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Specification and method for the determination of performance of automated liquid handling systems

*Spécification et méthode pour la détermination de performance des
systèmes automatisés de manipulation de liquides*



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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).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: [Foreword - Supplementary information](#)

The committee responsible for this document is ISO/TMBG, *Technical Management Board Groups*.

Specification and method for the determination of performance of automated liquid handling systems

1 Scope

This International Workshop Agreement (IWA) specifies methods for testing the volumetric performance of air-displacement, system-liquid filled and positive displacement automated liquid handling systems (ALHS), including an estimation of measurement uncertainties and established traceability to reference standards (preferably, traceability to SI Units). The testing methods specified in this document may also be used to measure the volumetric performance of automated liquid delivery systems which do not aspirate the test liquid.

This IWA also specifies statistical methods for the determination of random and systematic errors (including intra-plate and inter-plate comparisons), analysis of measured results when using multichannel dispensing heads, and analysis depending on dispense patterns. It further defines terms and formulae to be used for summarizing test results.

This IWA also specifies the information to be provided to users of ALHS, including the display of summary results and performance claims.

This IWA 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 microplates. Manipulation of the microplates on the deck of the automated liquid handling system may be achieved automatically, semi-automatically, or manually.

This IWA addresses the needs of:

- suppliers of ALHS, as a basis for quality control including, where appropriate, the issuance of supplier's declarations;
- test houses and other bodies, as a basis for independent certification and calibration;
- users of the equipment, to enable calibration, verification, validation, and routine testing of trueness and precision.

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

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories*

3 Terms and definitions

For purposes of this document, the following terms and definitions apply.

3.1 Definitions

3.1.1

accuracy

<automated liquid handling system> closeness of agreement between a delivered volume and the target volume

Note 1 to entry: The concept 'accuracy' is not a quantity and is not given a numerical quantity value. A liquid delivery is said to be more accurate when it is accomplished with a smaller liquid handling error.

[SOURCE: ISO/IEC Guide 99:2007, 2.13, modified]

3.1.2

air displacement

liquid handling principle in which a body of air is contained between the piston and the test liquid

Note 1 to entry: It is possible to have a large air gap (piston systems), or smaller air gap between the test liquid and the system liquid (liquid filled systems).

3.1.3

ALHS uncertainty

non-negative parameter characterizing the dispersion of the measured volumes relative to the target volume

Note 1 to entry: Uncertainty is inversely related to accuracy, and is a quantity value. This value should be expressed in accordance with ISO/IEC Guide 98-3.

[SOURCE: ISO/IEC Guide 99:2007, 2.26, modified]

3.1.4

aliquot

<automated liquid handling system> single delivery during a multi-dispense

3.1.5

automated liquid handling system

ALHS

system with a complete, installed liquid handling device, including tips and other essential parts needed for delivering a specified volume without human intervention into microplates

Note 1 to entry: Examples of automated liquid handling systems include automated pipetting systems (APS), and automated dispensing systems (ADS).

3.1.6

calibration

<automated liquid handling system> operation that, under specified conditions, establishes a relation between the target volume of the ALHS and the delivered volume

Note 1 to entry: A calibration may be expressed by a statement, a calibration curve or a calibration table. It may include a correction, but correction or adjustment is not a required element of a calibration.

[SOURCE: ISO/IEC Guide 99:2007, 2.39, modified]

3.1.7

dead air volume

captive air volume

air gap

<piston-operated automated liquid handling systems> air volume between the lower part of the piston and the surface of the aspirated liquid

Note 1 to entry: It is possible to have a large air gap (piston systems), or smaller air gap for liquid filled systems. Sometimes called captive air volume.

Note 2 to entry: Commonly, an air gap can be adjusted through ALHS system parameters, while the dead air volume or captive air volume cannot be adjusted (see [4.2.1](#)).

3.1.8

delivered volume

quantity delivered by a liquid handling system

Note 1 to entry: Delivered volume is a conceptual term and cannot be known with complete certainty due to measurement error.

3.1.9

dispense height

height at which the test liquid is dispensed relative to a stated reference

3.1.10

dispensing system

system for delivering liquids from a pre-filled liquid reservoir

3.1.11

disposable tip

tip, which is attached once and after use, as defined by the manufacturer, detached and intended to be discarded

Note 1 to entry: Disposable tips are usually made of plastic.

Note 2 to entry: Disposable tips are in contrast to fixed tips, which are described in [4.3.4](#).

3.1.12

dry contact dispensing

dispensing of liquid while tip is in contact with the dry target

3.1.13

factory acceptance testing

internal vendor testing to ensure ALHS performance to specifications

3.1.14

forward mode pipetting

direct mode pipetting

pipetting mode where the entire aspirated volume is delivered

3.1.15

immersion depth

depth of the tip below the liquid surface

Note 1 to entry: Immersion depth can be applied to both aspiration and dispensing (wet contact).

[SOURCE: Toolpoint Photometric Volume Check Procedure:2008, modified]

3.1.16

individually controlled channel

liquid handling channel that can be operated independently of other channels

3.1.17

labware

<automated liquid handling systems> materials used in conjunction with liquid handling operations

Note 1 to entry: Labware includes disposable tips, reservoirs, receiving vessels, adapters and microplates.

3.1.18

maximum permissible error

upper or lower permitted extreme value for the deviation of the dispensed volume from the target volume

3.1.19

maximum specified volume

largest volume for which the manufacturer offers specifications

Note 1 to entry: The maximum specified volume may vary depending on instrument configuration (e.g. disposable tip size, syringe size).

