure 2) pipetting, the pipette must be calibrated that way too. It might be that your customer wants to have the calibration data expressed at a nominal temperature of 20 °C as described in [6].

Maintenance

Pipettes are sensitive to wear. Pipette maintenance is a regular part of the services provided by a calibration lab; for this, use original parts supplied by the manufacturer. If replacement of O-ring, seal, or other components is required, ISO/IEC 17025:2005 (clause 5.10.4.3) only requires pre-maintenance or pre-adjustment calibration results when the pipette is to be used for calibration. ISO/IEC 17025:2017 (7.8.4.1d) is more particular and requires pre-adjustment and repair data when available for all instruments (i.e., including those not to be used for calibration). When customers want to save money by eliminating the pre-adjustment calibration, the data need not be reported, but the new ISO/IEC 17025 does require the data to be "readily available." Note that ISO/IEC 17025 does not specify that the calibration procedures before and after adjustment must be the same. Both procedures need to be agreed upon between customer and calibration laboratory (2017 clauses: 7.1.3, 7.2.1.4).

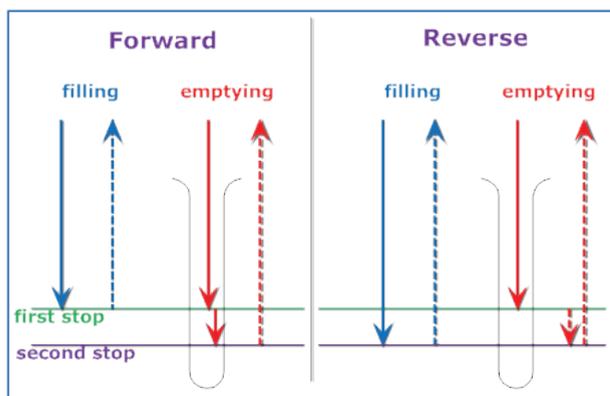


Figure 2. Forward versus reverse pipetting. To the left (forward pipetting): fill the pipette to the first stop and empty it by pushing all the liquid out. To the right (reverse pipetting): fill the pipette to the maximum extent and then empty the pipette only by pushing to the first stop.

Measurement Volumes and Number of Measurements

The measurement volumes must be agreed upon with the customer. ISO 8655-6:2002 describes a minimum of three volumes: the nominal volume, 50 % of the nominal volume, and the greatest of 10 % of the nominal volume and the lower limit of the useful volume range. For pipette calibrations, the repeatability of the calibration dominates the uncertainty budget for most of the pipette ranges. The number of repeat measurements at each volume is advised to be ten; the coverage factor must be calculated from the effective degrees of freedom that results from the uncertainty analysis as explained in UKAS M3003 [8] and other basic documents on uncertainty analysis. In order to minimize the effect from hysteresis in the volume adjusting mechanism of the micropipette, agree with the customer that the volume is always set in the same direction—preferably from higher volume down to the required volume.

Tip

Agree on the pipette tip (make and type) to be used during the calibration with the customer. Use the same pipette tip that the customer uses. Besides possible leakage when the pipette tip does not properly fit on the pipette, the pipette tip material and surface roughness have significant influence on the volume of water taken in by the pipette.

Decision Rule

The decision rule is a new item in ISO/IEC 17025:2017 and a major revision compared to the 2005 version of this standard. This rule refers to the way it is decided if a calibrated item conforms to a defined specification or not. The new ISO/IEC 17025 is much more specific in requiring

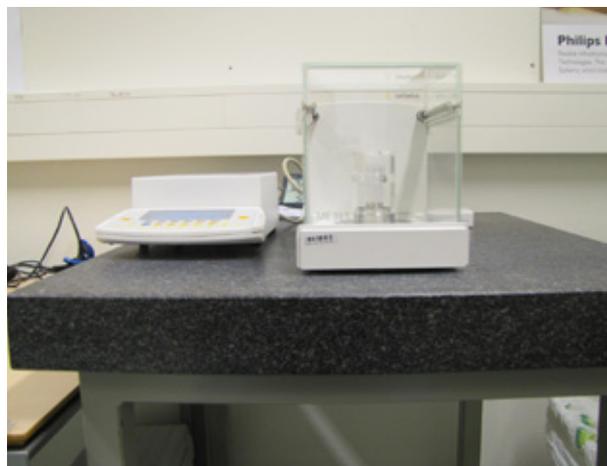


Figure 3. A balance set up for micropipette calibrations with an evaporation trap to minimize the effect of evaporation on the calibration. The balance is placed on a vibration free table. (Courtesy Gilson Netherlands)

