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Automated liquid handling systems —

Part 2: Measurement procedures for the determination of volumetric performance

Systèmes automatisés de manipulation de liquides —

Partie 2: Procédures de mesure pour la détermination des performances volumétriques

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 48, *Laboratory equipment*.

This first edition of ISO 23783-2, together with ISO 23783-1 and ISO 23783-3, cancels and replaces IWA 15:2015.

A list of all parts in the ISO 23783 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Globalization of laboratory operations requires standardized practices for operating automated liquid handling systems (ALHS), communicating test protocols, as well as analysing and reporting of performance parameters. IWA 15:2015 was developed to provide standardized terminology, test protocols, and analytical methods for reporting test results. The concepts developed for, and described in, IWA 15 form the foundation of the ISO 23783 series.

Specifically, this document addresses the needs of:

— users of ALHS, as a basis for calibration, verification, validation, optimization, and routine testing of trueness and precision;

— manufacturers of ALHS, as a basis for quality control, communication of acceptance test specifications and conditions, and issuance of manufacturer's declarations (where appropriate);

— test houses and other bodies, as a basis for certification, calibration, and testing.

The tests established in this document should be carried out by trained personnel.

Automated liquid handling systems —

Part 2: Measurement procedures for the determination of volumetric performance

1 Scope

This document specifies procedures for the determination of volumetric performance of automated liquid handling systems (ALHS), including traceability and estimations of measurement uncertainty of measurement results.

This document is applicable to all ALHS with complete, installed liquid handling devices, including tips and other essential parts needed for delivering a specified volume, which perform liquid handling tasks without human intervention into labware.

NOTE For terminology and general requirements of automated liquid handling systems, see ISO 23783-1. Determination, specification, and reporting of volumetric performance of automated liquid handling systems is described in ISO 23783-3.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 8655-6, *Piston-operated volumetric apparatus – Part 6: Gravimetric reference measurement procedure for the determination of volume*

ISO 23783-1, *Automated liquid handling systems — Part 1: Terminology and general requirements*

ISO 23783-3, *Automated liquid handling systems — Part 3: Determination, specification, and reporting of volumetric performance*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 23783-1 apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

4 Abbreviated terms

For the purposes of this document, the abbreviated terms given in ISO 23783-1 apply.

5 Measurement methods

5.1 Overview of methods suitable for measuring ALHS performance

When choosing a test method for an ALHS, its suitability for the specific test situation shall be evaluated. This evaluation shall consider the systematic and random error requirements of the ALHS to which the test method is being applied. The selected test method shall be adequate to evaluate whether the ALHS performance is fit for its intended purpose.

NOTE 1 Fitness for purpose is a foundational concept and closely related to the process of metrological confirmation as described in ISO 9000 and ISO 9001.

The test method shall have a sufficiently small measuring system uncertainty (MSU) for the specific test situation. The MSU should be determined in accordance with a suitable approach (see 8.2 for more detail).

NOTE 2 The measurement model approach for estimating MSU is described in ISO/IEC Guide 98-3 and the measurement system approach is described in EURACHEM/CITAG Guide CG 4 [4].

Table 1 is intended to provide an overview of methods suitable for determining the volumetric performance of ALHS. It provides cross-references between the method abstracts from 5.2 to 5.5, and the corresponding procedures in Annexes B to I. It further describes the volume ranges, plate and liquid types which can be used for testing ALHS performance with a given method. It also lists typical systematic and random errors achievable if a test procedure is exactly followed as described in its respective annex. The suitability of a method for a given test situation may also be determined by the required equipment or environmental conditions under which it needs to be carried out.

Only key test equipment is listed in Table 1, while test equipment to monitor liquid and air temperatures, relative humidity, and barometric pressure is required for each procedure, as specified in the corresponding annexes.

Table 1 — Test methods for ALHS

Method Ref.	Method	Liquid type	Plate type	Volume range	Typical systematic error [a]	Typical random error [a]	Environmental conditions [b]	Test equipment
			wells	µl	%	%		
Photometric methods								
5.2.1 Annex B	Dual-dye ratiometric photometric method	Aqueous, DMSO [c]	96	0,1 to 350,0	2,0 to 3,0	0,15 to 0,25	Temperature: Aqueous: 15 °C to 30 °C DMSO [c]: 19 °C to 30 °C RH [d]: 20 % to 90 %	— Microplate absorbance reader capable of measuring absorbance at 520 nm and 730 nm; — dimensionally characterized 96- or 384-well microplates with optically clear bottom; — calibration plate for plate reader; — microplate shaker; — balance; — spectrophotometer capable of measuring absorbance at 520 nm and 730 nm; — pH meter; — volumetric flasks.
			384	0,01 to 55,0	2,5 to 5,5	0,35 to 0,55		

[a] Typically, larger test volumes lead to smaller errors.

[b] The minimum temperature of the test environment shall be above the melting point of the test liquid, ensuring that it will not solidify at any point during the test. The relative humidity of the test environment shall be non-condensing.

[c] Dimethylsulfoxide.

[d] Relative humidity.

Table 1 *(continued)*

Method Ref.	Method	Liquid type	Plate type wells	Volume range µl	Typical systematic error [a] %	Typical random error [a] %	Environmental conditions [b]	Test equipment
5.2.2 Annex C	Single-dye photometric method	Aqueous	96	1,0 to 100,0	3	1,5	Temperature: 15 °C to 30 °C RH [d]: 40 % to 70 %	— Microplate absorbance reader capable of measuring absorbance at 492 nm and 620 nm; — 96- or 384-well microplates with optically clear bottom; — balance; — magnetic stirrer; — microplate shaker; — pH meter; — manual pipettes; — volumetric flasks.
			384	0,25 to 20,0	3	1,5		

[a] Typically, larger test volumes lead to smaller errors.

[b] The minimum temperature of the test environment shall be above the melting point of the test liquid, ensuring that it will not solidify at any point during the test. The relative humidity of the test environment shall be non-condensing.

[c] Dimethylsulfoxide.

[d] Relative humidity.

Table 1 *(continued)*

Method Ref.	Method	Liquid type	Plate type wells	Volume range µl	Typical systematic error [a] %	Typical random error [a] %	Environmental conditions [b]	Test equipment
5.2.3 Annex H	Fluorescence method	Aqueous, DMSO [c]	384	0,001 to 0,015	<8	<8	Temperature: 17 °C to 27 °C RH [d]: non-condensing	— Microplate fluorescence reader with excitation wavelength at 494 nm and emission analysis at 521 nm; — 384- or 1536-well fluorescence microplates; — balance; — bulk liquid dispenser or multi-channel pipette; — microplate shaker; — pH meter; — manual pipettes; — volumetric flasks.
			1 536	0,001 to 0,015	<8	<8		

[a] Typically, larger test volumes lead to smaller errors.

[b] The minimum temperature of the test environment shall be above the melting point of the test liquid, ensuring that it will not solidify at any point during the test. The relative humidity of the test environment shall be non-condensing.

[c] Dimethylsulfoxide.

[d] Relative humidity.

Table 1 *(continued)*

Method Ref.	Method	Liquid type	Plate type wells	Volume range µl	Typical systematic error [a] %	Typical random error [a] %	Environmental conditions [b]	Test equipment
Gravimetric methods								
5.3.1 Annex D	Single channel analysis	Any	n/a	0,5 to <20	≤1,4	≤0,6	Temperature: 17 °C to 30 °C RH [d]: 45 % to 80 %	— Balance; — density meter; — anti-electrostatic equipment; — anti-vibration table; — temperature- and humidity control for environment; — draft shield or draft-free environment for balance.
				20 to <200	≤0,9	≤0,3		
				200 to 1 000	≤0,9	≤0,3		
5.3.2 Annex E	Regression analysis	Any	n/a	<0,015	20 to 50	<10	Temperature: 17 °C to 27 °C RH [d]: 45 % to 80 % Barometric pressure: 600 hPa to 1 100 hPa	— Balance; — density meter with 6 decimal places; — anti-electrostatic equipment; — anti-vibration table; — temperature- and humidity control for environment; — draft shield or draft-free environment for balance.
				0,015 to <0,050	2 to 5	2,5 to 5		
				0,050 to 1	0,5 to 2	≤0,5		

[a] Typically, larger test volumes lead to smaller errors.

[b] The minimum temperature of the test environment shall be above the melting point of the test liquid, ensuring that it will not solidify at any point during the test. The relative humidity of the test environment shall be non-condensing.

[c] Dimethylsulfoxide.

[d] Relative humidity.

Table 1 *(continued)*

Method Ref.	Method	Liquid type	Plate type wells	Volume range µl	Typical systematic error [a] %	Typical random error [a] %	Environmental conditions [b]	Test equipment
Photometric/gravimetric hybrid method								
5.4 Annex F	Tartrazine as chromophore	Aqueous	96	1,0 to 300,0	0,2 to 0,8	0,5 to 1,0	Temperature:	— Balance; — microplate absorbance reader capable to measure absorbance at the following wavelengths, depending on the chromophore used:
			384	1,0 to 20,0	0,4 to 1,0	0,9 to 1,5	17 °C to 30 °C	— 4-nitrophenol: 405 nm and 620 nm, — Tartrazine: 450 nm and 620 nm, — Orange G: 492 nm and 620 nm
	4-nitrophenol as chromophore	Aqueous	96	10 to 1 000 5 to 250	<1 to 5	1 to 2	Temperature stability: < ±0,5 °C	
			96, 384	1 to 60 0,5 to 25	<1 to 5	1 to 2	RH [d]: 45 % to 80 % RH [d] stability: < ±10 %	
			384, 1 536	0,1 to 8	<2 to 10	2 to 5		— microplate shaker; — 96- or 384-well microplates with optically clear bottom; — manual pipettes; — centrifuge tubes 1,5 ml; — anti-vibration table; — temperature- and humidity control for environment; — draft shield or draft-free environment for balance.
	Orange G as chromophore	Aqueous	96	1 to 100	<1 to 5	1,5		
			384	1 to 50	<1 to 5	1,5		

[a] Typically, larger test volumes lead to smaller errors.

[b] The minimum temperature of the test environment shall be above the melting point of the test liquid, ensuring that it will not solidify at any point during the test. The relative humidity of the test environment shall be non-condensing.

[c] Dimethylsulfoxide.

[d] Relative humidity.

Table 1 *(continued)*

Method Ref.	Method	Liquid type	Plate type wells	Volume range µl	Typical systematic error [a] %	Typical random error [a] %	Environmental conditions [b]	Test equipment
Dimensional methods								
5.5.1 Annex G	Optical image analysis of droplets	Any	n/a	Free flying droplets of V <0,5 µl	<5	<2	Temperature: (20 ± 3) °C or (27 ± 3) °C RH [d]: 50 % to 80 %	— Stroboscopic camera or high-speed camera; — automatic image detection software.
5.5.2 Annex I	Optical image analysis of capillaries	Any	n/a	0,1 to 1,0	<10	<7	Temperature: 15 °C to 35 °C RH [d]: 15 % to 90 %	— Flatbed scanner; — image analysis software; — specialized plates with capillaries.
				>1,0 to 1 000	<5	<4		

[a] Typically, larger test volumes lead to smaller errors.

[b] The minimum temperature of the test environment shall be above the melting point of the test liquid, ensuring that it will not solidify at any point during the test. The relative humidity of the test environment shall be non-condensing.

[c] Dimethylsulfoxide.

[d] Relative humidity.

5.2 Photometric methods

5.2.1 Dual-dye ratiometric photometric method

This method allows the determination of volumes of aqueous test liquids from 0,1 µl to 350 µl in 96-well plates, and from 0,01 µl to 55 µl in 384-well plates. Volumes of dimethylsulfoxide (DMSO)-based test liquids can be determined from 0,11 µl to 10 µl in 96-well plates, and from 0,01 µl to 2,5 µl in 384-well plates.

This method is suitable to determine the performance of ALHS with up to 384 channels. The operating environment for this method is 15 °C to 30 °C (19 °C to 30 °C for DMSO liquids), and it is not dependent on the ambient relative humidity and barometric pressure at the test location. Further information on the effect of relative humidity and barometric pressure on this method can be found in Reference [5].

Traceability of the measurement results to the International System of Units (SI) is achieved through the use of a calibrated microplate absorbance reader, dimensionally characterized microplates, calibrated balance, and calibrated volumetric glassware.

The procedure for the dual-dye ratiometric photometric method specified in Annex B shall be followed.

5.2.2 Single-dye photometric method

This method is suitable for evaluating the volumetric performance of ALHS with up to 384 channels using aqueous test liquids. Volumes from 1 µl to 100 µl can be measured in 96-well plates, and from 0,25 µl to 20 µl in 384-well plates.

Traceability of the measurement results to the SI is achieved through the use of a calibrated balance, calibrated pipettes, a calibrated microplate absorbance reader, and calibrated volumetric glassware.

The procedure for the single-dye photometric method specified in Annex C shall be followed.

5.2.3 Fluorescence method

This method is suitable to evaluate the volumetric performance of ALHS delivering volumes smaller than 15 nl. The fluorescence of the test liquid of fluorescein in DMSO is measured in 384-well or 1536-well microplates, which are specifically suited for fluorescence measurements.

This method is intended to be used for non-contact liquid delivery devices (e.g. acoustic, dispensing valves, or inkjet-type technology) that deliver the liquid volume as free flying droplets or jets into the wells of the microplate.

Traceability of the measurement results to the SI is achieved through the use of a calibrated fluorescence microplate reader, calibrated balance, calibrated pipettes, and calibrated volumetric glassware.

The procedure for the fluorescence method specified in Annex H shall be followed.

5.3 Gravimetric methods

5.3.1 Single channel method

This method describes the apparatus, procedure and reference material for recording measurements with the gravimetric method. A single pan balance is used to take a measurement from a single channel at a time. The following accommodations shall be made:

— placement of the balance and the weighing vessel which reduce draft and vibrations to a suitable level;

— control of the environmental conditions affecting the mass to volume conversion of the measurement (temperature and relative humidity);

— monitoring of the barometric pressure, which affects the mass to volume conversion.

Traceability of the measurement results to the SI is achieved through the use of a calibrated balance and accounting for test liquid density and air buoyancy.

The procedure for the single-channel gravimetric method specified in Annex D shall be followed.

5.3.2 Regression analysis

The gravimetric regression method (GRM) is suitable for the measurement of very small liquid volumes, between 0,005 µl and 1 µl. The method is based on a gravimetric balance as the primary measurement device.

This method is intended to be used for non-contact liquid delivery devices (e.g. dispensing valves, acoustic, or inkjet-type dispensing) that deliver the liquid volume as free flying droplets or jets to the balance receptacle.

The key difference to traditional gravimetric methods used for the measurement of larger volumes is the determination of the target volume: a series of balance readings is recorded over a period of time before and after the device under test has delivered the liquid to be measured into the receptacle on the balance. The measurement result of the delivered test liquid is then determined as the difference between two linear regression lines fitted to the recorded balance data before and after the liquid delivery. This method allows measurement of balance drift due to evaporation and other disturbances of the measurement (e.g. by vibrations during the data acquisition), so that these can be compensated for in the measurement calculation (see Reference [6] for more details).

Accommodations regarding the placement of the balance and environmental control and monitoring given in 5.3.1 shall be made.

Traceability of the measurement results to the SI is achieved through the use of a calibrated balance.

The procedure for the gravimetric regression method specified in Annex E shall be followed.

5.4 Hybrid photometric/gravimetric method

The photometric / gravimetric hybrid method allows the evaluation of volumetric performance of ALHS by a combination of a gravimetric measurement with subsequent photometric measurements. Test liquid containing a chromophore is delivered into 96-well or 384-well microplates. The systematic error is determined by gravimetry of the aggregate deliveries into the microplate. Subsequently, the random error of volume deliveries is determined photometrically by measuring the relative absorbances of each well of the microplate.