3.1.20

measured volume

quantity reported by a volume measuring system

Note 1 to entry: In practice, all measurements contain some measurement error. The measured volume is a quantity value and serves as an estimate of the delivered volume which is not known with complete certainty.

3.1.21

measurement method

measurement procedure

detailed description of a measurement according to one or more measurement principles

Note 1 to entry: The source document (ISO/IEC Guide 99) draws a distinction between 'measurement method' and 'measurement procedure' and that distinction is ignored here. In this IWA, the terms are used interchangeably.

Note 2 to entry: The measurement method descriptions in this IWA detail the steps needed to make a volume measurement and calculate certain descriptive statistics. Additional details needed to operate the ALHS are part of the 'test process' as defined in [3.1.43](#). In this IWA, the measurement method is one of the components of a 'test process.'

[SOURCE: ISO/IEC Guide 99:2007, 2.6, modified]

3.1.22

measurement uncertainty

<measured volume> non-negative parameter characterizing the dispersion of the measured volumes relative to the delivered volume

Note 1 to entry: Uncertainty is inversely related to accuracy, and is a quantity value. This value should be expressed in accordance with the ISO/IEC Guide 98-3.

[SOURCE: ISO/IEC Guide 99:2007, 2.26, modified]

3.1.23

metrological traceability

property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty

Note 1 to entry: Additional information can be found in the notes to definition (ISO/IEC Guide 99:2007, 2.41) and the related term 'metrological traceability chain' (ISO/IEC Guide 99:2007, 2.42).

[SOURCE: ISO/IEC Guide 99:2007, 2.41]

3.1.24

microplate

flat plate with an array of wells

Note 1 to entry: Some dimensions of microplates are defined in ANSI/SLAS standards.^[1-5]

3.1.25

minimum specified volume

smallest volume for which the manufacturer offers specifications

Note 1 to entry: The minimum specified volume may vary depending on instrument configuration.

3.1.26**multichannel head**

group of liquid handling channels operated in common

Note 1 to entry: Common arrangements of multichannel heads include 8, 96, 384, and 1536 channel heads. Other arrangements are possible, e.g. 2 channel to 1536 channel configurations.

Note 2 to entry: Pipetting channels in a multichannel head may be controlled by a single, common drive, or each channel may be controlled individually.

3.1.27**multi dispense**

repeat dispense

sequential dispense

<automated pipetting systems> a collection of dispenses without intervening aspiration

Note 1 to entry: First dispense can be different, and is frequently wasted.

Note 2 to entry: Repeat dispenses usually dispense repeatedly the same volume, while sequential dispenses usually dispense different volumes.

3.1.28**non-contact dispensing**

contact-free dispensing

free-jet dispensing

dispensing of the liquid while tip is in air and without contacting the target or the liquid contained in the target

3.1.29**outlier**

member of a set of values which is inconsistent with the other members of that set

3.1.30**pipetting system**

system for aspirating and dispensing a specified volume of liquid

[SOURCE: Toolpoint Gravimetric Volume Check Procedure:2008, modified]

3.1.31**positive displacement**

direct displacement

liquid handling principle in which a mechanical actuator is in direct contact with the test liquid

3.1.32**precision**

<of liquid handling system> the closeness of agreement between the measured volume of independent delivered volumes under stipulated conditions

Note 1 to entry: Precision is conceptual and not a quantity value.

Note 2 to entry: Measurement precision is usually expressed numerically by measures of random error, such as standard deviation, variance, or coefficient of variation under the specified conditions of measurement.

Note 3 to entry: The 'stipulated conditions' can be, for example, repeatability conditions of measurement, intermediate precision conditions of measurement, or reproducibility conditions of measurement (see ISO 5725-1:1994).

[SOURCE: ISO 5725-1:1994, 3.12, modified]

3.1.33

random error

<automated liquid handling systems> component of liquid handling error that in replicate deliveries varies in an unpredictable manner

[SOURCE: ISO/IEC Guide 99:2007, 2.19, modified]

3.1.34

reservoir

liquid container

vessel that contains the liquid

3.1.35

reverse mode pipetting

pipetting mode in which excess volume is aspirated and remains in the tip after delivery

3.1.36

single dispense

individual dispense

<automated pipetting systems> single dispense per aspiration

3.1.37

site acceptance testing

in-situ testing at the user's site, typically part of the installation process

3.1.38

supplier's declaration

document by which a supplier gives written assurance that an ALHS conforms to the requirements of one or more commonly accepted industry standards

Note 1 to entry: This IWA can be referenced as an applicable industry standard.

3.1.39

systematic error

<automated liquid handling system> component of volumetric error that in replicate deliveries remains constant or varies in a predictable manner

Note 1 to entry: Systematic error is estimated by calculating the average volume of a series of deliveries and comparing it to the indicated volume of the automated liquid handling system. Frequently this result is expressed as a percentage of the indicated volume.

[SOURCE: ISO/IEC Guide 99:2007, 2.17, modified]

3.1.40

system liquid

liquid used to transmit energy between a mechanical actuator and the test liquid

Note 1 to entry: System liquids can reduce or completely eliminate system dead air volume.

Note 2 to entry: System liquid is usually deionized water. For special applications organic solvents such as DMSO or aqueous solutions such as saline (e.g. 0,9 % NaCl) can be used.