this than the preceding 2005 version. While the old standard only requires that you consider the uncertainty when stating conformity to specification on a certificate, the new standard wants you to understand the level of risk of a false statement when the measurement is within the uncertainty's range from the specification limit. Current practice is explained by ILAC [7] such that you cannot make a statement on conformity if the measurement is within uncertainty's reach of the specification limit. The new ISO/IEC 17025 allows you to follow your customer's "decision rule" to reach a conformity to specification decision. Particularly for low volume micropipettes, this can solve the problem you might have experienced when a decisive conclusion on conformity is not possible because the uncertainty is about as large as or even larger than specified tolerances. This means that not only do you have to agree on the specification tolerances with your customer but also the decision rule. For more information on conformity, read clauses 7.1.3 and 7.8.6 of the ISO/IEC 17025:2017. Further information on decision rules can be found in UKAS M3003, annex M [8], and a document published by BIPM on this subject [9].

Equipment

For proper gravimetric calibration of pipettes, you need a balance placed on a stable weighing table, a thermometer to measure water temperature, a hygrometer for measurement of ambient temperature and relative humidity, a barometer, sufficient pipette tips, pure water and a container to contain this water, a weighing vessel with either a lid or as part of an evaporation trap system to reduce evaporation influence on the measurement, and a timer to measure the time for evaporation measurement.

Balance

For calibration of a pipette, you need an accurate (analytical) balance that must be levelled prior to the measurement and placed on a vibration free bench or table. The balance must have resolution of 10 μg for pipettes between 10 μl and 100 μl and 1 μg for pipettes with smaller volumes. Larger volumes can be calibrated with 0.1 mg balances. For best uncertainties, the calibration of the balance requires particular attention to the linearity of the balance in its lowest ranges. After all, when using the balance, you use the balance to measure volumes between 1 μl (approximately 1 mg) up to, for example, 100 μl (100 mg on your balance). The balance you use for this calibration will have a maximum reading of some 5 grams. Make sure you know the linearity of the balance over the lowest milligrams as well, as that is where you are actually using the balance! Table 4.1 of ISO 8655-6 [2] gives the balance requirements.

Balance Calibration

Calibration of the balance may be done by an external service agent or by yourself. The balance uncertainty contributes quite (if not most) significantly to the calibration of a pipette at lower volumes (typically below 100 μl).

If you calibrate the balance yourself, use a set of mass

pieces ranging from 1 mg to 100 g. Not all weights in this set need to be calibrated. You could use two calibrated reference weights for consistency, but also if only one of the reference weights is calibrated, the linearity can be derived from differential measurements.

For this, add the weights on the weighing pan in the same order as you remove them. This order may be: 1 mg, 2 mg, 10 mg, 20 mg, 100 mg, 1 g, etc. The added mass for each weight should correspond with the reduced mass when you remove the same weight. Note that the weight is not added at the same load as when it is removed. Comparing the mass changes for each weight shows the nonlinearity of the balance (see Figure 4). For this measurement, not all weights need to be calibrated, as stability is the important feature that this method uses. Figure 4 shows that at higher loads, the calibration of the weights become an important contributor to the bias (difference between reading and calibrated value of the reference weight). This effect drops out if you compare the measured mass differences rather than using the bias. The reference weight(s) fix(es) the traceability of your balance, while the linearity measurements relate the other scale readings to the bias of the reference weight. The bias is larger than the non-linearity due to the uncertainties of the class E2-weights that drop out in the subtractions explained in Figure 4.