Chromophores suitable for this method are Tartrazine, Orange G, and 4-nitrophenol. The procedure described in Annex F is suitable for test volumes between 1 µl and 200 µl in 96-well plates, and 1 µl and 50 µl in 384-well plates.

Accommodations regarding the placement of the balance and environmental control and monitoring given in 5.3.1 shall be made.

Traceability of the measurement results to the SI is achieved through the use of a calibrated balance, calibrated pipettes, and calibrated volumetric glassware.

The procedure for the hybrid method specified in Annex F shall be followed.

5.5 Dimensional methods

5.5.1 Optical image analysis of droplets

This method measures the volume of delivered liquids by analysing images acquired by a high-speed camera and stroboscopic illumination during the liquid delivery cycle. It is suitable for ALHS,

which deliver liquid volumes as a sequence of discreet micro droplets (for further information on the determination of droplet volumes, see Reference [7]).

Traceability of the measurement results to the SI is achieved through calibration of the length scale of the acquired images.

The procedure for the optical image analysis of droplets specified in Annex G shall be followed.

5.5.2 Optical image analysis of capillaries

The method is based on the optical analysis of images acquired of capillaries of known and calibrated geometry. For the image acquisition, a flatbed scanner is used. The method provides a direct determination of the volume by the optical measurement of one or multiple lengths of the calibrated capillaries.

The method can be used to measure volumes between 0,1 µl and 1 000 µl for ALHS with 1 to 384 channels. The measurement uncertainties only minimally depend on the environmental conditions and are reliable in a broad range of environmental conditions without error corrections.

Traceability of the measurement results to the SI is achieved by using calibrated capillaries and a calibrated image acquisition device.

The procedure for the optical image analysis of capillaries specified in Annex I shall be followed.

6 Equipment and preparation

6.1 Test equipment

Test equipment used for volumetric performance measurements according to the procedures described in this document shall conform to the minimum performance requirements given in Tables 2, 3, 4, and 5, unless different minimum performance requirements are described within a specific test procedure. Balances shall be allowed to settle for at least 6 s before reading the indicated value.

NOTE 1 The balance repeatability and expanded uncertainty in use given in Table 2 are harmonized with Table 3 but the weighing ranges are restricted to improve accuracy when weighing an amount of dry chemicals.

NOTE 2 The balance requirements given in Table 3 are based on ISO 8655-6 and allow multiple replicate deliveries of test liquid into the same weighing vessel without emptying it out.

Table 2 — Minimum requirements for balances for weighing dry materials

Smallest amount to be weighed g	Readability mg	Repeatability mg	Expanded uncertainty in use [a] (k = 2) mg
<1,0	0,001	0,006	0,012
1,0	0,01	0,025	0,05
10	0,1	0,2	0,4
100	1	2	4
1 000	10	20	40

[a] Uncertainty in use can be determined according to ASTM E898-20[8] and EURAMET CG-18[9] at the minimum mass listed in the table.

Table 3 — Minimum requirements for balances for weighing liquids

Delivered volume of test liquid [a] µl	Readability mg	Repeatability mg	Expanded uncertainty in use [b] (k = 2) mg
<0,5	0,000 1	0,000 5	0,001
0,5 ≤ V < 20	0,001	0,006	0,012
20 ≤ V < 200	0,01	0,025	0,05
200 ≤ V ≤ 10 000	0,1	0,2	0,4

[a] Assumes one delivery of test liquid from a single channel.

[b] Uncertainty in use can be determined according to ASTM E898-20[8] and EURAMET CG-18[9] at the value of the largest volume of the listed range, assuming a single channel delivery.

Table 4 — Minimum performance requirements for absorbance microplate readers

Parameter	Requirement
Photometric resolution	0,001 AU [b]
Photometric trueness [a] from 0 AU to 1,0 AU	0,005 AU
Photometric trueness [a] from > 1,0 AU to 2,0 AU	0,010 AU
Photometric repeatability from 0 AU to 2,0 AU	0,005 AU
System linearity between 0 AU and 2,0 AU	0,010 AU
Wavelength accuracy	< ± 1,5 nm

[a] Photometric trueness is sometimes called photometric accuracy.

[b] Absorbance unit.

Table 5 — Minimum performance requirements for other test equipment

Instrument	Resolution	Expanded uncertainty (k = 2)
Thermometer for liquids	0,1 °C	0,2 °C
Thermometer for ambient air	0,1 °C	0,2 °C
Hygrometer	1 % RH [a]	5 % RH [a]
Barometer	0,1 kPa	1 kPa
Timing device	1 s	not relevant

[a] Relative humidity.

6.2 Manually operated single- and multi-channel pipettes

Single-channel and multi-channel pipettes shall be calibrated and shall fulfil the minimum performance requirements given in ISO 8655-2. Operator impact on manually pipetted volumes shall be considered in the calculation of errors and measurement uncertainty.

6.3 Preparation for testing

The ALHS under test, including all exchangeable parts to be used during the test, and all test equipment and test liquids shall be in thermal equilibrium (±2 °C) for at least 2 h prior to the start of testing. During the time of testing, the environmental conditions shall not change more than ±1 °C and 5 % relative humidity (RH). Requirements for environmental conditions are described in each test procedure. Laboratory instrumentation is designed to be operated under non-condensing humidity conditions.

All test equipment shall be calibrated according to the test procedure in this document, or according to the manufacturer's instructions if the calibration is not specifically explained in the procedure.

The ALHS under test and test equipment shall be powered on and allowed sufficient time to equilibrate for proper functionality according to the manufacturer's instructions.

Liquid reservoirs of ALHS shall be filled immediately before testing begins. ALHS which require priming shall be primed according to the manufacturer's instructions immediately before testing begins.

The tips of piston-operated ALHS should be pre-rinsed with the test liquid at least five times, whereby the dispensed test liquid is discarded to waste. This step is required every time a tip is changed.

7 Thermal expansion

If the test temperature is different from the temperature of adjustment of the ALHS, and if the cubic thermal expansion coefficient γ of the volumetric apparatus is known, Formula (1) may be used to correct the measured volume of test liquid for thermal expansion:

$$V_{L,tc} = V_L \times [1 - \gamma \times (t_T - t_{ref})] \quad (1)$$

where

$V_{L,tc}$ is the delivered volume of test liquid, corrected for thermal expansion of the ALHS;

V_L is the delivered volume of test liquid measured at the test temperature;

γ is the cubic thermal expansion coefficient of the volumetric apparatus;

t_T is the temperature at which the test is performed;

t_{ref} is the reference temperature of adjustment of the ALHS.

8 Traceability and measuring system uncertainty

8.1 Traceability

All measurements by relevant test equipment used for the calibration of an ALHS shall be traceable to the International System of Units (SI). The uncertainty of relevant test equipment affects the error of the reported volumetric results.

NOTE Relevant test equipment can include but is not limited to: balances, thermometers, pH meters, hygrometers, plate readers, spectrophotometers, calibrated pipettes, volumetric glassware, size calibration charts, and timing devices.

8.2 Estimation of measuring system uncertainty

8.2.1 Whole system approach

Measuring system uncertainty (MSU) may be estimated by statistical evaluation of results produced by the entire measuring system. Precision and bias studies, measurement system analysis, and inter-laboratory comparisons are some of the means by which this is achieved. Detailed approaches are described in Reference [4].

8.2.2 Measurement model approach

This approach estimates MSU based on an analysis of each input to a measurement model. This approach is detailed in Reference [3].

9 Reporting

Measurement results, traceability, and measurement uncertainty shall be reported in accordance with ISO 23783-3.

Annex A
(normative)

Calculation of liquid volumes from balance readings

A.1 Calculation of liquid volume from the balance reading

A.1.1 General formula for volume

For the conversion of the balance reading of the mass, m, to volume, V, at the test temperature, a correction for the liquid's density and air buoyancy is necessary. The calculation of the liquid volume at the test temperature is given by Formula (A.1).

In case the test liquid is water and the calculations given in Clause A.1 are not feasible, the balance indications may be converted to volume using the correction factors given in Clause A.2.

NOTE 1 Formula (A.1) is based on Formula (1) of ISO 4787:2021, however, the thermal expansion of the ALHS is neglected and only the thermal expansion of the test liquid is accounted for.

$$V_L = (m_L - m_E) \times \frac{1}{\rho_L - \rho_A} \times \left(1 - \frac{\rho_A}{\rho_B}\right) \quad \text{(A.1)}$$

where

V_L is the calculated volume of test liquid at the test temperature, in ml;

m_L is the balance reading of the weighing vessel after test liquid delivery, in g;

m_E is the balance reading of the weighing vessel before test liquid delivery, in g ($m_E = 0$ in case the balance was tared with the weighing vessel);

ρ_A is the density of air, in g/ml, see Formula (A.2);

ρ_B is the actual or assumed density of the weights used to calibrate the balance, in g/ml;

NOTE 2 Stainless steel weights of density 8,0 g/ml are typically used for balance calibration.

ρ_L is the density of the test liquid at the test temperature t (in °C), in g/ml.

For water, ρ_L can be calculated with the "Tanaka" Formula (A.3); the density of other test liquids should be determined with sufficiently small uncertainty (see A.1.4).

A.1.2 Calculation of air density

Formula (A.2) for air density can be used at temperatures between 15 °C and 27 °C, barometric pressure between 600 hPa and 1 100 hPa, and relative humidity between 20 % and 80 %.

$$\rho_A = \frac{1}{1\ 000} \times \frac{0{,}34\ 848 \times p - 0{,}009 \times h_r \times e^{(0{,}061 \times t)}}{t + 273{,}15} \quad \text{(A.2)}$$

where

ρ_A is the air density, in g/ml;

t is the ambient temperature, in °C;

p is the barometric pressure, in hPa;

h_r is the relative air humidity, in %.

The uncertainty of this formula can be calculated according to OIML R 111-1:2004, section C.6.3.6 [11]. At other environmental conditions, Formula (A.2) shall be replaced with the current CIPM air density equation.

NOTE The air density calculation valid at the time of publication of this document is CIPM-2007 and is described in Reference [12].

A.1.3 Calculation of water density

The density of pure water is normally calculated using formulae given in the literature. Formula (A.3), as published by Tanaka[13] provides a good basis for standardization:

$$\rho_W = a_5 \times \left[1 - \frac{(t_W + a_1)^2 \times (t_W + a_2)}{a_3 \times (t_W + a_4)} \right] \quad \text{(A.3)}$$

where

ρ_W is the density of water, in g/ml

t_W is the water temperature, in °C

a_1 = −3,983 035 °C

a_2 = 301,797 °C

a_3 = 522 528,9 (°C)^2

a_4 = 69,348 81 °C

a_5 = 0,999 974 950 g/ml

A.1.4 Test liquids other than water

When the test liquid is not pure water, the density of the test liquid ρ_L cannot be calculated by Formula (A.3). For these test liquids, density shall be measured or obtained from literature values.

NOTE The error associated with density measurements or use of literature values can range from negligible to significant.

A.2 Correction factors for the conversion of balance readings to volume

If the test liquid is distilled water, another possible procedure for the conversion of mass to volume is using the Z correction factors specified in Table A.1.

Convert each mass m_i obtained from balance readings by applying the Z correction factors at the mean temperature and barometric pressure measured during the measurement procedure, and using Formula (A.4):

$$V_i = m_i \times Z \quad \text{(A.4)}$$

where

V_i is the delivered test volume, in µl;

m_i is the measured mass of the delivered test volume, in mg;

Z is the Z correction factor from Table A.1, in µl/mg.

Table A.1 — Z correction factors for distilled water (air-saturated) in units of µl per mg

Temperature °C	Barometric pressure hPa						
	800	850	900	950	1 000	1 013	1 050
15,0	1,001 7	1,001 8	1,001 9	1,001 9	1,002 0	1,002 0	1,002 0
15,5	1,001 8	1,001 9	1,001 9	1,002 0	1,002 0	1,002 0	1,002 1
16,0	1,001 9	1,002 0	1,002 0	1,002 1	1,002 1	1,002 1	1,002 2
16,5	1,002 0	1,002 0	1,002 1	1,002 1	1,002 2	1,002 2	1,002 2
17,0	1,002 1	1,002 1	1,002 2	1,002 2	1,002 3	1,002 3	1,002 3
17,5	1,002 2	1,002 2	1,002 3	1,002 3	1,002 4	1,002 4	1,002 4
18,0	1,002 2	1,002 3	1,002 3	1,002 4	1,002 5	1,002 5	1,002 5
18,5	1,002 3	1,002 4	1,002 4	1,002 5	1,002 5	1,002 6	1,002 6
19,0	1,002 4	1,002 5	1,002 5	1,002 6	1,002 6	1,002 7	1,002 7
19,5	1,002 5	1,002 6	1,002 6	1,002 7	1,002 7	1,002 8	1,002 8
20,0	1,002 6	1,002 7	1,002 7	1,002 8	1,002 8	1,002 9	1,002 9
20,5	1,002 7	1,002 8	1,002 8	1,002 9	1,002 9	1,003 0	1,003 0
21,0	1,002 8	1,002 9	1,002 9	1,003 0	1,003 1	1,003 1	1,003 1
21,5	1,003 0	1,003 0	1,003 1	1,003 1	1,003 2	1,003 2	1,003 2
22,0	1,003 1	1,003 1	1,003 2	1,003 2	1,003 3	1,003 3	1,003 3
22,5	1,003 2	1,003 2	1,003 3	1,003 3	1,003 4	1,003 4	1,003 4
23,0	1,003 3	1,003 3	1,003 4	1,003 4	1,003 5	1,003 5	1,003 6
23,5	1,003 4	1,003 5	1,003 5	1,003 6	1,003 6	1,003 6	1,003 7
24,0	1,003 5	1,003 6	1,003 6	1,003 7	1,003 7	1,003 8	1,003 8
24,5	1,003 7	1,003 7	1,003 8	1,003 8	1,003 9	1,003 9	1,003 9
25,0	1,003 8	1,003 8	1,003 9	1,003 9	1,004 0	1,004 0	1,004 0
25,5	1,003 9	1,004 0	1,004 0	1,004 1	1,004 1	1,004 1	1,004 2
26,0	1,004 0	1,004 1	1,004 1	1,004 2	1,004 2	1,004 3	1,004 3
26,5	1,004 2	1,004 2	1,004 3	1,004 3	1,004 4	1,004 4	1,004 4
27,0	1,004 3	1,004 4	1,004 4	1,004 5	1,004 5	1,004 5	1,004 6
27,5	1,004 5	1,004 5	1,004 6	1,004 6	1,004 7	1,004 7	1,004 7
28,0	1,004 6	1,004 6	1,004 7	1,004 7	1,004 8	1,004 8	1,004 8
28,5	1,004 7	1,004 8	1,004 8	1,004 9	1,004 9	1,005 0	1,005 0
29,0	1,004 9	1,004 9	1,005 0	1,005 0	1,005 1	1,005 1	1,005 1
29,5	1,005 0	1,005 1	1,005 1	1,005 2	1,005 2	1,005 2	1,005 3
30,0	1,005 2	1,005 2	1,005 3	1,005 3	1,005 4	1,005 4	1,005 4

Annex B
(normative)

Dual-dye ratiometric photometric procedure

B.1 General

The delivered volumes into wells of microplates are determined using two chromophore solutions with known concentrations of each chromophore. Ponceau S and copper(II) chloride have absorbance maxima at 520 nm and 730 nm, respectively. All liquids contain the same concentration of copper(II) chloride, which is used to determine the fill height of each well. The test volume of Ponceau S test liquid is delivered into each well, mixed, and absorbances are read at 520 nm and 730 nm to calculate the delivered test liquid volume.