Note 3 to entry: System liquid can be used for flushing and rinsing tips to minimize cross contamination.

3.1.41

target volume

indicated volume

selected volume

volume which is intended to be delivered

3.1.42**test liquid**

liquid used for the volume measurement

Note 1 to entry: May be aqueous or other solvents. Aqueous test liquids can be pure water or contain other compounds such as buffers, dyes or salts. The chemical composition of the test liquid can vary significantly depending on method.

3.1.43**test process**

<automated liquid handling system> detailed description of an ALHS testing procedure including system operation and measurement method

Note 1 to entry: The test process includes all details needed to reproduce the test or interpret the results. The measurement method is defined in [3.1.21](#), and is only a part of the test process.

3.1.44**test report**

document reporting the result of the testing

Note 1 to entry: Details regarding information contained in test reports is specified in [Clause 8](#).

3.1.45**test result**

value of a characteristic obtained by carrying out a specified test method

Note 1 to entry: Test result is a broader concept than measured volume. The test result can be a single measured volume, a set of measured volumes, or descriptive statistics such as the mean or standard deviation of multiple measurements. The test method should specify what form the test results take.

[SOURCE: ISO 5725-1:1994, 3.2]

3.1.46**traceability**

metrological traceability

property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty

[SOURCE: ISO/IEC Guide 99:2007, 2.41]

3.1.47**trueness**

<automated liquid handling system> closeness of agreement between the average volume delivered in a large series of deliveries and the target volume

Note 1 to entry: Trueness is inversely related to systematic error, but is not related to random error.

[SOURCE: ISO/IEC Guide 99:2007, 2.14, modified]

3.1.48**validation**

<automated liquid handling system> confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled

Note 1 to entry: The term “validated” is used to designate the corresponding status.

Note 2 to entry: The test protocol for this testing should reflect the liquid volumes and instrument settings, at which the ALHS will be operated.

Note 3 to entry: A product may meet all of its specifications (verification), but that does not ensure that it will work in the operating paradigm (validation).

3.1.49**verification**

<automated liquid handling system> confirmation, through provision of objective evidence, that volumetric performance specifications have been fulfilled

Note 1 to entry: The term “verified” is used to designate the corresponding status.

Note 2 to entry: Volumetric performance specifications may vary depending on the environment where the ALHS is used, e.g. factory and field specifications may be different.

[SOURCE: ISO/IEC Guide 99:2007, 2.44, modified]

3.1.50**wet contact dispensing**

dispensing of the test liquid while tip is in contact with liquid present in the target

3.2 Abbreviated terms

See [Table 1](#).

Table 1 — Abbreviated terms used in this document

Abbreviated term	Explanation
ADS	Automated Dispensing System
ALHS	Automated Liquid Handling System
APS	Automated Pipetting System
C2C	Channel-to-Channel
CV	Coefficient of Variation
DI	De-ionized
DMSO	Dimethylsulfoxide
GA	Grand Average
HVAC	Heating, Ventilation, and Air Conditioning
IEC	International Electrotechnical Commission
IWA	International Workshop Agreement
MU	Measurement Uncertainty
MW	Molecular weight
NaOH	Sodium hydroxide
OA	Over All
OD	Optical Density
<i>p.a.</i>	<i>pro analysi</i> (purity grade of chemicals)
<i>p</i> -NP	<i>para</i> -nitrophenol
RH	Relative Humidity [%]
RSE	Relative Systematic Error
rcf	Relative centrifugal force
rpm	Revolutions per minute
SD	Standard Deviation
SMOW	Surface Mean Ocean Water

4 Operation of automated liquid handling systems

4.1 Types of automated liquid handling systems

The purpose of this clause is to provide examples of some types of ALHS. This list is not intended to be a comprehensive list as technologies continue to evolve.^[6]

4.1.1 Types of piston operated automated liquid handling systems

Automated liquid handling systems can operate as follows:

- variable volume; designed by the manufacturer to aspirate and dispense volumes selectable by the user within the specified useful volume range of the dispense head and selected tips, for example between 10 µl and 100 µl.
- a larger volume may be aspirated into the tips, followed by a series of subsequent dispenses of smaller aliquots.

The piston can

- either have a body of air contained between the piston and the surface of the liquid (air-displacement), or
- be in direct contact with the surface of the liquid (positive or direct displacement), or
- be in contact with a system liquid.

The system can

- have a single tip, or
- have multiple tips, operated by individual pistons, or
- have multiple tips, operated by a single, common drive or moving plate with multiple pistons simultaneously driven by a common drive.

The tip can be

- permanently attached to the dispense channel of the ALHS, or
- disposable, and used for one or more aspirate and dispense sequences.

4.1.2 Types of other (pump operated) automated liquid handling systems

Pump operated automated liquid handling systems can operate as follows:

variable volume; designed by the manufacturer to dispense volumes selectable by the user within the specified useful volume range of the dispense head.

The dispense head can

- be permanently attached to the instrument, or
- be exchangeable, e.g. to change the usable volume range or number of channels.

The system can

- have a peristaltic or diaphragm pump to aspirate liquid from a reservoir, or
- have a pressurized liquid reservoir and separate liquid valves to control the liquid delivery.

4.2 Adjustment

4.2.1 Need for adjustment

A standard parameter set for a given fluid class may need to be adjusted for optimizing the ALHS performance. ALHS parameters may need to be adjusted in one or more of the following situations:

- to accommodate liquid-specific properties;
- following the replacement of system components;
- following change of labware components; or
- following a change of the location of operation sites (e.g. at the factory vs. the end user's location).