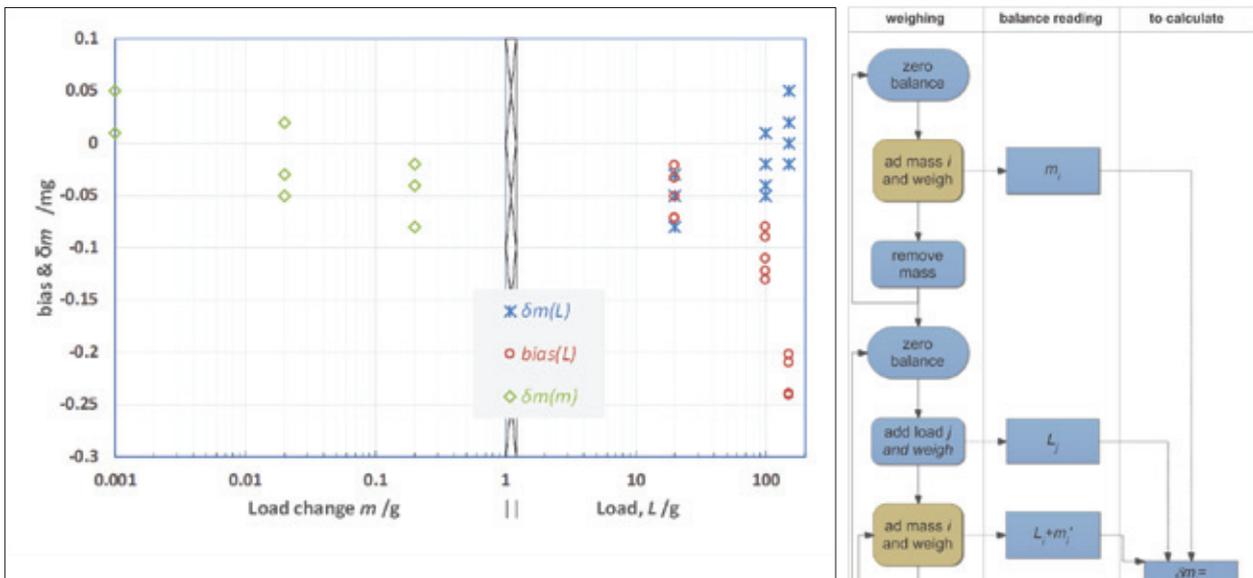


Figure 4. An analytical balance (total range 215 g, resolution 10 μg) was calibrated adding 1 mg, 20 mg and 200 mg weights (m) to a balance loaded with 20 g, 100 g and 150 g (L). The bias is equal to the balance reading minus the weight certificate values. For an ideal balance, the subtraction of the balance reading after and before adding a weight should be independent of the prior load. Differences δm between these subtractions for different loads are caused by balance nonlinearity. This nonlinearity is shown both in terms of the Load, L , and in terms of the applied weights, m .

Ambient Conditions

You shall need two thermometers: one to measure air temperature and one for the water temperature of the water in the container containing the source water. The water temperature measurement is the most demanding on accuracy of measurement and contributes more significantly than the other ambient condition parameters. The thermometer may not be so heavy that it makes the water container fall over. The thermometer should be calibrated to within 0.1 °C uncertainty. The air thermometer is to measure the difference between air and water temperature and must also be calibrated to within 0.1 °C.

The relative humidity must be measured even though it has no direct influence on the analysis. The humidity does influence the evaporation in the weighing vessel on the balance. The evaporation causes the weight on the balance to drift. This can be minimized by using a lid on the weighing vessel after adding each volume of water or by using an evaporation trap. The ambient humidity must be higher than 50 %rh and must be measured to within an uncertainty of 5 %rh. For measurement of the evaporation, and allowing a correction and/or the evaluation of its uncertainty, a timer is needed. This timing device should have an uncertainty of 1 s or better.

The ambient pressure must be measured with a barometer with a resolution of 1 hPa and an uncertainty of better than 5 hPa.

Calibration Procedure

As part of the preparation, make sure both pipettes and tips, water, and all associated measurement equipment are sufficiently climatized prior to the measurement.

Calibrate the balance once per day using a calibrated weight and adjust the balance using its internal adjustment system regularly to make sure the balance is corrected for ambient temperature drift. Make sure the balance setting does not automatically do this, ruining your measurement in the middle of a measurement cycle. Also verify that the drift setting of the balance is off.