B.2 Test equipment

B.2.1 General

All test equipment shall be chosen such that the required measuring system uncertainty (MSU) can be obtained. The MSU can be estimated according to the approach given in ISO/IEC Guide 98-3.

B.2.2 Microplate absorbance reader

A microplate absorbance reader meeting the minimum performance requirements according to Table 4 shall be used. Bandpass filters with centre wavelengths of (520 ± 1) nm and (730 ± 2) nm shall be installed in the plate reader. Alternatively, plate readers with monochromators suitable to select (520 ± 1) nm and (730 ± 2) nm as centre wavelengths may be used.

B.2.3 Spectrophotometer

Absorbance values of the copper(II) chloride and Ponceau S liquids shall be measured on a spectrophotometer with the minimum performance specifications as listed in Table B.1. Bandpass filters with centre wavelengths of (520 ± 1) nm and (730 ± 2) nm shall be installed in the spectrophotometer.

A calibrated cuvette with an optical path length of 10 mm shall be used for the absorbance measurements.

Table B.1 — Minimum performance requirements of the spectrophotometer

Parameter	Requirement
Photometric resolution	0,000 1 AU [c]
Photometric repeatability at A = 0,0 AU [a]	0,000 15 AU
Photometric repeatability at A = 0,5 AU [a]	0,000 15 AU
Photometric repeatability at A = 1,0 AU [a]	0,000 15 AU
Photometric repeatability at A = 1,5 AU [a]	0,000 15 AU
System linearity between 0 AU and 1,5 AU [b]	0,000 25 AU

[a] Bandpass and signal averaging time may be increased to meet the photometric repeatability requirement.

[b] Corrections may be applied to meet the system linearity requirement.

[c] Absorbance unit.

B.2.4 Microplates

High quality ANSI/SLAS 96-well or 384-well plates with a flat, optically clear bottom shall be used. Microplates shall be characterized on a lot basis to determine the bottom diameter or width (depending on plate type), and side-wall taper angle. Additionally, wells should be characterized to correct for any non-uniformity in well dimensions for well-specific correction throughout that lot of plates.

B.2.5 Calibrator plate

A calibrator plate shall be used to calibrate the daily performance of the microplate reader. Optical standards used in the calibrator plate shall have stable absorbances at 520 nm and 730 nm, and shall be re-calibrated regularly, according to the manufacturer's instructions.

B.2.6 Microplate shaker

The microplate shaker used to mix the delivered liquids shall fulfil the minimum requirements given in Table B.2.

Table B.2 — Microplate shaker minimum requirements for standard ANSI/SLAS microplates

Plate type	Speed r/min	Orbit mm
96 wells	1 300	1
384 wells	2 600	1

B.2.7 Balance

Balances used for accurate weighing of dry reagents and preparation of solutions shall meet the requirements of Tables 2 and 3.

Weighing results for liquids shall be corrected for density, temperature and air buoyancy when determining volume (see Annex A).

B.2.8 Other test equipment

The test equipment used in this procedure shall conform to the minimum performance requirements given in Table 5.

B.2.9 Volumetric glassware, Class A

Known volumes of reagent solutions or test liquids shall be prepared by volumetric or gravimetric means. In case of gravimetric preparation, the densities of the liquids shall be known and properly accounted for. Calibrated Class A glassware may be used for the preparation of the liquids.

NOTE Specifications for one-mark volumetric pipettes can be found in ISO 648 and ASTM E969[15] and for one-mark volumetric flasks in ISO 1042 and ASTM E 288[17].

B.3 Reagents

B.3.1 General requirements

All components used in the preparation of reagent solutions shall be of at least 99 % analytical purity unless otherwise stated. The chemical abstracts service (CAS) numbers of the chemicals used in this procedure are summarized in Table B.3.

Table B.3 — Chemicals used in the dual-dye ratiometric photometric method

Name	Formula	CAS number
Copper(II) chloride dihydrate	$CuCl_2 \cdot 2H_2O$	10125–13–0
Disodium hydrogen phosphate dihydrate	$Na_2HPO_4 \cdot 2H_2O$	10028–24–7
Hydrochloric acid	HCl	7647–01–0
Ponceau S	$C_{22}H_{12}N_4Na_4O_{13}S_4$	6226–79–5
Potassium hydrogen phthalate	$HOOCC_6H_4COOK$	877–24–7
Sodium hydroxide	NaOH	1310–73–2
Tetrasodium ethylenediamine-tetraacetic acid dihydrate (EDTA)	$(NaOOCCH_2)_2NCH_2CH_2N(CH_2COONa)_2 \cdot 2H_2O$	10378–23–1
Water	H_2O	7732–18–5

B.3.2 Water

Water used for chromophore solutions shall comply with grade 1 in accordance with ISO 3696.

B.3.3 Stability of solutions

If reagents or test liquids are to be stored for any length of time, they shall be tested for chemical stability. Preservatives may be added, if needed, to prevent microbiological growth. Such preservatives shall not alter the liquid properties or interfere with the absorbance properties of the liquids. All liquids shall be stored protected from light.

B.3.4 Preparation of reagent solutions

B.3.4.1 Buffer

Prepare phthalate buffer from 4,08 g/l of potassium hydrogen phthalate and 3,74 g/l of tetrasodium EDTA. Adjust to pH = 6,0 with either HCl or NaOH as needed and filter the solution through a 0,2 µm filter.

B.3.4.2 Copper(II) chloride solution

Prepare copper(II) chloride solution by dissolving 1,12 g of $CuCl_2 \cdot 2H_2O$ per litre of phthalate buffer (B.3.4.1), and subsequent filtration through a 0,2 µm filter. Measure and record the absorbance a_b at 730 nm in a cuvette with a 10 mm path length.

B.3.4.3 Ponceau S test liquids

Dissolve Ponceau S in copper(II) chloride solution (see B.3.4.2). Table B.4 lists Ponceau S test liquids for various ranges of test volumes (V_T) depending on the plate type used and indicates the amount of Ponceau S chromophore (which can contain up to 15 % of water) per litre of Ponceau S test liquid. Ponceau S test liquids No. 5 and No. 6 can be prepared by dissolving Ponceau S directly in phthalate buffer (B.3.4.1). Filter each Ponceau S test liquid through a 0,2 µm filter.

Table B.4 — Ponceau S test liquids

Ponceau S test liquid No.	Test volume range in 96-well plates	Test volume range in 384-well plates	Ponceau S chromophore [a]
	µl	µl	g/1 000 ml
1	$200 < V_T \leq 350$	n/a	0,040 0
2	$50 \leq V_T \leq 200$	$10 \leq V_T \leq 55$	0,079 7
3	$10 \leq V_T < 50$	$2,5 \leq V_T < 10$	0,318 8

[a] Amounts listed in this table are target values. Actual amounts may vary up to ±5 % from the target value.

Table B.4 *(continued)*

Ponceau S test liquid No.	Test volume range in 96-well plates µl	Test volume range in 384-well plates µl	Ponceau S chromophore [a] g/1 000 ml
4	$2 \leq V_T < 10$	$0,5 \leq V_T < 2,5$	1,594 1
5	$1 \leq V_T < 2$	$0,3 \leq V_T < 0,5$	3,936 7
6	$0,1 \leq V_T < 1$	$0,03 \leq V_T < 0,3$	15,697 0

[a] Amounts listed in this table are target values. Actual amounts may vary up to ±5 % from the target value.

For each Ponceau S test liquid, determine the absorbance per unit path length (a_r) at 520 nm by measuring the absorbance in a cuvette with a 10 mm path length. Ponceau S test liquids may be carefully diluted for these measurements so that a_r can be calculated.

B.4 Test conditions

The ambient temperature should be between 15 °C and 30 °C, and the relative humidity should be between 20 % and 90 % (non-condensing). When using DMSO as a solvent for the test liquids, the ambient temperature should be between 19 °C and 30 °C to prevent DMSO from solidifying.

B.5 Procedure

B.5.1 Preparation

a) Calibrate the plate reader using the calibrator plate. Re-calibrate the plate reader if the temperature has changed more than ±2 °C since the last calibration.

b) Fill a microplate with the clear buffer and shake on the microplate shaker according to Table B.5. Measure the baseline absorbance of each well at 520 nm and 730 nm. Measure a new set of baseline absorbances each time the plate reader has been calibrated.

Table B.5 — Baseline absorbance

Plate type	Fill volume µl	Shaking time s
96 wells	200	60
384 wells	55	60

c) Fill a liquid reservoir appropriate to the ALHS under test with the copper(II) chloride solution, and one or more liquid reservoirs with the Ponceau S test liquids corresponding to the volumes to be tested (see Table B.4). Keep the reservoirs covered when not in use to protect from evaporation. Liquids should be left exposed for no longer than 20 min to 60 min, depending on the exposed surface area, reservoir fill volume and environmental conditions.

B.5.2 Dry delivery test procedure

a) Using the ALHS under test, deliver the target volume of Ponceau S test liquid into the empty wells of the microplate.

b) Post-fill copper(II) chloride solution into the microplate so that the combined total volume is approximately 200 µl per well in 96-well plates, and approximately 55 µl per well in 384-well plates. The amount of copper(II) chloride solution required is only approximate and can vary by ±10 %.

NOTE The copper(II) chloride solution can be delivered from any ALHS or other device that is capable of delivering the approximate required volume.

c) Inspect the filled plates for excessive droplets of solution near the well top, or bubbles trapped in the wells. If either occurs, then the plate should be centrifuged at 1 000 r/min to 2 000 r/min for 30 s to 60 s, or until the droplets have been pushed into the wells, and the bubbles have been pushed out.

d) Shake the filled plates on a microplate shaker with a rotary orbit of 1 mm, at a speed of 1 300 r/min to 1 500 r/min for 60 s for 96-well microplates. For 384-well microplates, shake at a speed of 2 600 r/min to 2 700 r/min for 120 s.

e) Insert the microplate into the plate reader and measure the absorbances at 520 nm and 730 nm.

B.5.3 Wet delivery test procedure

a) Pre-fill copper(II) chloride solution into the microplate so that the combined total volume after addition of the Ponceau S test liquid is approximately 200 µl per well in 96-well plates, and approximately 55 µl per well in 384-well plates. The amount of copper(II) chloride solution required is only approximate and can vary by ±10 %.

NOTE The copper(II) chloride solution can be delivered from any ALHS or other device that is capable of delivering the approximate required volume.

b) Using the ALHS under test, deliver the target volume of Ponceau S test liquid into the pre-filled wells of the microplate.

c) Follow steps c), d), and e) according to B.5.2.

B.6 Volume Calculations

The amount of test liquid volume delivered is calculated in three steps:

a) Since the concentration of copper(II) chloride is the same in the Ponceau S test liquids and the copper(II) chloride solution, Formula (B.1) can be used to determine the liquid depth (l) in each well.

$$l = \frac{A_{730}}{a_b} \tag{B.1}$$

where

l is the path length;

A_{730} is the measured absorbance at 730 nm;

a_b is the absorbance per unit path length of the copper(II) chloride solution.

b) Use Formula (B.2) to calculate the total volume of liquid in the well if the wells of the micro plate are round. At this point, well-specific geometrical corrections may be applied to reduce well-to-well variability.

$$V_W = \pi \times l \times \frac{D^2}{4} + \pi \times D \times l^2 \times \frac{\tan\theta}{2} + \pi \times l^3 \times \frac{\tan^2\theta}{3} \tag{B.2}$$

where

V_W is the total liquid volume in the well,

D is the diameter of the well bottom,

θ is the side wall taper angle.

c) Use Formula (B.3) to calculate the total volume of liquid in the well if the wells of the micro plate are square shaped. At this point, well-specific geometrical corrections may be applied to reduce well-to-well variability.

$$V_W = l \times w_B^2 + \frac{l^2 \times w_B \times (w_T - w_B)}{h} + \frac{l^3 \times (w_T - w_B)^2}{3 \times h^2} \quad \text{(B.3)}$$

where

w_B is the bottom width of the well;

w_T is the top width of the well;

h is the height of the well.

d) The calculation of the delivered volume of Ponceau S test liquid (V_T) is given by Formula (B.4):

$$V_T = V_W \times \left(\frac{a_b}{a_r} \right) \times \left(\frac{A_{520}}{A_{730}} \right) \quad \text{(B.4)}$$

where

V_T is the volume of delivered test liquid;

a_r is the absorbance per unit path length at 520 nm of the used Ponceau S test liquid;

A_{520} is the measured absorbance at 520 nm.

B.7 Traceability

Traceability of measurement results from this ratiometric photometric procedure to the International System of Units (SI) occurs through three main measurement paths:

a) Characterized microplates. Microplates shall be characterized using traceable dimensional measurements of each well.

b) Characterized reagent solutions. Traceable absorbance measurements shall be made using a spectrophotometer which performance is maintained against calibrated neutral density (ND) glass and calibrated wavelength standards. A cuvette with calibrated internal path length shall be used to provide a known path length.

c) Calibrated plate reader absorbance measurements. A calibration plate shall be used to provide the traceability path to the measured absorbance values from the plate reader.

Annex C
(normative)

Single dye photometric procedure

C.1 General

This procedure is suitable for testing ALHS with up to 384 channels, using aqueous, dimethylsulfoxide (DMSO), or water/DMSO mixtures as test liquids. The solubility of Orange G in water/DMSO mixtures and DMSO is similar to its solubility in aqueous liquids. This procedure can be used to test volumes of 1 µl to 100 µl in 96-well microplates, and volumes between 0,25 µl and 20 µl in 384-well microplates.

Buffering all solutions at pH 7 is important as the absorbance properties of Orange G are pH dependent.

C.2 Applicable volume ranges

Depending on the test volume and microplate type used, the appropriate Orange G test liquids according to Table C.1 shall be prepared.

Table C.1 — Orange G test liquids

Orange G concentration	Test volumes in 96-well microplates	Test volumes in 384-well microplates
0,2 g/l	10 µl to 100 µl	2 µl to 20 µl
1,6 g/l	2 µl to 10 µl	–
2,0 g/l	–	0,25 µl to 2 µl
8,0 g/l	1 µl to 2 µl	–

C.3 Test equipment

C.3.1 General

All test equipment used for performing this procedure shall be calibrated and serviced according to its manufacturer's instructions. The uncertainty of the test equipment shall be known so that the MSU of this procedure can be estimated. Test equipment shall fulfil the minimum requirements given in Table 5.

C.3.2 Manual pipettes

Pipettes with a nominal volume as close as possible to the desired transfer-volume shall be used.

C.3.3 Balances

Balances used in this procedure shall fulfil the minimum requirements given in Table 2.

C.3.4 Microplate reader

The microplate absorbance reader shall fulfil the minimum requirements given in Table 4. Bandpass filters with a centre wavelength of 492 nm and 620 nm shall be used. Alternatively, plate readers with monochromators suitable to select 492 nm and 620 nm as centre wavelengths may be used.

C.3.5 Microplates

High quality ANSI/SLAS microplates with a flat and optically clear bottom shall be used.

C.3.6 Volumetric glassware

Calibrated Class A glassware may be used for the preparation of the solutions.

NOTE Specifications for one-mark volumetric flasks can be found in ISO 1042 and ASTM E 288[17].

C.4 Reagents

C.4.1 Chemical substances used

All components used in the preparation of reagent solutions shall be of at least 99 % analytical purity unless otherwise stated. Chemicals used in this procedure are listed in Table C.2.