The performance of an ALHS can be corrected and optimized by adjusting system parameters such as the aspiration and dispense speeds, immersion depth of the tip, dispense height, air gaps, and others.

The scope of adjustable system parameters varies between ALHS models and the manufacturer's adjustment instructions should be followed.

Some ALHS can be used at volumes outside of the volume range specified by the manufacturer for the installed system components. In this case, the ALHS performance at these volumes shall be validated with a test method (see [Clause 6](#)) suitable for these used volumes.

4.2.2 Liquid classes

An automated liquid handling system is adjusted by its manufacturer for the delivery of its selected volume (or multiple volumes as specified by the manufacturer). The manufacturer shall report the test solution, instrument settings, and environmental basis used for defining the standard liquid class. Users of ALHS who define liquid classes and test the volumetric performance of the ALHS shall report the test liquid, instrument settings, and environmental basis for each tested liquid class.

4.2.3 Adjustment of ALHS settings

Some automated liquid handling systems have a provision for adjustment by the user when, for example, it is found in routine calibration that the volume delivered is not within specification. Such user adjustment shall be made according to the manufacturer's instructions and by reference to one of the methods for the determination of measurement error specified in this IWA.

4.3 Tips

4.3.1 General

The dispensing orifice of the tip shall be shaped in such a way that consistent dispensing of the liquid is achieved. When the pipetting operation is completed, any amount of liquid remaining in or around the dispensing orifice of the tip shall be consistent.

In the case of sterilizable tips, the sterilization procedures indicated as appropriate by the manufacturer in user information or on packaging shall not negatively affect the metrological characteristics of the tips such as shape, seal and wettability.

NOTE This requirement can be assessed by comparing errors of measurement using tips which have and have not been sterilized.

4.3.2 Air-displacement tips

4.3.2.1 Air-displacement tips shall be disposable parts, usually made of plastic, which fit on the ALHS dispensing head and prevent the instrument from contact with the aspirated liquid.

4.3.2.2 Disposable air-displacement tips shall be fitted in accordance with the ALHS supplier's instructions to form a good seal between the tip and the dispensing head.

Disposable tips should not be cleaned or reused unless their metrological characteristics are confirmed and they are shown fit for use in the specific application (validation).

NOTE Variability of the amount of externally retained liquid or an incomplete seal will contribute to poor precision when testing with one of the methods described in this IWA.

4.3.3 Positive displacement tips

4.3.3.1 Positive displacement tips shall consist of a plunger and a capillary which fit on the tip holder of the dispensing head of the automated liquid handling system. Various materials may be used for the plunger, such as metal, plastic, or ceramic and for the capillary, such as plastic or glass. These pipette tips may be reusable or disposable (both plunger and capillary are changed together, per manufacturer's instructions).

4.3.3.2 The shape and material of the plunger and capillary shall confer a good seal of the tip, as well as a smooth action between the plunger and the capillary, to ensure consistent dispensing of the liquid.

4.3.4 Fixed tips

4.3.4.1 Description and materials

Fixed tips can be manufactured from various materials, such as stainless steel or polymeric materials. These tips may be coated for inertness to pipetted fluids or for specific functionality, e.g. conductivity to sense contact with fluids in receptacles on the deck of the ALHS.

4.3.4.2 Development of cleaning protocol and testing / confirmation of metrological characteristics

Functionality and metrological performance of fixed tips should be tested at regular intervals. It is recommended to follow the manufacturer's cleaning protocol and use instructions for best performance of fixed tips.

4.3.4.3 Maintenance and exchange of fixed tips

Fixed tips should be examined for damage and tested for proper functionality at regular intervals according to the manufacturer's instructions, which should contain protocols for the maintenance and replacement of such tips.

4.4 Environmental conditions

4.4.1 Discussion and recommendations

Changes in temperature, relative humidity, and barometric pressure can cause changes in the volumetric performance of piston-operated automated liquid handling systems. It is recommended that temperature and relative humidity be monitored at all locations where operational performance testing of ALHS is conducted.

4.4.2 Factory acceptance testing

It is recommended that factory acceptance testing is performed in a location where the temperature and relative humidity can be controlled. It is good practice to equilibrate all equipment at least 2 h prior to testing in an environment as defined by the ALHS manufacturer. Both the ALHS and the test method can have specific sensitivity to environmental conditions. The relevant environmental conditions and their upper and lower limits (e.g. temperature, RH, barometric pressure) shall be identified and recorded.

This equipment includes the ALHS, and may include a balance, plate reader, pipettes, test liquids, weight calibration standards, etc. Any deviations from the recommended conditions shall be recorded and reported with the test results. Estimates of the measurement uncertainty shall be based on the actual test conditions.

4.4.3 Site acceptance and user testing

It is recognized that automated liquid handling systems are frequently installed and used in locations where temperature, relative humidity, and barometric pressure differ from factory testing conditions. It is recommended that site acceptance testing be performed at prevailing local conditions, which should be stable within the requirements of the test method and the manufacturer's specifications before and during the time of testing. For reference, the temperature and relative humidity with their minimum and maximum values during the time of testing should be recorded. At a minimum, the temperature or temperature range during the time of testing shall be reported with the test results for all equipment used during this testing (e.g. ALHS, balance, plate reader, test liquids, etc.), and the estimate of measurement uncertainty shall reflect the actual test conditions.