Verify that the difference between water temperature and ambient temperature is not bigger than 0.2 °C. During the calibration, the temperature should not drift by more than 0.5 °C.

At the beginning of the measurement cycle at each volume, a tip shall [2] be used only to pre-wet the air inside the pipette. For this, fill the pipette with water from the reservoir five times and empty the water to waste. This will more or less "saturate" the air inside the pipet so that evaporation during the calibration is minimized. Evaporation would cause a significant increase in volume of the air cushion of the pipette, displacing the water from the pipette tip. Before the calibration, remove the tip and replace it by a new one that you now carefully fill only once emptying this also to waste. Tare the balance. It is only now that you are ready

to measure.

A measurement cycle consists of 10 measurements; if the customer prefers (to cut cost of calibration) less measurements per volume, the uncertainty will be larger because of the required correction of the coverage factor. Tare the balance after the measurement and before adding a new filling in the weighing vessel.

Take notice of the way that water is to be taken in the pipette and to be delivered in the weighing vessel as described in [2 section 7.2]. In Germany, DAkkS requires a slightly deviating approach for this [10 sections 7.2 and 7.3]. While ISO 8655-6 [2] requires a new tip for every measurement, Guideline DKD-R 8-1 "Calibration of piston-operated pipettes with air cushion" [10] allows, under conditions, that the same pipette tip may be used for the full measurement cycle. It is advised to at least replace the tip for each different volume. Pre-wet a newly fitted tip once after replacement.

For evaporation measurements, the time is to be measured for a measurement cycle. Then over this time, measure the drift on the balance due to evaporation. Divide this by ten to get the evaporation for a single measurement. Repeat this measurement a number of times to allow for an uncertainty estimate of this effect.

Z-factor and Pipette Calibration Data

The analysis of a pipette calibration is not a straightforward calculation. The widely-used gravimetric calibration method (described in ISO8655-6 [2]) relies on an amount of pure water to be transferred into the vessel placed on the balance. The weight of this water relates to the volume of the transferred water by a formula that incorporates the density of the water, density of air and the buoyancy effect affecting the weight of the water on the balance. This formula is, in practice, replaced by the so-called Z-factor, that depends on water temperature, air temperature, and pressure and can be found in table A1 of ISO 8655-6. ISO 8655 takes the air temperature to be equal to the water temperature. For more detail, see Figure 5 and accompanying text.

Uncertainty Analysis

Z-factor Uncertainty

For proper uncertainty analysis the sensitivity of the Z-factor to the influence parameters (temperatures, pressure, but also humidity) is to be calculated. This can be done analytically as in ISO/TR 20461 [6] but also by numerical variation of the influence parameters. The uncertainty basics are laid out in ISO/TR 20461.

As stated before, the uncertainty from relative humidity is negligible. The Z-factor dependence can be simply found by looking at the Z-dependence on pressure or temperature in table A.1 in ISO 8655 [2]. These so-called sensitivity factors are used to multiply the thermometer and barometer uncertainties in the budget for the calculation

of the uncertainty contributions from these ambient measurements on the calibrated volume. For more accurate calculation of the uncertainty the ISO/TR 20461 approach is better calculating separate sensitivity coefficients for ambient temperature and water temperature.

ISO/TR 20461 explains the temperature dependence of the pipette and includes an uncertainty component in the budget for the heating of the pipette by the operator.

Balance Uncertainty

The calibration of the balance results in two components for the uncertainty. One is the linearity component the other is the uncertainty following from the calibration using the reference weight.

Smaller contributions are given as the reproducibility of the balance and the readability of the balance. It can be argued, however, that these components are already part of the balance calibration. As these are smaller than the balance calibration contribution, these components have no significant influence in the combined uncertainty.

Temperature drift sensitivity of the balance results in another generally small contribution per degree ambient temperature instability. This can be calculated using the maximum allowed temperature deviation being 0.5 °C.

Water Purity

The water purity can be verified by boiling out a weighed volume of water and weighing the residual. As very pure (distilled) water should be used, the uncertainty from this effect should be negligible.