Table C.2 — Chemicals used

Chemicals/solutions	CAS Number [a]	Formula	Molecular mass
Orange G	1936–15–8	$C_{16}H_{10}N_2Na_2O_7S_2$	452,36 g/mol
Disodium hydrogen-phosphate dihydrate	10028–24–7	$Na_2HPO_4{\cdot}2H_2O$	177,99 g/mol
Sodium hydroxide, 1 M	1310–73–2	NaOH	40,0 g/mol
Deionised water	7732–18–5	H_2O	18,0 g/mol
[a] Chemical abstracts service number.			

C.4.2 Buffer 0,1 M

In a 2 000 ml volumetric flask, place (35,6 ± 0,1) g of disodium hydrogen phosphate dehydrate and 2 000 µl of 1 M sodium hydroxide solution. Make up to 2 000 ml with deionized water and stir with a magnetic stir bar until the disodium hydrogen phosphate dehydrate is completely dissolved (this can take up to 30 min), for a final pH of approximately 7. Filter the buffer through a 0,2 µm filter and store in a polyethylene bottle.

Measure and record the pH of the buffer prior to using it for the preparation of Orange G test liquids.

C.4.3 Orange G test liquid 8,0 g/l

In a 500 ml volumetric flask, place (4,0 ± 0,05) g of Orange G and add buffer until the meniscus reaches just the neck of the flask. Add a magnetic stir bar and stir the liquid until Orange G is completely dissolved. Remove the magnetic stir bar, rinse it with buffer into the flask, and add buffer to the score mark of the flask. Close the volumetric flask and mix the liquid several times by careful inversion of the flask. Filter the test liquid through a 0,2 µm filter and store it in an amber glass bottle.

C.4.4 Orange G test liquid 2,0 g/l

In a 1 000 ml volumetric flask, place (2,0 ± 0,01) g of Orange G and add buffer until the meniscus reaches just the neck of the flask. Add a magnetic stir bar and stir the liquid until Orange G is completely dissolved. Remove the magnetic stir bar, rinse it with buffer into the flask, and add buffer to the score mark of the flask. Close the volumetric flask and mix the liquid several times by careful inversion of the flask. Filter the test liquid through a 0,2 µm filter and store in an amber glass bottle.

C.4.5 Orange G test liquid 1,6 g/l

Measure 100 ml of the 8 g/l Orange G test liquid with a volumetric flask. Transfer this amount into a 500 ml volumetric flask. Rinse the 100 ml flask with buffer and add these rinses to the 500 ml flask.

Add buffer to the score mark, close the volumetric flask and mix the liquid several times by careful inversion of the flask. Place the Orange G test liquid in an amber glass bottle.

C.4.6 Orange G test liquid 0,2 g/l

Measure 50 ml of the 2 g/l Orange G test liquid with a volumetric flask. Transfer this amount into a 500 ml volumetric flask. Rinse the 50 ml flask with buffer and add these rinses to the 500 ml flask. Then add buffer up to the score mark, close the volumetric flask and mix the liquid several times by careful inversion of the flask. Place the Orange G test liquid in an amber glass bottle.

C.5 Test conditions

The ambient temperature in the test room should be between 15 °C and 30 °C, and the relative humidity between 40 % and 70 % (non-condensing).

C.6 Test procedure

C.6.1 Calibration curves

Prepare calibration curves as follows:

a) Carefully pipette the volumes of Orange G test liquids and buffer as indicated in Table C.3 into test tubes, which can be tightly capped. Mix the contents of each test tube carefully.

b) For 384-well plates, pipette at least 5 replicates of 75 µl of each liquid in a row of a 384-well microplate using a calibrated manual pipette.

c) For 96-well plates, pipette at least 5 replicates of 200 µl of each liquid in a row of a 96-well microplate using a calibrated manual pipette.

d) Centrifuge the plate for 60 s at 164 rcf (relative centrifugal force).

e) Measure the absorbance of the microplate at 492 nm for Orange G, and at 620 nm for reference. Subtract the reference absorbance at 620 nm from the absorbance at 492 nm.

f) Calculate the average of 5 replicates of the same liquid.

g) Plot the test volumes versus absorbance values for the same concentration of Orange G. The measured absorbance shall not exceed 2,0 AU.

h) Calculate the regression curve for each volume range; i.e. for the same concentration of Orange G.

i) Each point represents the average of 5 replicates, which were manually pipetted.

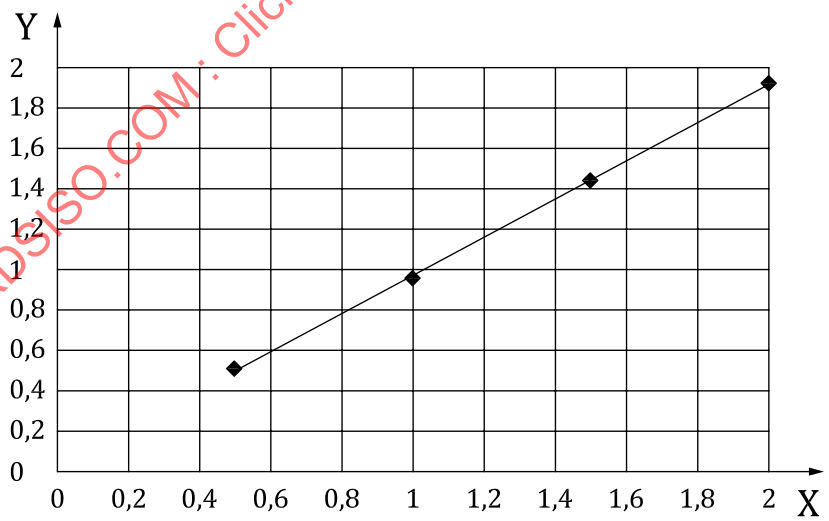
Figure C.1 shows an example of a calibration curve for the volume range 0,5 µl to 2,0 µl in a 96-well plate.

Table C.3 — Calibration points for all volumes in 96-well and in 384-well microplates

Plate format	Concentration of Orange G test liquid g/l	Volume of Orange G test liquid µl	Buffer	Combined volume ml	Test volume equivalent µl
384	0,2	50	1 ml + 450 µl	1,5	2,5
384	0,2	60	1 ml + 440 µl	1,5	3,0
384	0,2	100	1 ml + 400 µl	1,5	5,0
384	0,2	200	1 ml + 300 µl	1,5	10,0

Table C.3 *(continued)*

Plate format	Concentration of Orange G test liquid g/l	Volume of Orange G test liquid µl	Buffer	Combined volume ml	Test volume equivalent µl
384	0,2	400	1 ml + 100 µl	1,5	20,0
384	2,0	10	2 ml + 990 µl	3,0	0,25
384	2,0	20	2 ml + 980 µl	3,0	0,50
384	2,0	40	2 ml + 960 µl	3,0	1,00
384	2,0	80	2 ml + 920 µl	3,0	2,00
96	0,2	100	1 ml + 900 µl	2,0	10,0
96	0,2	300	1 ml + 700 µl	2,0	30,0
96	0,2	500	1 ml + 500 µl	2,0	50,0
96	0,2	700	1 ml + 300 µl	2,0	70,0
96	0,2	1 000	1 ml	2,0	100,0
96	1,6	20	1 ml + 980 µl	2,0	2,0
96	1,6	30	1 ml + 970 µl	2,0	3,0
96	1,6	50	1 ml + 950 µl	2,0	5,0
96	1,6	70	1 ml + 930 µl	2,0	7,0
96	1,6	100	1 ml + 900 µl	2,0	10,0
96	8,0	5	1 ml + 995 µl	2,0	0,5
96	8,0	10	1 ml + 990 µl	2,0	1,0
96	8,0	15	1 ml + 985 µl	2,0	1,5
96	8,0	20	1 ml + 980 µl	2,0	2,0
96	8,0	25	1 ml + 975 µl	2,0	2,5



Key

X target volume in µl

Y absorbance in AU

Figure C.1 — Example calibration curve for test volumes 0,5 µl to 2,0 µl in 96-well microplates

C.6.2 Test procedure

With the ALHS under test, the following protocol shall be performed.

C.6.2.1 96-well microplates

a) Deliver the target volume of the appropriate Orange G test liquid into the wells of the 96-well microplate.

b) Add the complementary amount of buffer to fill the well to a final volume of 200 µl; e.g. with a calibrated multi-channel pipette.

c) Shake the plate on a microplate shaker for 30 s at 1 000 r/min (revolutions per minute), to achieve complete mixing of the solutions.

d) Centrifuge the microplate for 60 s at 164 rcf (relative centrifugal force) to remove air bubbles and establish a defined meniscus.

e) Measure the absorbance of each well at 492 nm and 620 nm for Orange G and reference, respectively (if the plate reader uses a pulsed light source, use at least 10 flashes per measurement). The absorbance per measurement shall not exceed 2,0 AU.

C.6.2.2 384-well microplates

a) Deliver the target volume of the appropriate Orange G test liquid into the wells of the 384-well microplate.

b) Add the complementary amount of buffer to fill the well to a final volume of 75 µl; e.g. with a calibrated multi-channel pipette.

c) Shake the plate on a microplate shaker for 15 s at 2 000 r/min (revolutions per minute), to achieve complete mixing of the solutions.

d) Centrifuge the microplate for 60 s at 164 rcf (relative centrifugal force) for removing air bubbles and establishing a defined meniscus.

e) Measure the absorbance of each well at 492 nm and 620 nm for Orange G and reference, respectively. If the plate reader uses a pulsed light source, use at least 10 flashes per measurement. The absorbance per measurement shall not exceed 2,0 AU.

C.7 Calculation of volume

Determine the delivered volume of test liquid in each well by using the linear regression formula of the calibration curve appropriate for the delivered volume, together with the measured absorbance value. Solve the regression formula for x to determine the delivered volume in each well.

EXAMPLE Based on the calibration curve shown in Figure C.1, the absorbance of one well has been measured as 1,442 6 AU. The linear regression formula in this example is: $y = 0{,}944\ 2x + 0{,}026\ 3$. Solving the formula for x in this case yields $x = 1{,}5$ µl of test liquid.

C.8 Traceability

Measurement results of the single-dye photometric method are traceable to the International System of Units (SI) by means of the calibrated balance, calibrated volumetric flasks, and calibrated pipettes.

Annex D
(normative)

Gravimetric procedure, single channel measurement

D.1 General

This method describes the apparatus, procedure and reference material for determining measurement error using the gravimetric method. A single pan balance is used to take a measurement from a single channel at a time.

D.2 Test equipment

D.2.1 Balance

A balance of appropriate capacity for the test volume and fulfilling the minimum requirements of Table 3 shall be used.

D.2.2 Liquid reservoir

The liquid reservoir shall be large enough to accommodate all test liquid required for the complete series of tests.

D.2.3 Weighing vessel

Mass loss due to evaporation shall be taken into consideration when selecting an appropriate weighing vessel.

It is recommended that the weighing vessel be supplied with a lid to mitigate mass loss due to evaporation. This is particularly important if the vessel is moved at any time during the weighing process. If the vessel remains stationary throughout the weighing process and a lid is not used, then a height-above-fluid to diameter ratio of 3:1 is recommended.

The gravimetric regression method can also be used to quantify mass loss due to evaporation. This method is only recommended when using an open weighing vessel that is not moved during the measurement process. See Annex E for further details.

D.2.4 Environmental condition test equipment

The environmental conditions of the test room shall be monitored using test equipment according to Table 5.

D.3 Test liquid

The reference test liquid for the gravimetric method is distilled or deionized water conforming to grade 3 as specified in ISO 3696, degassed or air-equilibrated. The test liquid shall be in thermal equilibrium with the ALHS under test and the test equipment.

Other test liquids may be used provided the sensitivity of the given test liquid to environmental conditions is known and accounted for where significant.

D.4 Test conditions

In order to control the variability of the results, the test environment should be kept as stable as possible. Table D.1 gives recommended temperature and relative humidity ranges and permitted changes during the test. The change in recorded value over the duration of the testing session should remain within the recommended value.

Table D.1 — Test conditions

Environmental condition	Range	Change during the test
Water temperature	17 °C to 30 °C	≤1 °C
Air temperature	17 °C to 30 °C	≤3 °C
Relative humidity	45 % to 70 %	≤10 %

The balance shall be placed in a draft-free environment on a stable, vibration-free surface within the same test environment as the ALHS under test.

D.5 Procedure

D.5.1 Preparation

Environmental conditions shall be recorded at the beginning and the end of the weighing procedure. The difference between the two readings shall be compared against Table D.1.

D.5.2 Evaporation

D.5.2.1 Weighing vessel

In order to yield a consistent mass loss due to evaporation across all measurements, the weighing vessel shall be prepared with an initial volume of test liquid sufficient to completely cover the bottom of the vessel.

D.5.2.2 Determine mass loss due to evaporation

The environmental conditions of the test room, the time taken to perform a single measurement, and the handling of the weighing vessel during a measurement will affect the mass loss due to evaporation.

Before using the ALHS to deliver the test liquid, a series of at least 10 blank measurements shall be taken.

a) Record the tare weight of the weighing vessel b_0, or tare the balance to zero ($b_0 = 0$).

b) If the weighing vessel needs to be moved to a delivery position, move the weighing vessel into that position and remove the lid, if a lid is used. When positioning the weighing vessel, the following conditions shall be met:

   — test liquid shall not be delivered to the weighing vessel;

   — the ALHS liquid delivery apparatus shall not come into physical contact with the weighing vessel;

   — if it is not possible to avoid liquid delivery or contact of the liquid delivery apparatus with the weighing vessel in order to perform a blank measurement, place the weighing vessel next to the delivery location and deliver the blank measurement to a waste vessel.

c) Perform a blank delivery. Follow the same cycle time as with a test liquid delivery.

d) Record the mass of the weighing vessel as b_i. A single measurement of mass loss due to evaporation is calculated using Formula (D.1):

$$b'_i = b_i - b_0 \tag{D.1}$$

where

b'_i is the mass loss due to evaporation;

b_i is the mass of the weighing vessel;

b_0 is the tare mass of the weighing vessel.

e) Obtain the average mass loss due to evaporation by calculating the average of all blank measurements according to Formula (D.2).

$$\overline{b'} = \sqrt{\left(\frac{\sum_{i=1}^{n} b'_i}{n}\right)^2} \tag{D.2}$$

where

$\overline{b'}$ is the average mass loss due to evaporation;

n is the number of blank measurements.

D.5.3 Volume measurement

a) Record the test room environmental conditions (temperature, relative humidity, and barometric pressure) and the temperature of the test liquid at the beginning of testing.

b) If the weighing vessel must be removed from the balance weighing pan in order for the ALHS to deliver the test liquid, remove the weighing vessel and place where appropriate for the ALHS.

c) If the weighing vessel has a lid, remove the lid. Deliver the test liquid to the weighing vessel at the currently selected test volume. Replace the lid if applicable.

d) If the weighing vessel must be moved to the balance weighing pan in order to take a measurement, move the weighing vessel to the balance weighing pan.

e) Record the balance reading as m_i.

f) Tare or zero the balance and begin a new measurement.

g) Perform as many measurements as required.

h) Record the test room environmental conditions and test liquid temperature at the end of testing.

D.6 Volume calculations

D.6.1 Correction for evaporation

The average mass loss due to evaporation shall be added to each balance measurement. The resultant mass will be referred to as the corrected mass or m'_i as shown in Formula (D.3):

$$m'_i = m_i + \overline{b'} \tag{D.3}$$

where

m'_i is the mass of test liquid, corrected for evaporation;

m_i is the balance reading.

D.6.2 Conversion of corrected mass to volume

The values m_i obtained in accordance with D.5.3 are balance readings. A correction taking into account test liquid density and air buoyancy is necessary for the conversion of the balance readings m_i to volumes V_i. The balance readings shall be converted to volume in accordance with Annex A.