Regardless of available environmental controls, it is recommended that automated liquid handling systems be situated in an appropriate environment that reduces temperature extremes (e.g. away from windows with direct sunlight exposure, or concentrated heat sources such as autoclaves, HVAC systems and vents, or high voltage installations). Manufacturer's recommendations for the installation and use environment of ALHS should be followed.

Deviations from ideal/recommended test conditions need to be reflected in the measurement uncertainty (MU) estimate.

5 Volumetric performance

5.1 Introductory discussion

Automated liquid handling systems (ALHS) are designed to deliver amounts of liquid at a target volume. The target volume is typically set using software or other digital control. Volumetric performance is assessed by measuring the volume of each liquid delivery and evaluating the data.

Volumetric performance is typically assessed by suppliers as part of the manufacturing process quality control or a supplier's service offering. Volumetric performance can also be assessed by users, as well as by third party testing and calibration service providers.

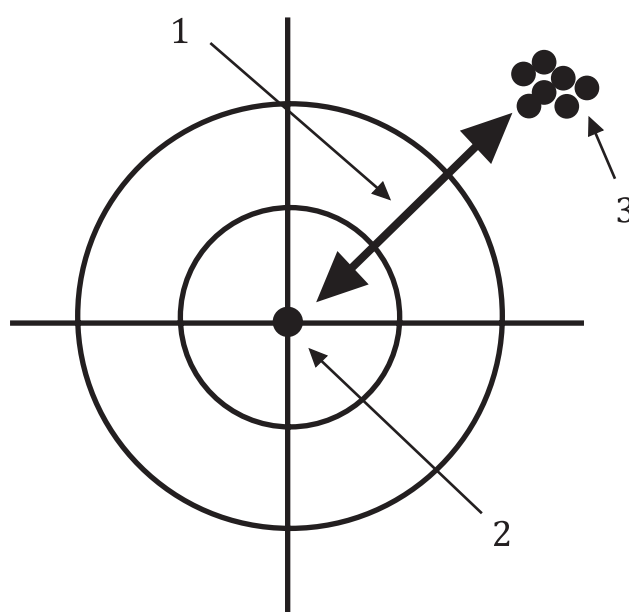
Automated liquid handling systems are designed to handle a variety of liquids of differing physical properties such as density, viscosity, surface tension and contact angle against solid surfaces. The volumetric performance of the ALHS can vary depending on these physical properties, so a description of the test liquid shall be included when reports of volumetric performance are made. This description of the test liquid may be made in terms of chemical composition, physical properties, or both.

Manufacturers of ALHS can make performance claims at various volumes for a particular instrument configuration. The maximum specified volume and minimum specified volume establish a liquid handling range over which the system manufacturer has established volumetric performance specifications. However, in some systems it is possible for the user to program the system to deliver volumes which are outside of this range (i.e. greater than the maximum specified volume or less than the minimum specified volume).

In preparing for a volumetric performance test, the ALHS will be set to deliver a particular target volume. For testing by the supplier, the target volume will frequently be identical to one of the manufacturer's specified volumes. Operators may decide to test at any target volume they wish. During testing each delivered volume is expected to be slightly different from the target volume. The delivered volume is a conceptual quantity because it cannot be known with certainty and can only be approximated by measurement.

In order to evaluate volumetric performance, measurements are made of individual delivered volumes. The measured volume is a quantity which consists of a numerical value and units. The recommended units are microlitres (μl) though related units such as millilitres (ml) and nanolitres (nl) are sometimes used and are also acceptable. The measured volume is an estimate of the delivered volume and departs slightly from the true delivered volume due to measurement uncertainty (MU). Measurement uncertainty reflects the fact that no measurement is perfect.

In [Figure 1](#), a set of measured volumes are represented as points on a target. The target volume is represented conceptually as the centre of the target and each measured volume as a circular mark. The width of the circular mark represents the measurement uncertainty and the true value of the delivered volume is believed to be somewhere within each circular mark. In [Figure 1](#), each mark is some distance from the centre and this distance of each mark from the centre is proportional to the error of the liquid handling system. Taken together, the set of measured volumes is related to the accuracy of the liquid handling system. Accuracy improves as each member of the data set moves closer to the centre of the target.



Key

- 1 ALHS error
- 2 target volume
- 3 measured volumes

Figure 1 — Relationships between target volume, measured volumes and ALHS error

Accuracy may be improved by improving precision and trueness. These concepts are illustrated in [Figure 2](#). Improving precision brings the cluster of the results into a smaller bunch, while improved trueness occurs when the centre of the cluster is closer to the centre of the target.

Accuracy, precision and trueness are conceptual terms. Quantitative expressions of these concepts are given in terms of uncertainty, random error and systematic error, respectively.