Repeatability

Every measurement is repeated a number, n , of times with n normally being 10 [2]. The repeatability is calculated from the standard deviation s . This standard deviation can be compared with the required value in the specification. The component for repeatability of the measurement in the uncertainty budget is the standard deviation of the mean $\bar{s} = s/\sqrt{n}$. Generally this component is the dominant contributor to the uncertainty. The operator has a very large influence on the calibration result.

Further Uncertainty Components:

Pressure vs. Altitude

Thorough German research has shown that this uncertainty calculation is not complete [11,12]. There are some other dominant factors contributing to the uncertainty that are not sufficiently identified in ISO/TR20461. These relate to pressure effects that become significant when working with the pipette on a different altitude than where the pipette was calibrated. DKD document [10] describes an elegant formula (3) allowing correction of a calibration result to a different pressure. An uncertainty component of 20 hPa is advised on the Z-factor to account for this effect.

Buoyancy Effect and the Z-factor

When using an ordinary (analytical) balance for pipette calibration, the balance was calibrated by a stainless steel reference weight with density $\rho_r = 8000 \text{ kg m}^{-3}$. This density is a fixed value within the required accuracy. During pipette calibration the balance weighs water with a density of approximately 1000 kg m^{-3} and compares this with the weight of the stainless steel reference weight previously used for the calibration. The density of water depends on the temperature of the water [3].

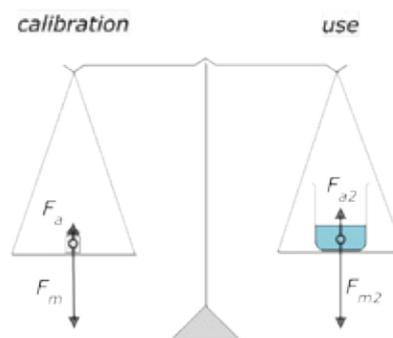


Figure 5. The upwards buoyancy effect is caused by the weight of displaced air by the volume of the stainless steel weight (on the left) vs. the larger amount of displaced air by the volume of water on the right. The weight on both loads is partly compensated by the weight of the displaced air.

The upwards buoyancy effect is caused by the weight of displaced air by the volume of the stainless steel weight (on the left) versus the larger amount of displaced air by the volume of water on the right. The weight on both loads is partly compensated by the weight of the displaced air. The water having larger volume for the same weight experiences larger upward force because of the larger volume of displaced air. The air density is approximately 1.0 kg m^{-3} and depends on air temperature, pressure, and to a lesser extent, on relative humidity; the equation for this can be found in [4]. Part of the equation in reference [4] is the molar gas constant. This value has very recently undergone a very small change as a consequence of the redefinition of the SI units. As part of the revision of the SI, new values have been defined for the Boltzmann constant and the Avogadro constant. The product of these is equal to the gas constant. The effect of this change to the Z-factor is however very small and insignificant for the calculation of the Z-factor [5].

Knowing the density of water $\rho_w(t_{90,w})$ as a function of water temperature, the density of air $\rho_a(t_{90,a}, p_r, x_v)$ as a function of temperature, humidity and pressure, and the correction factor from buoyancy can be calculated from [6]:

$$Z(t_{90,w}, t_{90,a}, p_r, x_v) = \frac{1}{\rho_r} \times \frac{\rho_r - \rho_a}{\rho_w - \rho_a}$$

As the dependence of this Z-factor from humidity is insignificant in comparison with the other parameter dependencies, the humidity dependence is neglected.