D.7 Traceability

Measurement results of the gravimetric method are traceable to the International System of Units (SI) through the use of a calibrated balance, calibrated test equipment for monitoring the environmental conditions, and properly accounting for the density and air buoyancy of the test liquid.

NOTE 1 Annex A describes the mass to volume conversion for water.

NOTE 2 EURAMET CG-19[18] includes information on the estimation of the MSU for this procedure.

Annex E
(normative)

Gravimetric regression procedure

E.1 General

The gravimetric regression procedure is applicable to liquid volumes delivered as droplets or jets by the device under test (DUT), e.g. by non-contact nanodispensing technologies or inkjet-type printheads. This method is suitable for volumes between 1 nl and 100 µl per droplet or jet.

This procedure is based on the gravimetric principles for quantitative volume determination described in ASTM E542[19] and ISO 4787 which are adapted to the nano- and microlitre range. This method performs a linear regression on balance readouts, which are continuously acquired before and after the addition of the delivered test liquid. This approach corrects for the mass loss due to evaporation during measurement, which is significant for very small delivered volumes.

E.2 Test equipment

E.2.1 General requirements

Balances used for this method shall conform to the minimum performance requirements given in Table 3. Other test equipment shall conform to the requirements given in Table 5.

E.2.2 Test liquids

The gravimetric measurement is largely independent of the test liquid used. The effective density of the test liquid ρ_L at temperature, t , shall be known to relate the measured mass to the delivered volume. The density can be determined either from literature or by direct measurement. Since the temperature influences the density of the liquid, it should be carefully measured and recorded during measurement. For density measurements, the test equipment shall have the minimum performance requirements given in Table E.1.

Table E.1 — Minimum requirements for density measurements

Equipment	Resolution	Expanded uncertainty (k = 2)
Thermometer	0,2 °C	1 °C
Hygrometer	0,1 % RH	3 % RH
Barometer	0,5 hPa	4 hPa
Density meter	0,000 01 g/ml	$3{,}04 \times 10^{-6}$ g/ml

Test liquids should have a low vapour pressure to reduce evaporation of liquid from the droplet during the whole measurement time.

NOTE A suitable test liquid is grade 1 water according to ISO 3696.

E.3 Environmental conditions

The measurements should be performed under the following conditions: barometric pressure between 600 hPa and 1 100 hPa, ambient temperature between 17 °C and 27 °C and relative humidity between

45 % and 80 %. The environmental conditions (temperature, humidity, pressure) shall be recorded for consideration in the volume calculations.

NOTE Ambient conditions influence the gravimetric measurement mainly through the air density ρ_A determination and the evaporation rate of liquid from the droplet as well as from the balance.

E.4 Measurement setup

The gravimetric setup shall consist of a high precision laboratory micro balance. The balance as well as the DUT shall be placed on a vibration-isolated or -compensated table to reduce mechanical vibrations.

a) A micro weighing vessel shall be filled with a minimum of 200 µl of the used test liquid such that the bottom of the weighing vessel is completely covered and shall be placed in the centre of the weighing pan. The vessel and weighing pan should be covered by a shield with a circular opening.

NOTE Weighing vessels made from electrically conductive material (e.g. metal) are preferable to avoid electrostatic charging.

b) The nozzle of the DUT shall be positioned concentrically above the weighing pan.

c) A draft shield shall be placed over the entire measurement setup to isolate it from surrounding air drafts.

E.5 Procedure

E.5.1 Mass measurements

The following steps shall be followed to perform the mass measurements.

a) Record the environmental conditions (temperature, relative humidity, and barometric pressure) at the beginning of the weighing procedure.

b) Record balance readings n times before, during, and after the test liquid delivery. This results in a series of balance readouts I_i at time t_i. Each balance readout I_i corresponds to the mass m_i of the filled weighing vessel and an independent error ε_i which represents a random measurement error (see E.6.3).

E.5.2 Volume determination

Under ideal laboratory conditions (constant temperature, humidity and air pressure), the evaporation rate of the test liquid can be assumed to be constant, and m_i is expected to have a linear relation to time t_i (for more information on the evaporation rate of water, see Reference [20]). To compensate for evaporation effects, the balance readouts recorded in E.5.1 before and after the test liquid delivery shall be processed as follows:

a) Perform a linear regression for both data sets (before and after test liquid delivery).

Directly after the delivery of the test liquid, there is a sudden increase in mass as seen from the balance readings in Figure E.1. This abrupt rise settles down after a short time so that the balance only measures the evaporation of the liquid. Therefore, a settling time shall be defined after delivery of the liquid, during which the balance readouts are not included in the calculation of the linear regression after delivery (see Figure E.1).

b) Calculate the difference between these two linear regression values ($m_{after} - m_{before}$) at the time of test liquid delivery (t_{del}). This difference corresponds to the mass m of the delivered test liquid as shown in Formula (E.1):

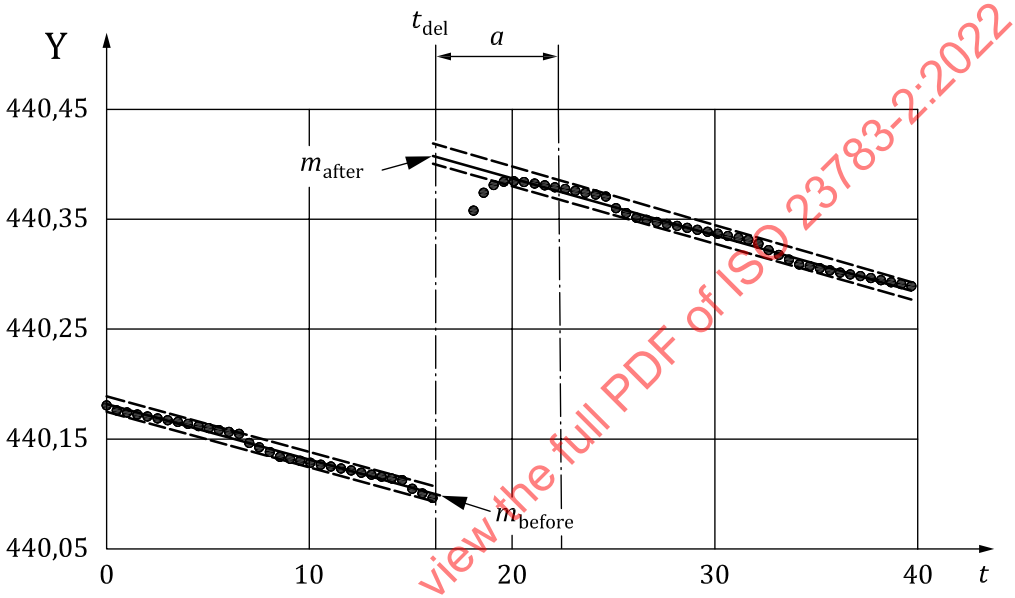
$$m = m_{after} - m_{before} \quad \text{(E.1)}$$

where

m is the mass of the delivered test liquid;

m_{after} is the mass calculated by linear regression of the recorded balance readings after the delivery at the time of delivery (t_{del});

m_{before} is the mass calculated by linear regression of the recorded balance readings before the delivery at the time of delivery (t_{del}).



Key

t time in s

Y mass in mg

. balance readouts

—— linear regression

– – prognosis interval

a settling time of the balance (mass recorded during this time is not used in calculations)

Figure E.1 — Graph of a typical measurement — linear regressions of the recorded mass before and after test liquid delivery shown as a function of time

E.6 Calculation of delivered volume

E.6.1 Delivered volume

The delivered volume at the test temperature is calculated from the calculated mass, m, according to Formula (E.2):

$$V_L = m \times \frac{1}{\rho_L - \rho_A} \times \left(1 - \frac{\rho_A}{\rho_C}\right) \quad \text{(E.2)}$$

where

V_L is the volume of test liquid at the test temperature;

m is the mass of the delivered test liquid calculated from linear regression values by Formula (E.1);

ρ_L is the density of the test liquid at the test temperature;

ρ_A is the density of air at the test temperature;

ρ_C is the density of the weights used to calibrate the balance at the test temperature.

The air density is calculated according to Formula (A.2). If the test liquid is distilled water, its density is calculated according to Formula (A.3), and delivered volumes are determined in accordance with Annex A. If a test liquid other than grade 1 water is used, its density at the test temperature shall be determined experimentally or taken from a literature reference.

The volume of test liquid at a reference temperature is calculated according to Clause 7.

E.6.2 Systematic error

The mass values m_{before} and m_{after} are subject to a measurement error resulting from the error of the used gravimetric balance. The systematic balance measurement error ϑ_{bal} is provided by the manufacturer and is part of each balance readout. If two values m_b and m_a with the same balance error (ϑ_{bal}) are subtracted from each other, the result m_d and the resulting error Δm_d follow the Gaussian Law of error propagation according to Formula (E.3):

$$m_d \pm \Delta m_d = (m_a - m_b) \pm \sqrt{2 \times (\vartheta_{bal})^2} \tag{E.3}$$

where

m_d is the difference between two measured masses on the balance;

Δm_d is the error of the mass difference m_d;

m_a is the first measured mass;

m_b is the second measured mass;

ϑ_{bal} is the systematic error of the balance.

E.6.3 Random error

The random error ε_i occurs due to external influences like the evaporation of the liquid or external vibration. Each balance readout I_i includes the mass m_i of the weighing vessel filled with liquid and a random error ε_i. Assuming a constant evaporation rate, the mass has a linear relationship to time as shown in Formula (E.4):

$$m_i = a - b \times t_i \tag{E.4}$$

where

m_i is the i-th measured mass on the balance;

t_i is the time at which the i-th mass was measured;

a is the regression parameter a (see Formula (E.6));

b is the regression parameter b (see Formula (E.7)).

By taking the random error into account, the balance readings can be expressed as shown in Formula (E.5). For more information of the random error calculation, see Reference [6].

$$I_i = m_i + \varepsilon_i = a - b \times t_i + \varepsilon_i \tag{E.5}$$

where

I_i is the i-th balance readout;

ε_i is the random error of the i-th balance readout.

Assuming that each random error ε_i obeys an independent distribution with variance of σ_i^2, the regression parameters a and b can be calculated according to Formulae (E.6) and (E.7):

$$a = \frac{\sum_{i=1}^{n} \frac{t_i^2}{\sigma_i^2} \times \sum_{i=1}^{n} \frac{I_i}{\sigma_i^2} - \sum_{i=1}^{n} \frac{t_i}{\sigma_i^2} \times \sum_{i=1}^{n} \frac{t_i \times I_i}{\sigma_i^2}}{\sum_{i=1}^{n} \frac{1}{\sigma_i^2} \times \sum_{i=1}^{n} \frac{t_i^2}{\sigma_i^2} - \left(\sum_{i=1}^{n} \frac{t_i}{\sigma_i^2} \right)^2} \tag{E.6}$$

$$b = \frac{\sum_{i=1}^{n} \frac{1}{\sigma_i^2} \times \sum_{i=1}^{n} \frac{t_i \times I_i}{\sigma_i^2} - \sum_{i=1}^{n} \frac{t_i}{\sigma_i^2} \times \sum_{i=1}^{n} \frac{I_i}{\sigma_i^2}}{\sum_{i=1}^{n} \frac{1}{\sigma_i^2} \times \sum_{i=1}^{n} \frac{t_i^2}{\sigma_i^2} - \left(\sum_{i=1}^{n} \frac{t_i}{\sigma_i^2} \right)^2} \tag{E.7}$$

where

n is the total number of balance readings;

σ_i^2 is the variance of the error ε_i.

The change of mass due to evaporation, with respect to the previous measurement ($m_i - m_{i-1}$), can be assumed to be the same at each time t_i when assuming a constant evaporation rate b and constant time intervals ($t_i - t_{i-1}$) between the balance readouts. The approximation of σ_i^2 by a constant value is therefore justified and the regression line provides a reasonable estimate of the continuously decreasing mass value. The linear regression parameters (a_{before} and b_{before}), as well as (a_{after} and b_{after}) are determined according to Formulae (E.6) and (E.7), based on two series of balance readouts before and after liquid delivery. With the linear regression parameters (a_{before}, b_{before}) and (a_{after}, b_{after}), the projected mass values at the time of delivery (t_{del}), $m_{\text{LR,before}}$ (t_{del}) and, $m_{\text{LR,after}}$ (t_{del}) can be readily calculated according to Formula (E.8) and Formula (E.9).

$$m_{\text{LR,after}}(t_{\text{del}}) = a_{\text{after}} + b_{\text{after}} \times t_{\text{del}} \tag{E.8}$$

$$m_{\text{LR,before}}(t_{\text{del}}) = a_{\text{before}} + b_{\text{before}} \times t_{\text{del}} \tag{E.9}$$

where

$m_{\text{LR,after}}$ is the projected mass value at the time of delivery, based on the linear regression of post-delivery mass values;

$m_{\text{LR,before}}$ is the projected mass value at the time of delivery, based on the linear regression of pre-delivery mass values;

a_{after} is the linear regression parameter a after the delivery;

a_{before} is the linear regression parameter a before the delivery;

b_{after} is the linear regression parameter b after the delivery;

b_{before} is the linear regression parameter b before the delivery;

t_{del} is the time of the test liquid delivery.

The mass of the delivered liquid is calculated as the difference between the two linear regression values at the delivery event according to Formula (E.10):

$$m = m_{LR,after}(t_{del}) - m_{LR,before}(t_{del}) \quad \text{(E.10)}$$

E.7 Traceability

The measurement results of the gravimetric regression method are traceable to the International System of Units (SI) through a calibrated micro balance, calibrated test equipment for monitoring the environmental conditions, calibrated density determination of the test liquid, and accounting for the air buoyancy of the test liquid.

NOTE 1 Annex A describes the mass to volume conversion for water.

NOTE 2 EURAMET CG-18[9] includes information on the estimation of the MSU for this procedure.

Annex F
(normative)

Photometric/gravimetric hybrid procedure

F.1 General

The hybrid test procedure involves two measurement steps, which are carried out successively. The first step involves a gravimetric measurement (see F.7.1), which is followed by a photometric analysis (see F.7.2) of the delivered volumes in the microplate.

The delivered volume from either a single channel, or the sum of all channels, is measured gravimetrically, which serves as a reference for subsequent photometric measurements of individual channels.

F.2 Test equipment

F.2.1 General

The microplate absorption reader and the analytical balance shall be calibrated at regular intervals, and the test conditions shall be adhered to strictly.

F.2.2 Labware

The following labware is necessary for performing the photometric/gravimetric hybrid procedure:

— calibrated class A glassware;

— micro centrifuge tubes of 1,5 ml capacity;

— high quality, optically clear, flat-bottom 96-well, 384-well, or 1 536-well plates, non-treated, made of polystyrene (PS);

— reagent reservoirs for multi-channel pipettes of 100 ml and 300 ml capacity.

NOTE Specifications for one-mark volumetric pipettes can be found in ISO 648 and ASTM E969,[15] and for one-mark volumetric flasks in ISO 1042 and ASTM E288[17].

F.2.3 Microplate reader

The microplate absorbance reader shall be capable of measuring the absorbance in 96-well, 384-well, or 1536-well plates at 620 nm and at the wavelength corresponding to the chromophore used for the photometric measurement. Table F.1 lists chromophores, which may be used for absorbance measurements and the corresponding absorbance wavelengths for the measurements.

Table F.1 — Chromophores for absorbance measurements

Chromophore or reference	4-nitrophenol	Tartrazine	Orange G	Water/blank
Wavelength	405 nm	450 nm	492 nm	620 nm

The microplate absorbance reader used for this procedure shall meet the minimum performance requirements given in Table F.2.