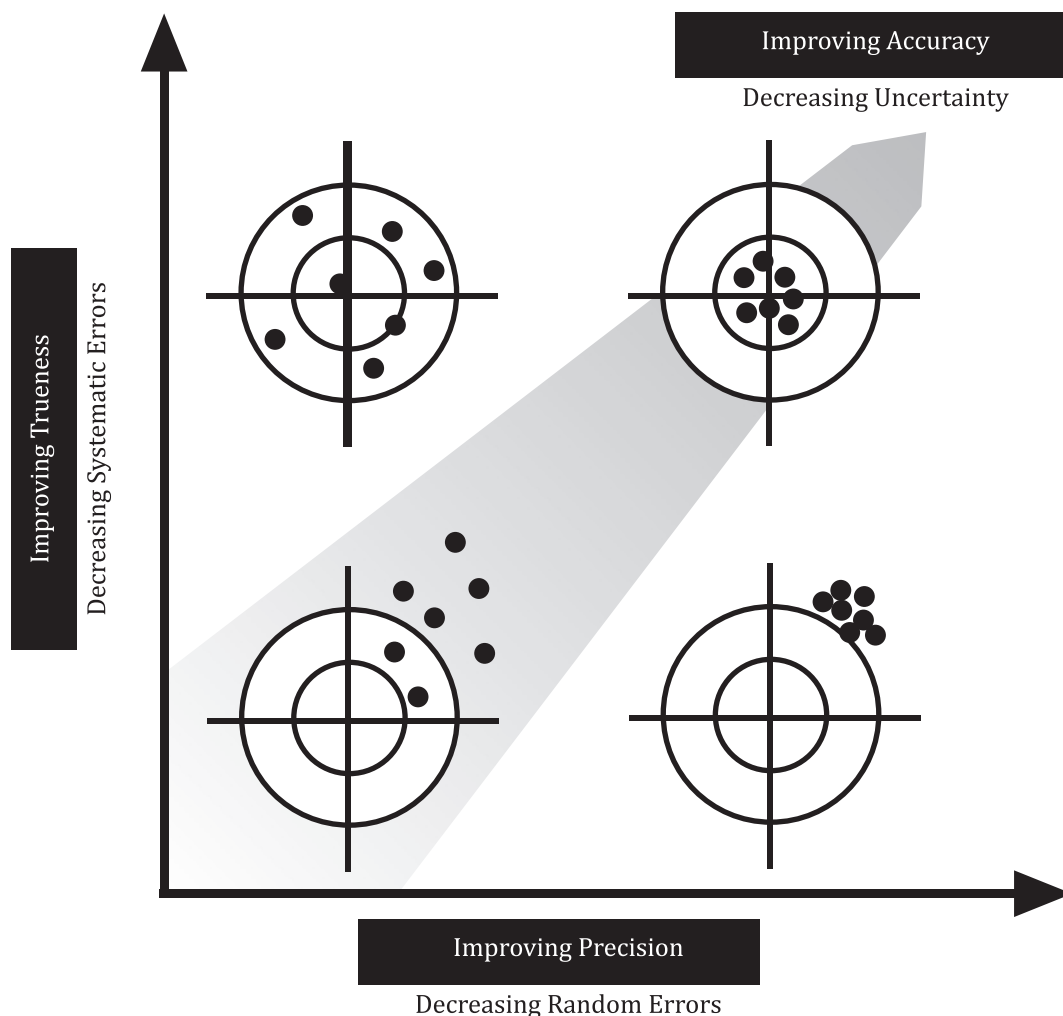


Figure 2 — Relationship between trueness, precision, and accuracy of an ALHS

Test results include data sets of individual measured volumes, and also descriptive statistics which summarize the data sets. Systematic error and random error are two examples of descriptive statistics which are commonly employed in the testing of ALHS.

5.2 Data collection and examination

Each delivered volume can be measured to determine the measured volume V . Volume measurement methods are described in [Clause 6](#).

Prior to calculating descriptive statistics, it is recommended that the measured volumes be visually examined for evidence of outliers, trending, or patterns. Such features may indicate the need for more detailed analysis, optimization, or additional testing to determine the cause. For purposes of this IWA, outliers are considered to be unusual results that cannot be reliably repeated. Trending refers to results that vary in a regular way when viewed by time or dispense order. Patterns might be observed when viewing data in a spatial arrangement such as examining results distributed in a plate arrangement. Visualization aids such as heat mapping may be used to help identify patterns. The presence of outliers, trending or patterns might indicate the need for further investigation, including optimization or repair of the ALHS.

NOTE Statistical consideration of outliers is beyond the scope of this IWA, but is discussed in detail elsewhere. [\[7,8\]](#)

5.3 Indexing to track data

With multiple different channels, replicates and experimental possibilities, an identification scheme is needed to keep track of the data.

5.3.1 Indexing from the channel perspective

Viewed from the perspective of the liquid handler, each volume delivery can be given an index number in the form of an ordered triplet of integers (l,m,n) where:

l is an index for the dispensing channel and ranges from 1 to L ;

m is an index for run, and ranges from 1 to M ;

n is an index for delivery order within a single run and ranges from 1 to N .

NOTE 1 The variable L is the number of dispensing channels per ALHS. L can be as small as 1 for the case of a single channel device, to 384 or greater.

NOTE 2 The variable M is used to track different runs, which can be different experiments under different conditions, or which may be replicates of prior experiments for the purposes of assessing reproducibility or drift over longer time periods.

NOTE 3 The variable N is the number of replicates in a repeatability test where the volumes are delivered in a short period of time under nearly identical conditions. This IWA does not specify a minimum number of replicates to be used. However, the number of replicates (N) should be reported when repeatability data are used to calculate averages or standard deviations as the reliability of these descriptive statistics depends on the number of replicates.

In this way, a measured volume V of the n -th delivery by the l -th channel, during the m -th run is given by the symbol $V_{(l,m,n)}$.

The channel perspective is recommended for purposes of evaluating volumetric performance and determining whether particular channels are performing correctly. Alternative indexing systems such as the microplate perspective are described in 5.3.2. Examples illustrating these systems are provided in Annex A.