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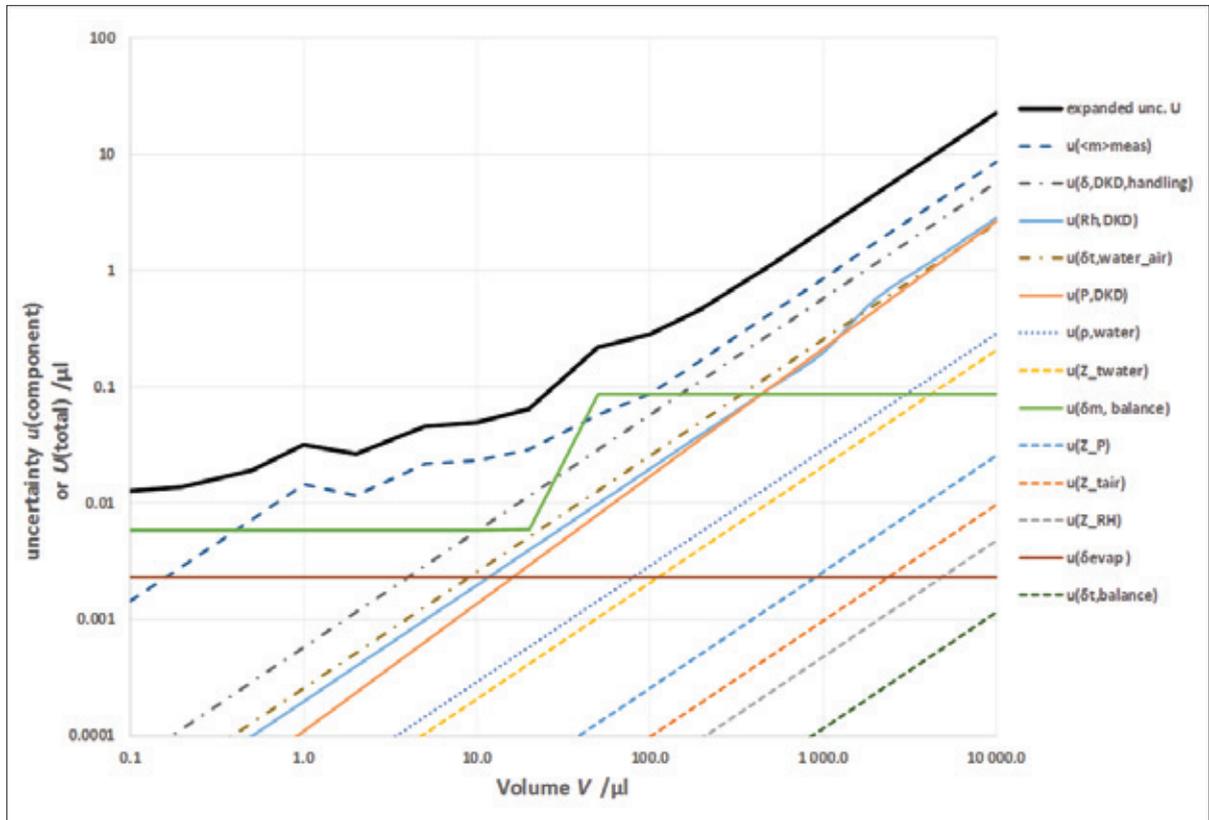


Figure 6. CMC example uncertainty based on a combination of relevant terms from ISO/TR 20461 and DKD R8-1 (see Table 1 on the following page).

nominal volume:		20 μl						
quantity / unit	value	uncertainty contribution	distribution	standard uncertainty	degrees of freedom	sensitivity factor	standard uncertainty	
$X [X]$	x_i	$u'(X)$	$F\{X_i\}$	$u(X_i)$	ν_i	$[Y/X]$	$u(Y_i)$	
1	$\delta_{m, balance}$ [mg]	0	0.0103 Uniform_sqrt3	0.006	1000	1.003023	0.00596416	
2	$\delta t_{balance}$ [$^{\circ}\text{C}$]	0	0.2000 Uniform_sqrt3	0.115	1000	2.00E-05	0.00000231	
3	δ_{evap} [mg]	0.010	0.0040 Uniform_sqrt3	0.002	150	1.003023	0.00231638	
ambient conditions working on Z-factor								
4	t_{water} [$^{\circ}\text{C}$]	20.9	0.096 Normal_k=1	0.096	2574	4.33E-03	0.00041481	
5	t_{air} [$^{\circ}\text{C}$]	21.1	0.252 Normal_k=1	0.252	2864	-7.73E-05	0.00001948	
6	P [hPa]	999	2.453 Normal_k=1	2.453	1260	2.08E-05	0.00005106	
7	RH [%rh]	58%	4.88% Normal_k=1	4.88%	2221	-1.96E-04	0.00000954	
8	$\delta t_{water,air}$ [$^{\circ}\text{C}$]	0	0.20 Uniform_sqrt3	0.12	1000	4.40E-02	0.00508072	
9	ρ_{water} [$\text{kg}\cdot\text{m}^{-3}$]	998.02	0.050 Uniform_sqrt3	0.029	2081	2.00E-02	0.00057735	
10	RH_{pipet} [%rh]	58.0%	4.88% Normal_k=1	4.88%	1000	8.08E-02	0.00394296	
11	P_{pipet} [hPa]	999.0	20 Triangle	8.16	1000	3.57E-04	0.00291339	
12	m_{meas} [mg]	19.940	0.029 Normal_k=1	0.029	10	1.003023	0.02886772	
13	$\delta_{handling}$ [%]	0	0.10% Uniform_sqrt3	0.06%	1000	20.000	0.01154709	
resulting volume					20.010 μl			
					effective degrees of freedom		16.1	
					combined uncertainty $u(Y)$		0.033 μl	
					coverage factor k		2.169	
					expanded uncertainty $U(Y)$		0.071 μl	