Table F.2 — Microplate absorbance reader minimum performance requirements

Accuracy 0 to 2 AU [a]	< ±(1 % + 10 mAU)
Accuracy 2 to 3 AU	< ±2,5 %
Wavelength Accuracy	≤ ±1,5 nm
[a] Absorbance unit.	

F.2.4 Microplate shaker

An orbital motion microplate shaker should be used, with the performance characteristics given in Table F.3.

Table F.3 — Performance characteristics for the orbital motion microplate shaker

Orbit	0,9 mm to 1,1 mm
Speed	1 600 r/min to 1 800 r/min
Timer range	20 s to 2 min

F.2.5 Balance, thermometer, hygrometer, barometer, and timer

A precision analytical balance with the minimum performance requirements given in Table 2 for dry weights and Table 3 for weighing liquids shall be used. The minimum performance requirements for the thermometers, hygrometer, barometer, and timer are given in Table 5. The precision balance shall be verified before measurements with suitable weights.

NOTE ASTM Class 2 weights (according to ASTM E617[21]) or OIML E2 weights (according to OIML R 111-1[11]) are suitable for the verification of the precision balance.

F.3 Reagents

F.3.1 General requirements

The test liquids are prepared from the respective chromophore and distilled water, sodium hydroxide, or phosphate buffer according to the procedures in F.3.3.2, F.3.3.3 and F.3.3.4. Co-solvents or surfactants may be used to better reflect specific applications (e.g. dimethylsulfoxide (DMSO)). The influence of solvents other than pure water, co-solvents, or other additives on the liquid properties (density, surface tension, viscosity) shall be considered when converting mass to volume. Test liquids shall be free of any solid particles and may be passed through a 0,2 µm filter after preparation.

F.3.2 Chemicals used

All components used in the preparation of reagent solutions shall be of at least 99 % analytical purity unless otherwise stated. Chemicals used in this procedure and their corresponding chemical abstracts service (CAS) registration numbers are listed in Table F.4.

Table F.4 — Chemicals used in the photometric/gravimetric hybrid method

Name	Formula	CAS Number
disodium hydrogen phosphate dihydrate	$Na_2HPO_4 \cdot 2H_2O$	10028–24–7
4-nitrophenol	$C_6H_5NO_3$	100–02–7
Orange G	$C_{16}H_{10}N_2Na_2O_7S_2$	1936–15–8
sodium hydroxide	NaOH	1310–73–2
Tartrazine	$C_{16}H_9N_4Na_3O_9S_2$	1934–21–0
water	H_2O	7732–18–5

F.3.3 Test liquids

F.3.3.1 General

The concentration of the chromophore in the test liquid should be adjusted so that the absorbance is measured in the optimal dynamic range of the microplate reader (usually between 0,1 AU and 2,0 AU). Amounts of chromophores to be weighed may vary up to ±5 % from the target value.

F.3.3.2 to F.3.3.4 provide examples of recommended test liquids based on Tartrazine, 4-nitrophenol, and Orange G for measuring different test volumes in 96-well, 384-well, and 1 536-well microplates. The shelf life of these test liquids can be up to 6 months when stored in a tightly capped container at 20 °C and protected from direct light. The absorbance properties of test liquids, which have been stored for any period of time shall be verified prior to use.

Keep containers and reservoirs filled with test liquids covered to prevent evaporation; only uncover liquid reservoirs during the aspiration cycle when using piston-operated ALHS.

F.3.3.2 Tartrazine test liquids

Tartrazine has a molar mass of 534,3 g/mol. Nominal concentrations should be 96 µg/ml for measurements in 96-well plates and 24 µg/ml for measurements in 384-well plates. Pure deionized water is used as a solvent and for the pre-fill volume. Table F.5 gives values for common test volumes with Tartrazine test liquids. Other volumes may be tested by adjusting the values from Table F.5 accordingly.

Table F.5 — Test volumes with Tartrazine test liquids

	96-well microplates with 125 µl final volume/well			384-well microplates with 50 µl final volume/well		
Test volume	**Prefill volume**	**Tartrazine concentration**		**Prefill volume**	**Tartrazine concentration**	
µl	µl/well	mg/ml	mM	µl/well	mg/ml	mM
100	25	0,12	0,225	—	—	—
50	75	0,24	0,449	—	0,024	0,045
30	95	0,4	0,749	20	0,04	0,075
10	115	1,2	2,25	40	0,12	0,225
5	120	2,4	4,49	45	0,24	0,449
1	124	12	22,5	49	1,2	2,25

F.3.3.3 4-Nitrophenol test liquid

The molar mass of 4-nitrophenol is 139,1 g/mol. When using 4-nitrophenol as chromophore, 0,1 M NaOH is used as solvent and diluent to generate the optimal pH value of pH > 9,2 for the photometric measurement. The nominal concentration should be 16,7 µg/ml (0,12 mM) for measurements in 96-well, 384-well, and 1536-well plates. The solvent for the pre-fill volume is 0,1 M NaOH. Table F.6 gives values for common test volumes with 4-nitrophenol test liquid. Other volumes may be tested by adjusting the values from Table F.6 accordingly.

Table F.6 — Test volumes with 4-nitrophenol test liquid

	96-well microplates with 200 µl final volume/well			384-well microplates with 50 µl final volume/well			1 536-well microplates with 8 µl final volume/well		
Test volume	Prefill volume	4-nitrophenol concentration		Prefill volume	4-nitrophenol concentration		Prefill volume	4-nitrophenol concentration	
µl	µl/well	mg/ml	mM	µl/well	mg/ml	mM	µl/well	mg/ml	mM
100	100	0,033 4	0,24	—	—	—	—	—	—
50	150	0,066 8	0,48	—	0,016 7	0,12	—	—	—
20	180	0,167	1,20	30	0,041 7	0,30	—	—	—
10	190	0,334	2,40	40	0,083 5	0,60	—	—	—
5	195	0,668	4,80	45	0,167	1,20	3	0,026 7	0,192
2	198	1,669 3	12,00	48	0,417 3	3,00	6	0,066 7	0,480
1	199	3,34	24,00	49	0,835	6,00	7	0,133 5	0,960
0,5	199,5	6,677 3	48,00	49,5	1,669 3	12,00	7,5	0,267 1	1,920

F.3.3.4 Orange G test liquids

Orange G has a molar mass of 452,36 g/mol. Nominal concentrations should be 55 µg/ml for measurements in 96-well plates and 29,3 µg/ml for measurements in 384-well plates. When using Orange G as chromophore, a buffer solution of 17,8 g/l disodium hydrogen phosphate dihydrate and 1 ml/l of 1 M sodium hydroxide solution should be used as a solvent and diluent to generate the optimal pH value of pH = 7 for the photometric measurement. The pH value shall be checked and recorded before each measurement. Pure deionized water is used as a solvent for the pre-fill volume. Table F.7 gives values for common test volumes with Orange G test liquids. Other volumes may be tested by adjusting the values from Table F.7 accordingly.

Table F.7 — Test volumes with Orange G test liquids

	96-well microplates with 200 µl final volume/well			384-well microplates with 75 µl final volume/well		
Test volume	Prefill volume	Orange G concentration		Prefill volume	Orange G concentration	
µl	µl/well	mg/ml	mM	µl/well	mg/ml	mM
100	100	0,11	0,243	-	-	-
50	150	0,22	0,486	25	0,044	0,097 2
30	170	0,367	0,81	45	0,073	0,162
10	190	1,1	2,43	65	0,22	0,486
5	195	2,2	4,86	70	0,44	0,973
1	199	11	24,3	74	2,2	4,86

F.4 Test environment

All measurements shall be performed under the following environmental conditions:

— The test environment shall be draft free, and the balance shall be placed on a vibration-free support.

— Ambient temperature: (20 ± 3) °C or (27 ± 3) °C with a maximum variation of ± 0,5 °C during the test.

— Ambient relative humidity: between 45 % and 80 %.

F.5 Evaporation

During method validation, it shall be determined whether measurement results need to be corrected for evaporation. If evaporation needs to be taken into account, the effect shall be determined experimentally and compensated mathematically. The error due to evaporation shall also be reflected in the determination of the measuring system uncertainty, if reported.

The error due to evaporation may be determined with a pre-filled, uncovered microplate, which is subjected to the same timing and movements (inside and outside of the plate reader) as a microplate with test liquid during the actual volume measurement.

F.6 System linearity

The combined linearity of the microplate reader and chromophore solutions shall be determined. Within the absorbance range from 0,1 AU to 2,0 AU, the measured absorbance should be proportional to the test volume. The optimum chromophore concentrations given in Tables F.5, F.6 and F.7 can thus be used to cover a volume range of 30 % to 150 % of the nominal test volume.

F.7 Procedure

F.7.1 Gravimetric measurement step

F.7.1.1 General

At least five replicate measurements per test volume shall be taken.

F.7.1.2 Whole plate approach

This test shall be performed for the test volumes of interest, not exceeding the maximum usable well volume of 200 µl in 96-well microplates, 75 µl in 384-well microplates, and 8 µl in 1 536-well microplates.

a) Tare the empty balance;

b) Weigh and record the tare mass (m_E) of the microplate with lid:

   1) For test volumes >25 µl, weigh the empty microplate with lid;

   2) For test volumes ≤25 µl, weigh the microplate with pre-filled solvent volumes and its lid;

c) Deliver the test volume into the empty or pre-filled test plate and immediately place the lid on the test plate. When using piston-operated ALHS, preferably use reverse pipetting mode;

d) Start the timer;

e) Weigh the test plate immediately to avoid evaporation;

f) The time interval between delivering the test liquid and weighing the test plate shall not exceed 15 s, otherwise the error due to evaporation shall be corrected;

g) Record the gross mass of the test plate in grams;

h) Record the time between test liquid delivery and mass measurement;

i) Record the tare mass of the plate with its lid before delivering the second replicate into the plate;

j) Repeat steps c) to i) until the desired number (n) of replicate test liquid deliveries have been measured;

k) Calculate the mean mass of delivered test liquid per well ($\bar{m}$) for the test plate according to Formula (F.1):

$$\bar{m} = \frac{m_G - m_E}{N} \quad \text{(F.1)}$$

where

$\bar{m}$ is the average delivered test liquid mass per well;

m_G is the gross mass of the plate with the lid;

m_E is the mass of the empty plate with the lid;

N is the number of wells, which have been filled with test liquid, e.g. N = 8 for a liquid delivery with an 8-channel device.

l) Calculate the mean volume delivered per channel ($\bar{V}$) in microlitres for the test plate according to Formula (F.2):

$$\bar{V} = \bar{m} \times Z \times 1\,000 \quad \text{(F.2)}$$

where

$\bar{V}$ is the average delivered volume of test liquid per channel;

Z is the density conversion factor for water at the previously measured water temperature, see Annex A.

F.7.1.3 Single channel approach

The single channel gravimetric measurement is usually performed on a corner channel for ease of handling. This channel is considered the reference channel for all further measurements in this approach.

The micro centrifuge tubes shall be handled only with gloves to avoid the transfer and weighing of skin oils, as well as body warmth, which can increase the rate of evaporation.

a) Place a micro centrifuge tube on the balance and tare the balance.

b) If using disposable tips: pre-wet the reference channel five times.

c) Deliver the test volume into the micro centrifuge tube, which has been tared in step a), and close the tube's lid immediately after test liquid delivery to avoid evaporation;

d) Place the micro centrifuge tube on the balance. This first result is not recorded.

e) For piston-operated ALHS, use the same tip, and aspirate the test volume again by immersing the tip only as much as necessary below the surface of the test liquid in the reservoir (2 mm to 3 mm).

f) Deliver the test liquid to the micro centrifuge tube, touching the tip against the inside wall, and close the tube immediately.

g) Record the mass m_i in mg.

h) Repeat steps e) to g) at least four times to get n readings.

i) The values obtained by balance readings are in milligrams. Convert each mass obtained in step g) to volume V_i in µl by applying the correct Z correction factor as described in Annex A according to Formula (F.3):

$$V_i = m_i \times Z \times 1\,000 \tag{F.3}$$

where

V_i is the delivered volume of test liquid from the reference channel;

m_i is the mass of the test liquid volume delivered by the reference channel.

j) Calculate the mean volume $\bar{V}$ at the test temperature as shown in Formula (F.4):

$$\bar{V} = \frac{1}{n} \times \sum_{i=1}^{n} V_i \tag{F.4}$$

where

$\bar{V}$ is the mean test volume of n delivered volumes of test liquid;

V_i is the i-th delivered volume.

F.7.2 Photometric measurement step

F.7.2.1 General

After gravimetric testing over the entire volume range, this test should be performed at 10 % of the nominal volume of variable volume instruments. At least five replicate measurements shall be performed.

A reference absorbance measurement is performed at 620 nm for each microplate. The absorbance readings from the reference wavelength are subtracted from the readings of the absorbed wavelength of the test liquid (e.g. 450 nm for Tartrazine, 405 nm for 4-nitrophenol, and 492 nm for Orange G) to eliminate the background absorbance and to compensate for reader and microplate tolerances.

F.7.2.2 Conversion plate

A conversion plate needs to be created to correlate the absorbance as measured by the plate reader photometer to the delivered test liquid volume in microlitres. The conversion plate needs to be created using the same channel or channels used for the gravimetric measurement in F.7.1.

a) Prefill volume (if needed):

1) If working with disposable tips: prewet tips at least five times with the pre-fill volume of the appropriate pre-fill solvent.

2) Deliver pre-fill solvent into at least 8 wells in a plate. Make sure to keep the microplate covered to prevent evaporation.

b) If working with disposable tips: Load new tips to the pipetting head and prewet tips at least five times with the test liquid appropriate for the test volume.

c) Deliver the appropriate test liquid into the wells of the microplate.

1) Deliver test liquid to the entire microplate in the case of the whole plate approach.

2) Using the reference channel in the case of the single channel approach, deliver 8 replicates of test liquid into the wells containing the pre-fill volume of solvent [see step a)2)].

d) Place the microplate on the shaker, without lid. It is recommended to shake at 1 600 r/min to 1 800 r/min for 2 min. Confirm that no air bubbles are present in the wells.

e) Read the absorbances A_i corresponding to the used chromophore in the test liquid with the plate reader.

f) Subtract the reference readings at 620 nm from the absorbance readings. This step is sometimes performed automatically by the plate reader.

g) Calculate the mean absorbance according to Formula (F.5), where n = 8 in the case of the single channel approach, or n = 96 for a 96-well plate, n = 384 for a 384-well plate, or n = 1536 for a 1 536-well plate in the case of the whole plate approach:

$$\overline{A} = \frac{1}{n} \times \sum_{i=1}^{n} A_i \tag{F.5}$$

where

$\overline{A}$ is the mean absorbance,

n is the number of wells filled with test liquid and read by the plate reader,

A_i is the corrected absorbance per well, from step f).

h) Calculate the conversion factor C using the mean volume $\overline{V}$ from either the single channel or the whole plate gravimetric measurement according to Formula (F.6):

$$C = \frac{\overline{V}}{\overline{A}} \tag{F.6}$$

where C is the conversion factor between absorbance and volume of test liquid.

F.7.2.3 Test plate

Deliver the test liquid into all microplates to be measured successively with the ALHS under test:

a) Prefill volume (if needed):

1) If working with disposable tips: pre-wet tips five times with the pre-fill volume of the appropriate pre-fill solvent.

2) Deliver the pre-fill volume into each well of the microplate. Make sure to keep the microplate covered to prevent evaporation.

b) If working with disposable tips: Load new tips to the pipetting head and pre-wet the tips five times with the test volume of the test liquid.

c) Deliver the test volume of test liquid into each well of the microplate, and immediately cover the wells, which have been filled.

d) Place the microplate on the shaker, without a lid. It is recommended to shake at 1 600 r/min to 1 800 r/min for 2 min. Confirm that no air bubbles are present in the wells.

e) Read the absorbance of each well.

f) Subtract reference readings at 620 nm from the absorbance readings to obtain the corrected absorbance (A_i) of each well (i = 1 to 96 for 96-well plates, i = 1 to 384 for 384-well plates, and i = 1 to 1 536 for 1 536-well plates). This step is sometimes performed automatically by the plate reader.