5.3.2 Indexing from the microplate perspective

When volumes are dispensed into microplates for measurement, it is common to index by row, column, and plate. In 96- and 384-well microplates, it is common for rows to be designated by letters (e.g. A through H, and A through P, respectively) while columns are numbered (1 through 12, and 1 through 24, respectively). This viewpoint is of particular interest to users who may want to evaluate precision, trueness or accuracy from a plate perspective. Indexing schemes are not mutually exclusive. When volume measurements are made in microplates, knowledge of the liquid handling system programming allows the data from the rows, columns and plates to be translated into the channel, run, and dispense order.

NOTE 1 It is not necessary to consider different plates to be different experiments. For example, a 96 tip head could be tested by making a series of deliveries into three 96-well plates. In this case, plates 1, 2 and 3 could be considered to be dispense replicates $n = 1, 2$ and 3 , while all three plates are considered part of a single experiment. An example of this scenario is included in A.5.

NOTE 2 Users are frequently interested in “within plate” variation or variation and patterns across different plates. For example, when patterns are observed within a plate, the user may be interested in whether the pattern is repeatable across additional plates. Also, when evaluating different ALHS for a particular application, the user may wish to evaluate data from the plate perspective without regard to the arrangements of independent channels and thus simply compare whole plate precision of two different systems.

5.4 Descriptive statistics on an individual channel basis

Typically, N deliveries of a constant target volume are measured and averaged to identify the actual volume a liquid handler is dispensing. These N consecutive dispenses, referred to as a run, are usually dispensed in quick succession to avoid pause time effects. A run can be preceded by several pre-dispenses which are dispensed into the waste and therefore not measured or taken into account. Pre-dispenses allow for the dispensing system to adjust to new situations, for example, a new target volume, a change of reagent or the start of the dispensing procedure after an idle time. This way, a well-defined and reproducible initial situation is set before a run which helps to increase precision.

The **average volume** delivered by a particular channel during a particular run is given in Formula (1). This average volume can then be used in the formulae to calculate both systematic and random errors.

$$\bar{V}_{(l,m)} = \frac{1}{N} \sum_{n=1}^N V_{(l,m,n)} \quad (1)$$

where

$\bar{V}_{(l,m)}$ is the average of all measured volumes from channel ' l ' during run ' m ';

N is the number of replicate deliveries in the run;

$V_{(l,m,n)}$ is a single measured volume.

Systematic error (e_S) is estimated by the deviation of the measured mean volume from the target volume. For example, if the user seeks to deliver a reagent with a target of $V_T = 100 \mu\text{l}$ and the system delivers an actual volume of $97 \mu\text{l}$, the systematic error is $-3 \mu\text{l}$ (absolute error) or -3% (relative error). The determination of the systematic error of a single channel in a single run is given by Formula (2). This formula can be generalized and applied in any situation where it is desired to compare a measurement result to the target volume.

$$e_{S(l,m)} = \bar{V}_{(l,m)} - V_T \quad (2)$$

where

$e_{S(l,m)}$ is the systematic error of channel ' l ' during run ' m ';

V_T is the target volume, the volume intended to be delivered.

The systematic error can also be expressed in relative terms as shown in Formula (3):

$$e_{S(l,m)} = \frac{\bar{V}_{(l,m)} - V_T}{V_T} \times 100\% \quad (3)$$

Estimates of systematic error can be improved by increasing the number of measurements in the data set, either by increasing N , or by conducting multiple replicate runs and summing over both N

and M . Increasing N is accommodated in Formula (1), while summing over multiple experiments is shown in Formula (4).

$$\bar{V}_{(l)} = \frac{1}{MN} \sum_{m=1}^M \sum_{n=1}^N V_{(l,m,n)} \quad (4)$$

where

$\bar{V}_{(l)}$ is the mean measured volume from channel 'l';

M is the number of runs included in the average.

Formula (4) can be re-arranged as shown in Formula (5), and the identical result can be obtained by either Formula (4), or by taking the M replicated results of Formula (1), and averaging them together as shown in Formula (5).

$$\bar{V}_{(l)} = \frac{1}{M} \sum_{m=1}^M \frac{1}{N} \sum_{n=1}^N V_{(l,m,n)} = \frac{1}{M} \sum_{m=1}^M \bar{V}_{(l,m)} \quad (5)$$

Random error (S_r) of a channel is usually assessed by calculating the standard deviation of a series of N measured volumes under repeatability conditions, as shown in Formula (6).

$$S_{r(l,m)} = \sqrt{\frac{\sum_{n=1}^N (V_{(l,m,n)} - \bar{V}_{(l,m)})^2}{N-1}} \quad (6)$$

where $S_{r(l,m)}$ is the random error of channel 'l' during run 'm'.

This standard deviation can be divided by the average volume, and multiplied by 100 to convert to a percentage as shown in Formula (7). This is the recommended descriptive statistic for random error and is often called the coefficient of variation (CV).

$$C_{V(l,m)} = \frac{S_{r(l,m)}}{\bar{V}_{(l,m)}} \times 100 \% \quad (7)$$

where $C_{V(l,m)}$ is the coefficient of variation of channel 'l' during run 'm'.

Estimates of random error can also be improved by increasing the number of measurements in the data set, either by increasing N , or conducting multiple replicated experiments and combining the results. Increasing N is accommodated in Formula (6).