The measurement result would be reported as 20.010 $\mu\text{l} \pm 0.071 \mu\text{l}$ for approximately 95% corresponding coverage probability

Figure 7. Uncertainty budget for a volume corresponding with the CMC budget in Figure 6.

U	Expanded uncertainty at 95 % percent coverage probability.
$u(\langle m \rangle_{\text{meas}})$	Repeatability of measurement specification ISO 8655-2.
$u(\delta_{\text{DKD,handling}})$	DKD handling effect 0.07 % of volume.
$u(RH_{\text{DKD}})$	DKD relative humidity effect as described in text for 5 %rh uncertainty.
$u(\delta t_{\text{water,air}})$	Difference between air and water temperature at 0.2 °C.
$u(P_{\text{DKD}})$	DKD pressure effect at 0.01 % of volume per 1 %rh.
$u(\rho_{\text{water}})$	Density of water uncertainty estimated as 0.005%.
$u(Z_{\text{t,water}})$	Z-factor water temperature at uncertainty of 0.2 °C.
$u(\delta m_{\text{balance}})$	Balance calibration 0.01 mg for 5 g balance and 0.15 mg for other balance.
$u(Z_p)$	Z-factor pressure contribution at uncertainty of 10 hPa.
$u(Z_{\text{t,air}})$	Z-factor air temperature at uncertainty of 0.5 °C.
$u(Z_{RH})$	Z-factor relative humidity contribution at uncertainty of 10 %rh.
$u(\delta_{\text{evap}})$	Evaporation: 4 nl uncertainty in measured effect.
$u(\delta_{\text{t,balance}})$	Temperature sensitivity of balance for standard balance specification.

Table 1. UNCERTAINTY BUDGET components used to produce Figures 6 and 7.

The DKD document [11] also explains that this pressure effect depends on humidity. It is argued that while transferring the water from the container to the weighing vessel, water evaporates from the aspirated water into the air cushion. This increases the volume of and humidity in the air cushion and drives some of the liquid out of the pipette. An uncertainty contribution has been taken from studies to amount to 0.007 % of the transferred volume per change of humidity by 1 %rh for 100 μl and 1000 μl nominal pipette volumes. At 1 μl nominal pipette volume, this effect is 0.01 % of volume per change of humidity by 1 %rh. Prewetting to increase humidity inside the pipette becomes even more important.

Handling Component

DKD document [10] recognizes handling influences on the calibration through:

1. mechanical effects (hysteresis reproducibility of the piston stroke);
2. influences by the operator (waiting times, pace of work, pipette angle, operating force, immersion depth); and
3. hand warmth, as the thermometer is heated in the hand of the operator.

To account for these effects, German accredited laboratories are required to include a contribution of 0.1% of the calibrated volume in the budget for handling.

For readers wanting to do some calculations themselves, a GUM workbench example budget is available from our website (Kelvin.training).

References

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Martin de Groot (martindegroot@kelvin.training), Kelvin Training, Kampdijklaan 4, 5263 CJ Vught, Netherlands, <https://kelvin.training/>.