F.8 Calculation of delivered volume

Using the conversion factor C, convert the absorbance readings obtained in F.7.2.3 (step f) to the volume of test liquid according to Formula (F.7):

$$V_i = C \times A_i \tag{F.7}$$

where V_i is the volume of test liquid delivered into an individual well i (i = 1 to 96 for 96-well plates, i = 1 to 384 for 384-well plates, and i = 1 to 1 536 for 1 536-well plates).

F.9 Traceability

The measurement results of the photometric/gravimetric hybrid method are traceable to the International System of Units (SI) through a calibrated balance, calibrated volumetric flasks, calibrated pipettes, calibrated test equipment for monitoring the environmental conditions, and properly accounting for the density and air buoyancy of the test liquid.

NOTE 1 Annex A describes the mass to volume conversion for water.

Annex G
(normative)

Optical image analysis of droplets

G.1 General

This procedure is only applicable to liquid volumes provided as free flying liquid droplets, for example, generated by non-contact nanodispensing technologies or inkjet printheads. The recommended volume range for this method is 100 pl to 1 µl per droplet. Measurement of larger volumes is possible by consecutive measurements of a series of individual droplets.

This procedure is based on imaging liquid droplets in flight by a suitable digital optical setup, such as a calibrated high-speed camera or a stroboscopic imaging system. From the acquired grey-scale images of the droplet, the size and shape of the outline of the droplet are determined by conversion to a black and white image by the image processing algorithm described in G.6.4. The three-dimensional volume of the droplet is then reconstructed by rotating the two-dimensional projection extracted from the image around the axis defined by the flight path of the droplet, assuming rotational symmetry of the droplet shape.

G.2 Test Equipment

G.2.1 Requirements

A digital optical imaging system shall acquire one or several grey-scale images of a free-flying droplet generated by the volumetric delivery device under test (DUT). The imaging system shall consist of a digital detector, an optical lens system, and an illumination source. Table G.1 gives the minimum performance requirements for the components of the optical imaging system.

Table G.1 — Minimum requirements for the optical imaging system

Equipment	Minimum requirement
Image detector resolution	1 mega pixel
Optical lens system magnification	V_{drop} > 0,5 µl: 0,5 × magnification V_{drop} > 0,1 µl: 0,7 × magnification V_{drop} > 0,01 µl: 1 × magnification
Optical lens system distortion	Smaller than 2 % for the entire lens system
Depth of focus	500 µm
Light source brightness	1000 lumen
Maximum exposure time for image capture	50 µs
Minimum frame rate of high-speed video camera	10 000 frames per second (fps)

The required optical magnification depends on the size of the droplets and can vary for different volume ranges. The detector resolution and the magnification shall achieve a magnification factor α of smaller or equal to 5, such that the two-dimensional projection of the droplet visible in the processed black and white image shall consist of at least 200 pixels.

G.2.2 Image contrast

The contrast of the recorded images shall exhibit a contrast-to-noise ratio (R_{CN}), also referred to as Rose criterion, of larger or equal than 9 for a clear distinction between the object (droplet) and background

in the image (see Figure G.1 for examples). The R_{CN} is an object measure based on the droplet grey-scale value, the background grey-scale value and the background noise. It is calculated by Formula (G.1), in which the noise of the background can be approximated by the standard deviation of the greyscale values of the background pixels.

NOTE Additional information about image contrast can be found in References [22] and [23].

$$R_{CN} = \frac{\left|\bar{\chi}_{drop} - \bar{\chi}_{bg}\right|}{\sigma_{bg}} \quad \text{(G.1)}$$

where

R_{CN} is the contrast-to-noise ratio;

$\bar{\chi}_{drop}$ is the mean greyscale value of the droplet, in arbitrary units of the detector;

$\bar{\chi}_{bg}$ is the mean greyscale value of the background, in arbitrary units of the detector;

σ_{bg} is the noise of the background, in arbitrary units of the detector.

a) Droplet image with acceptable image quality (R_{CN} = 15,9) **b) Droplet image with insufficient image quality (R_{CN} = 8,7)**

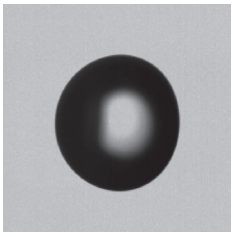
Figure G.1 — Different image quality in droplet images

G.2.3 Image blur

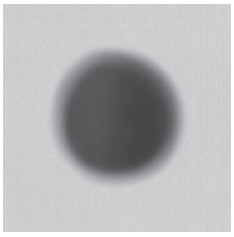
A sharp image of the droplet is required to achieve precise measurements. The image of the droplet can appear blurred if the image of the droplet is recorded out of focus of the optical system or the exposure time is too long. Therefore, the depth of focus of the optical system should be sufficiently large to reduce the blur due to defocusing and the exposure time shall be sufficiently short to reduce the motion blur. High speed camera systems with more than 10 000 frames per second (fps) or stroboscopic imaging systems with less than 50 µs exposure time are typically suitable to achieve sufficiently sharp images. The combined blur of the droplet in the acquired grey scale image resulting either from optical defocusing and/or motion blur should be as small as possible.

The sharpness of the image shall be determined in terms of the between-class-variance (BCV) of the droplet image compared to the background image. The BCV is an object measure based on the variation of grey scale values at the transition from the background to the object. The normalized BCV value (φ) shall be larger than 0,93 to ensure acceptable image quality (see Figure G.2 for examples).

NOTE Additional information on image blur and between-class-variance can be found in References, [23], [24], [25], [26] and [27].



a) Typical droplet image exhibiting sufficiently small blur (φ = 0,961)



b) Droplet image captured out of focus with an unacceptable blur (φ = 0,925)

Figure G.2 — Droplet image quality

The BCV (σ_B^2) shall be calculated for each single droplet image as follows:

If the image is represented by R grey levels [0, 1, 2, ..., R-1] and the two classes correspond to C1 = [0, 1, ... d] comprising grey levels of all pixels belonging to the background, and C2 = [d+1, d+2, ..., R-1] comprising grey levels of all pixels belonging to the droplet, then the between-class-variance, σ_B^2, is given by Formula (G.2):

$$\sigma_B^2 = p_1 \times p_2 \times [\mu_1 - \mu_2]^2 \tag{G.2}$$

$$p_1 = \sum_{i=0}^{d} p(i) \tag{G.3}$$

$$p_2 = 1 - p_1 \tag{G.4}$$

where

σ_B^2 is the between-class-variance;

p_1 is the class 1 probability with the mean grey level of μ_1;

p_2 is the class 2 probability with the mean grey level of μ_2;

μ_1 is the mean grey level of class 1;

μ_2 is the mean grey level of class 2.

The total mean corresponding to the entire data set (μ_T) is given by Formulae (G.5) and (G.6):

$$\mu_T = \mu_1 + \mu_2 \tag{G.5}$$

$$\mu_T = \sum_{i=0}^{R-1} i \times p(i) \tag{G.6}$$

$$\mu_1 = \sum_{i=0}^{d} \frac{i \times p(i)}{p_1} \tag{G.7}$$

$$\mu_2 = \sum_{i=d+1}^{R-1} \frac{i \times p(i)}{p_2} \tag{G.8}$$

where

μ_T is the total mean grey level of the entire data set;

R is the total number of grey levels in the image;

i is the index for the individual grey levels;

$p(i)$ is the probability of finding a pixel with the i-th grey level in the image (it can be estimated by dividing the total number of pixels having the i-th grey level by the total number of pixels in the image).

The normalized BCV (φ) is normalized by making it invariant to affine grey level transformations as shown in Formulae (G.9) and (G.10):

$$\varphi = \frac{\sigma_B^2}{\sigma_T^2} \tag{G.9}$$

$$\sigma_T^2 = \sum_{i=0}^{R-1} (i - \mu_T)^2 \times p(i) \tag{G.10}$$

where

φ is the normalized between-class-variance;

σ_T^2 is the total variance.

G.3 Measurement setup

In order to achieve short exposure times as well as high contrast, a strong illumination source (e.g. a high-power LED) shall be positioned opposite to the camera at a sufficiently large distance to illuminate the droplet. This setup will render the droplet as a clearly distinguishable dark shape in front of the background as shown in Figures G.1 and G.2. The illumination source should not radiate excessive heat towards the droplet, which would increase evaporation and loss of volume from the droplet.

An optical system with a large depth of focus (DOF) should be used to reduce measurement errors caused by droplets not located in the focal plane of the imaging system.

The quality criteria required for the recorded images in terms of R_{CN}, BCV, and the total number of pixels per droplet stated above shall be achieved by the used measurement setup to produce precise and valid results.

G.4 Test Liquids

Test liquids suitable to be used with this method may be opaque or optically clear and shall produce a clear contrast to the background image. Test liquids should have a low vapour pressure to reduce evaporation of liquid from the droplet during the time between its generation and imaging.

NOTE Mixtures of water and glycerol or dimethylsulfoxide (DMSO) exhibit a lower vapour pressure than pure water.

G.5 Environmental conditions

Constant ambient light conditions should be established (e.g. use of a flicker-free ambient light source).

Ambient temperature should be (20 ± 3) °C or (27 ± 3) °C with a maximum variation of ±0,5 °C during the test. Ambient relative humidity should be between 50 % and 80 %.

Ambient temperature, relative humidity, and barometric pressure should be reported.

All test equipment and test liquids shall be thermally equilibrated to the test room temperature.

G.6 Procedure

G.6.1 Installation of the DUT

The DUT shall be installed in front of the imaging system in such a manner that the flight path of the ejected droplets is parallel to the gravitational force and is located within the focal plane of the imaging system.

NOTE Once the droplet appears sharp, regardless of the position where it is captured within the image frame, the flight path and focal plane are properly aligned.

The nozzle orifice of the DUT shall be positioned at such a height above the optical axis of the imaging system that the droplet can be imaged in a fully detached state from the nozzle. A minimum distance of three times the droplet diameter between nozzle orifice and imaging position is recommended.

G.6.2 Calibration of the camera and optical system

The optical magnification system strongly influences the result of the measurement. The optical magnification factor, α, is the dominant source of error. Therefore, the optical system should be calibrated carefully. The optical magnification factor, α, of the imaging system is given by the ratio of the size of an imaged object (O_S) and the number of pixels the image of the object interrogates on the camera chip (n_p), see Formula (G.11). It is expressed in units of length [µm] and is therefore dependent on the properties of the camera chip as well as on the magnifying optical lens system.

$$\alpha = \frac{O_S}{n_p} \tag{G.11}$$

where

α is the optical magnification factor, in µm;

O_S is the real size of the imaged object, in µm;

n_p is the number of pixels the imaged object interrogates on the camera chip.

For a given setup, the magnification factor can be determined by the following procedure:

a) A suitable optical resolution test target is positioned in the focal plane in front of the detector in such a manner that the test target is parallel to the detector chip surface and the horizontal and vertical features on the test target are aligned parallel with the rows and columns of the pixels on the detector chip.

NOTE 1 A 1951 USAF resolution test target according to Reference [28] is an example of a suitable optical resolution target for this procedure.

NOTE 2 Proper alignment has been achieved when the features on the test target appear sharp throughout the image.

b) Use appropriate elements or groups of lines on the optical resolution test target for the calibration. The elements on the test target used for calibration should correspond to the size of the droplets to be measured.

NOTE 3 When using the 1951 USAF as the resolution test target, the lines in group 3, element 6, which have a combined total width of 174,85 µm can be used for the calibration.

c) Relate the known width of the lines, and the distance between the lines, respectively, on the test target to the corresponding number of pixels in the recorded image. Evaluate horizontal and vertical lines separately to determine the vertical and horizontal magnification factors α_x and α_y. Calculate the magnification factor α as the average of the vertical and horizontal magnification factors according to Formula (G.12):

$$\alpha = \frac{\alpha_x + \alpha_y}{2} \tag{G.12}$$

where

α is the magnification factor, in µm;

α_x is the vertical magnification factor, in µm;

α_y is the horizontal magnification factor, in µm.

G.6.3 Image acquisition

G.6.3.1 General

The calibrated optical system (G.6.2) shall be used to acquire calibrated images of the test liquid droplets in flight from which the shape, size, and circularity of the two-dimensional projection of the droplet can be extracted.

The delay time between ejection of the droplet from the DUT and capturing its image within the region of interest (ROI) and with an acceptable circularity shall be determined experimentally, so that all of the ejected liquid volume is captured within the image. Satellite droplets that can follow the main droplet under certain circumstances should not remain undetected.

The method can also be applied if more than one droplet is visible in the image, for example, when a satellite droplet is following the main droplet, or if short liquid jets are imaged, which decay into smaller droplets. Such liquid delivery situations should be avoided as the measurement error in these cases can increase significantly. Therefore, in case more than a single droplet is recorded, the number of droplets shall be reported in addition to the measurement result.

G.6.3.2 Still image acquisition

Two images should be recorded per measurement:

a) Record a grey scale image of the ROI shortly before the delivery of the droplet is triggered (referred to as “reference image” in this standard).

b) Record a grey scale image of the test liquid droplet in flight when it is inside the ROI (referred to as “droplet image” in this standard).

G.6.3.3 High-speed video imaging

If a high-speed video capture system is used, test liquid droplet images at different positions within the ROI, as well as reference images for the corresponding positions (right before the droplet reaches the respective position) should be extracted from the video recording.

Several images of the same droplet should be obtained at different positions in flight. These images, and the corresponding reference images, should be evaluated as if they were captured as still images (see G.6.3.2). The calculated values for the test liquid droplet volumes should be averaged to yield a more accurate volume result and estimation of the measurement error.

G.6.4 Image processing

The applied image processing algorithm influences the accuracy of this method, as it determines the outline and area of the droplet in the black and white image that is used for the volume calculation. Acquired images shall be processed by exactly following the steps below to obtain the black and white image, which is needed for the volume calculation (referred to as "processed image" in this standard).

The following steps of image processing shall be performed:

a) Apply a Gaussian blur filter to the reference and droplet images with a matrix size of at least 3 × 3. For more information on Gaussian blur filters, see Reference [29].

b) Subtract the grey scale value of each pixel of the processed reference image from the corresponding pixel of the processed droplet image.

c) Convert the difference in grey scale values to a black and white image using the Otsu algorithm (for more information on the Otsu algorithm, see Reference [27]). By convention, pixels belonging to the droplet image shall be coloured white, and pixels belonging to the surrounding background shall be coloured black.

NOTE 1 The Otsu-algorithm has been selected for several reasons: this algorithm is available free of any licence and its implementation is possible without any ambiguity as well as at low computational cost. Furthermore, it has been proven by experimental benchmarks in the range from 5 nl to 45 nl that the Otsu algorithm produces consistent results when compared to the gravimetric regression procedure described in Annex E.

d) Determine the number, M, of isolated white objects in the image. M should equal 1, otherwise the number of imaged objects shall be reported together with the measurement results.

e) Determine the contour of each white object and convert all pixels within this contour to white colour in order to remove glare spots, if any.

NOTE 2 Depending on the imaging system, a glare spot can appear inside the projected droplet image.

f) Determine the circularity, K, of each white object as described in Formula (G.13):

$$K = \frac{4\pi \times A_W}{P_W^2} \tag{G.13}$$

where

K is the circularity of the white object;

A_W is the area of the white object;

P_W is the perimeter of the white object.

If K is less than 0,88 for any of the white objects, the circularity value shall be reported together with the final measurement result as the estimated error of the measurement can be incorrect. For more information about circularity determination, see Reference [30].

g) Count the number of white pixels that make up each object. If any of the objects contains less than 200 pixels, the data shall be rejected and the evaluation shall be stopped with no result.

G.7 Calculation of delivered volume

G.7.1 General

In order to obtain precise measurement results, the test liquid droplet images shall be sufficiently round [i.e. K is larger or equal to 0,88 according to step f) in G.6.4] and shall consist of at least 200 pixels

[see step G.6.4 g)]. If these criteria are not met by any object in the processed image, the calculation of the volume as described below shall not be carried out, because the result could have an unacceptably high error.

In principle, the calculation of the delivered test liquid volume could also be executed when the criteria for circularity and resolution are not met. However, the results shall not be considered as valid and shall not be reported with reference to this standard in such cases.

G.7.2 Delivered volume

Using the number of white pixels, which have been extracted from the processed black and white image of the droplets and the magnification factor α, the total volume of test liquid of all detected objects measured at temperature, t, can be calculated according to Formula (G.14):

$$V(t) = \sum_{i=1}^{M} \left\{ \sum_{j=1}^{N_i} \left[\pi \times \left(\frac{n_{i,j}}{2} \right)^2 \times \alpha^3 \right] \right\} + \Delta V_{\text{evap}} + \Delta V_{\text{rot}} \tag{G.14}$$

where

$V(t)$ is the volume of test liquid of all detected objects in the ROI at temperature t, in µm^3;

M is the total number of objects in the processed black and white image;

N_i is the number of rows of white pixels forming the droplet image i;

$n_{i,j}$ is the number of white pixels in row j of the droplet image i;

ΔV_{evap} is the volume of test liquid that has evaporated before the image was taken, in µm^3;

ΔV_{rot} is the volume difference between the real droplet volume and the volume of a body reconstructed from a stack of thin spherical discs, which is generating the identical two-dimensional image as the real droplet, in µm^3.

NOTE 1 The volume ΔV_{evap} evaporating from a droplet during a certain time is a complex function of many parameters. It can be considered as a very small, negligible quantity in the context of this procedure, as the time between droplet ejection and imaging is usually very short (approx. 1 ms). The effectively evaporated volume is therefore approximated by $\Delta V_{\text{evap}} \approx 0$.

NOTE 2 The volume ΔV_{rot} represents the systematic error stemming from the assumed rotational symmetry and includes also the digitalization error due to conversion of the real image into a digital, pixel-based image. For the equipment and procedure described here, this error is negligible compared to other systematic and random errors.

G.7.3 Systematic error

The systematic error is influenced by:

a) imaging system (primarily by the error in the magnification factor α, and the total number of pixels per droplet);

b) image quality in terms of R_{CN} and BCV;

c) type and parameters of the image processing algorithm, if another algorithm than the Otsu-algorithm is used;

d) shape of droplets - if spherical symmetry of the droplet (cylindrical symmetry of the droplet slices) is not given;

e) evaporation from the droplet;

f) environmental effects that affect the image calibration;

g) satellite droplets, which are not captured in the image.

G.7.4 Random error

The random error is influenced by:

a) image artefacts (e.g. dust, glare spots) that can lead to artefacts in the black and white image;

b) position of the droplet within the image that can influence the number of interrogated pixels on the detector and associated effects of aliasing;

c) sharpness of the droplet image, which can change depending on the droplet position;

d) oscillations of the droplet that deform its shape and symmetry;

e) variations in illumination and vibrations.

G.8 Traceability

Traceability of the measurement results to the International System of Units (SI) is achieved through the calibrated droplet image for which the length quantity is used as scale. From the calibrated image, the volume of the droplet is calculated using Formula (G.14), giving the total volume as sum of individual cylindrical discs. The height of each disc is one pixel. This height can be converted into SI units by multiplication with the calibration factor α in the SI-unit of meter. Similarly, the diameter of the cylinder, measured by the number of pixels in the corresponding row, can be converted into SI units by multiplication with the calibration factor α. The total volume of each cylinder, as well as the droplet volume being the sum of all cylinders, is thus traceable to the SI by Formula (G.14). Thus, the measurement result is traceable to the SI, provided that the optical magnification factor α was properly determined in SI units.

Annex H
(normative)

Fluorescence procedure

H.1 General

This procedure is based on fluorescence measurements of dimethylsulfoxide (DMSO)-based fluorescein test liquids at 521 nm and is suitable for test volumes of smaller or equal to 15 nl in 384-well or 1 536-well microplates.

H.2 Test equipment

H.2.1 Fluorescence plate reader

The fluorescence microplate reader shall be capable of measuring the fluorescence of fluorescein above pH 8, with an excitation wavelength (λ_{ex}) of 494 nm and measuring emission (λ_{em}) at 521 nm. The unit may use filters or monochromators for both excitation and emission wavelength selection. The system shall register a signal of at least 10 times the background noise at the lowest concentration of fluorescein used.

System drift shall be compensated by running a 96-well, solid-phase, inorganic fluorescence standard before and after readings of individual plates. Large standard deviations in signals not associated with drift are unacceptable.

NOTE Since the total error squared of the entire analysis is equal to the square root of the summation of the squares of the individual errors, the error of the analysis will always be greater than the error of any individual source of error. This means that if the error of the reader is 10 %, the error of delivered volume will always be greater than 10 %.

H.2.2 Fluorescence reference plate

The excitation and emission wavelengths of the reference plate should closely match those of fluorescein, and the fluorescence standard should be suitably stable.

H.2.3 Bulk liquid handler

A calibrated system (automated or manual) capable of transferring 30 µl of 10 mM NaOH solution to all wells of a 384-well plate shall be used.

The error in the transfer of sodium hydroxide solution shall be included in the calculation of the cumulative error and MSU of this procedure since it affects the final assay concentration of fluorescein.

H.2.4 Microplates

The ANSI/SLAS 384-well or 1 536-well microplates shall be designed for fluorescence analysis on the fluorescence plate reader used. The microplates shall minimize crosstalk between the wells.

H.2.5 Plate shaker

Use a plate shaker to ensure complete mixing of reagents. The small volumes used in this technique will mix relatively rapidly at room temperature due to differences in the surface tension of the reagents.

H.2.6 Plate centrifuge

The centrifuge shall be capable of centrifuging microplates at 150 g for 1 min to remove air bubbles from the bottom of the wells.

H.2.7 Other test equipment

The balance and test equipment used in this procedure shall meet the minimum performance requirements given in Table 2 and Table 5, respectively.

H.2.8 Glassware

Calibrated class A glassware one-mark volumetric flasks shall be used:

— One 1 l volumetric flask;

— One 100 ml volumetric flask;

— Four 10 ml volumetric flasks (eight if performing optional fluorescein verification test);

— Funnels to fill volumetric flasks.

NOTE Specifications for one-mark volumetric flasks can be found in ISO 1042 and ASTM E288[17].

H.2.9 Pipettes

Calibrated pipettes shall be used for the following volume ranges:

— One pipette capable of delivering 3 ml.

— Pipettes capable of transferring 10 µl to 360 µl.

— Optional: multichannel hand-held pipettes capable of aspirating and transferring 30 µl and 50 µl.

NOTE A multichannel pipette will allow faster in-plate serial dilutions for the development of the standard curve.

H.2.10 Other materials

— Paraffin film.

H.3 Test liquids

H.3.1 General

All components used in the preparation of reagent solutions shall be of at least 99 % analytical purity unless otherwise stated. The chemicals used in this procedure are described in Table H.1.

Table H.1 — Chemicals for the fluorescent procedure

Name	Formula	CAS number [a]
Dimethyl sulfoxide (DMSO), anhydrous, ≥ 99 %	C_2H_6SO	67–68–5
Fluorescein, disodium salt, < 10 % water content	$C_{20}H_{10}Na_2O_5$	518–47–8
Sodium hydroxide	NaOH	1310–73–2
Water, distilled and deionised	H_2O	7732–18–5
[a] Chemical abstracts service number.		

H.3.2 Preparation of test liquids

H.3.2.1 General

Prepare the test liquids according to the procedures in the following subclauses.

H.3.2.2 Preparation of sodium hydroxide diluent

Pipet 1,00 ml of 10 N NaOH into a 1-l volumetric flask already containing approximately 500 ml of distilled, deionized water. Add distilled, deionized water to the volumetric flask to the fill mark, and seal the flask with paraffin film or another acceptable seal.

Mix the solution via multiple inversions of the volumetric flask. Alternatively, use a well-cleaned magnetic stir bar to mix the solution with a magnetic stirrer but be aware of recent studies on contamination by magnetic stir bars. Seal the volumetric flask from air during mixing. Small deviations in sodium hydroxide concentration will not affect the fluorescence of fluorescein, which plateaus above pH 8.

Allow the sodium hydroxide diluent to cool to room temperature prior to using it in this procedure.

H.3.2.3 Preparation of 0,5 mM fluorescein solution

Add 18,825 mg (±5 %) of freshly weighed disodium fluorescein to a 100 ml volumetric flask and fill to the mark with anhydrous DMSO from a septum-sealed bottle. Immediately stopper the flask and wrap the stopper and neck with a sheet of paraffin film to reduce exposure to atmospheric moisture.

NOTE Anhydrous DMSO is hygroscopic and rapidly absorbs water from the air.

H.3.2.4 Preparation of 0,15 mM fluorescein test liquids

Prepare four 0,15 mM fluorescein test liquids according to the following procedure if testing the ALHS performance at multiple DMSO hydration levels.

Label four 10 ml volumetric flasks as follows:

— 0,15 mM fluorescein in 100 % DMSO;

— 0,15 mM fluorescein in 90 % DMSO;

— 0,15 mM fluorescein in 80 % DMSO;

— 0,15 mM fluorescein in 70 % DMSO.

Add 3 ml of 0,5 mM fluorescein solution to each flask. Add either none, 1 ml, 2 ml, or 3 ml of distilled, deionized water to each flask as outlined in Table H.2 and then fill the flasks to the mark with 100 % anhydrous DMSO. Seal each flask and mix the contents by repeated inversions. Let test liquids cool to room temperature overnight in a dark location.

Table H.2 — Preparation of 0,15 mM fluorescein test liquids

DMSO concentration	0,5 mM fluorescein solution in anhydrous DMSO	Water (distilled and deionized)	DMSO, anhydrous
100 %	3 ml	-	7 ml
90 %	3 ml	1 ml	6 ml
80 %	3 ml	2 ml	5 ml
70 %	3 ml	3 ml	4 ml

Mixing water and DMSO is exothermic and the solutions will get hot. Care should be taken and corresponding precautions in order not to sustain burn injuries to the hands.

H.3.2.5 Preparation of 0,15 mM fluorescein test liquid in 70 % DMSO

Prepare the following 0,15 mM fluorescein test liquid if testing the ALHS at a single DMSO hydration level.

Label a 50 ml volumetric flask with a label indicating 0,15 mM fluorescein in 70 % DMSO. Add 15 ml of 0,5 mM fluorescein solution to the flask, followed by 15 ml of distilled, deionized water. Fill the flask to its calibration mark with 100 % anhydrous DMSO. Seal the flask and mix by repeated inversions. Let test liquid cool to room temperature overnight in a dark location.

H.3.2.6 Preparation of 150 nM fluorescein test liquids

Label four 10 ml volumetric flasks as follows:

— 150 nM fluorescein, 0,001 % DMSO;

— 150 nM fluorescein, 0,000 9 % DMSO;

— 150 nM fluorescein, 0,000 8 % DMSO;

— 150 nM fluorescein, 0,000 7 % DMSO.

Pipet 10 µl of each of the 0,15 mM fluorescein test liquids prepared in H.3.2.4 into the prepared 10 ml volumetric flasks. Fill to the mark with 10 mM NaOH solution. Seal and mix by repeated inversions. Store overnight in a dark location if not used immediately.

H.3.2.7 Preparation of 150 nM fluorescein test liquid in 0,000 7 % DMSO

Label one 50 ml volumetric flask with "150 nM fluorescein, 0,000 7 % DMSO." Pipet 50 µl of the 0,15 mM fluorescein test liquid prepared in H.3.2.5 into the prepared volumetric flask. Fill the flask to the mark with 10 mM NaOH solution. Seal and mix by repeated inversions. Store overnight in a dark location if not used immediately.

H.3.2.8 Test liquid stability and storage

Test liquids should be made fresh and used immediately. However, the 0,5 mM fluorescein solution can be stored in a sealed amber bottle covered with aluminium foil for several months without any detectable loss in fluorescence.

All other fluorescein test liquids should be made fresh immediately before the procedure. In case this is impractical, it shall be ensured that all test liquids have been prepared at the same time and have been stored for the same amount of time under identical storage conditions.

NOTE Solutions of sodium hydroxide slowly absorb carbon dioxide from the air. The concentration of 10 mM NaOH is high enough that the pH of analysis will remain above pH9 when the NaOH solution is stored in a sealed bottle.

The 0,5 mM fluorescein solution and sodium hydroxide solution should be stored for a maximum of one year.

Any stored solution or test liquid shall be checked for degradation of fluorescence or pH prior to use.

H.4 Environmental conditions

This procedure is designed to be performed at a temperature of 17 °C to 27 °C. The barometric pressure and relative humidity should be recorded.

NOTE Fluorescence results are based on relative fluorescence units. If there is no substantial change in barometric pressure or humidity during the volume transfer and analysis steps, these environmental parameters have little, if any effects on the results.

H.5 Procedure

H.5.1 General

The fluorescence procedure can be performed in 384-well or 1 536-well microplates. In the following, plate layouts for 384-well plates are described. When using 1 536-well plates, the plate layouts described for 384-well plates should be replicated four times, once per quadrant of the 1 536-well plate.

H.5.2 Drift and linearity test of fluorescence microplate reader

a) Allow the fluorescence plate reader to warm up for the time as specified by the manufacturer and set excitation and emission wavelengths to 494 nm and 521 nm, respectively.

b) Place the 96-well, solid-phase inorganic reference plate into plate reader. Read and record average fluorescence over the entire plate and note time. This is datum point $F_{ref,t0}$. Remove the reference plate from the reader.

c) After 10 min, reinsert the reference plate, re-read fluorescence and record with time. This is datum point $F_{ref,t10.}$ Remove the reference plate from reader.

d) Repeat step c) for 2 h. These are data points $F_{ref,tx}$, where "tx" is the time in minutes since the beginning of the experiment.

e) Plot fluorescence versus time. ($F_{ref,tx}$ vs. tx).

f) Often, the first datum point (time zero) will deviate significantly from the other results. If so, this point may be discarded.

g) Determine and record linear best fit and R^2 value.

h) Determine the coefficient of variation (C_V) of the signals. The C_V sets a minimum value for the coefficient of variation for the entire procedure.

i) If the R^2 value is greater than 0,90, the system can be used for the measurement of liquid deliveries.

j) Do not use the reader if it shows inconsistent drift (e.g. change in slope, discontinuities).

H.5.3 Fluorescein concentration test

H.5.3.1 General

This test uses hand-held pipettes to dispense the fluorescein test liquids and therefore allows the user to approximate the total error created by all steps of the process other than those involving the ALHS under test (see H.6.2).

H.5.3.2 Fluorescein test liquids of various hydration levels

a) Pipet 30 µl of each of the four test liquids (150 nM fluorescein) made in accordance with H.3.2.6 into four separate wells of a 384-well fluorescence assay plate. Place the test liquids in centrally located wells such as G11, G12, G13, G14, H11, H12, H13, H14, I11, I12, I13, I14, J11, J12, J13, J14 as shown in Figure H.1.