Averaging CV over multiple experiments can be accomplished using a root-mean-squares approach as shown in Formula (8) below. This formula is permissible when N is identical in each experiment.

$$C_{V(l)} = \sqrt{\frac{\sum_{m=1}^M C_{V(l,m)}^2}{M}} \quad (8)$$

where $C_{V(l)}$ is the coefficient of variation of channel 'l' combining data from multiple runs.

NOTE 1 CV results should not be combined by simple averaging (arithmetic mean).

NOTE 2 When combining or averaging random errors, the precise details of the mathematical operation matter, and, depending on the details may lead to a different value of the CV result. If a formula other than Formula (8) is used, the equations used should be described in sufficient detail to permit an unambiguous understanding.

Examples applying these channel statistics are included in [Annex A](#). Evaluation based on channel statistics is particularly useful from a supplier's perspective and is frequently used to determine whether channels are working properly.

5.5 Descriptive statistics on a run order basis

In some cases, it is useful to view data on a run order basis. While channel analysis is useful for determining whether an instrument requires repair or maintenance, run order analysis can be particularly valuable during method development to determine whether the liquid handling protocol is properly optimized to prevent systematic trending effects during the liquid delivery sequence. For example, some programming choices can result in a "first shot effect" where the $n = 1$ delivery is consistently greater or lesser than subsequent deliveries.

The average volume on a run order basis ($\bar{V}_{(n)}$) can be calculated by Formula (9).

$$\bar{V}_{(n)} = \frac{1}{LM} \sum_{l=1}^L \sum_{m=1}^M V_{(l,m,n)} \quad (9)$$

where

$\bar{V}_{(n)}$ is the mean measured volume from the n -th dispense of all channels and all runs;

L is the number of channels in the ALHS.

NOTE The case where only one run is performed ($M = 1$) can be accommodated within Formula (9) and Formula (10).

In addition to run-order volume, a run order random error can be calculated using Formula (10) and expressed as a CV using Formula (11). These statistics can be useful in determining whether the random error or CV changes during the dispense order. For example, when a disposable pipette tip is re-used a number of times, it is possible that the CV will eventually increase.

$$S_{r(n)} = \sqrt{\frac{\sum_{l=1}^L \sum_{m=1}^M (V_{(l,m,n)} - \bar{V}_{(n)})^2}{LM - 1}} \quad (10)$$

where $S_{r(n)}$ is the standard deviation of the n -th dispense across all channels, and combining data from multiple runs.

$$C_{V(n)} = \frac{S_{r(n)}}{\bar{V}_{(n)}} \times 100 \% \quad (11)$$

where $C_{V(n)}$ is the coefficient of variation of a particular n -th dispense across all channels, and combining data from multiple runs.

5.6 Descriptive statistics for entire data sets

The grand average volume is useful in determining the overall trueness at a particular target volume; it is the arithmetic mean of all measured volumes in the data set and calculated using Formula (12). The

grand average volume can be converted to a systematic error by analogy to Formula (2) and relative systematic error by analogy to Formula (3).

$$\bar{V}_{GA} = \frac{1}{LMN} \sum_{l=1}^L \sum_{m=1}^M \sum_{n=1}^N V_{(l,m,n)} \quad (12)$$

where $\bar{V}_{GA}$ is the grand average volume calculated from all channels, all runs and all replicates.

Overall CV (CV_{OA}) includes contributions from random error within each individual channel, and also contributions from systematic differences between channels. In some cases, the overall CV is of particular interest, and can be calculated using Formula (13).

$$C_{V,OA} = \frac{\sqrt{\frac{1}{(LMN-1)} \sum_{l=1}^L \sum_{m=1}^M \sum_{n=1}^N (V_{(l,m,n)} - \bar{V}_{GA})^2}}{\bar{V}_{GA}} \quad (13)$$

where $C_{V,OA}$ is the coefficient of variation of all measurements within the data set.

5.7 Differences between channels

Systematic differences between channels are of concern to users who wish to limit or compensate for variation in their experimental results, and also to ALHS manufacturers who wish to improve overall CV in their systems.

One way to limit differences between channels is to establish limits for systematic error and apply them to each channel. Therefore, when establishing or evaluating specifications for systematic error it is important to consider whether the limits apply to each channel evaluated individually, or only to the systematic error calculated from the grand average volume.

Channel-to-channel differences can also be quantified as a channel-to-channel CV ($C_{V,C2C}$), calculated as shown in Formula (14).

$$C_{V,C2C} = \frac{\sqrt{\frac{1}{(L-1)} \sum_{l=1}^L (\bar{V}_{(l)} - \bar{V}_{GA})^2}}{\bar{V}_{GA}} \quad (14)$$

where $C_{V,C2C}$ is the coefficient of variation of the mean volumes delivered by each channel.

The channel-to-channel CV need not be specified or calculated in all cases. However, it can be useful in determining whether channel-to-channel CV is a significant contributor to the overall CV.

5.8 Handling of sub-deliveries

Some systems deliver liquids by depositing multiple sub-deliveries to form a final delivered volume. For example, acoustic dispensers, ink jet dispensers, and some other systems may deliver in this way.^[9] For the purposes of this IWA, assessing the volumetric performance of such systems is based on the volume of the combined deliveries, without individual measurement of each sub-delivery.

6 Measurement methods

6.1 Overview of methods suitable for measuring ALHS performance

When choosing a test method for an ALHS, the user of this IWA shall evaluate its suitability for the specific test situation. This evaluation shall consider the requirements for systematic and random errors of the test method, which will be applied to the ALHS being tested. The user shall ensure that the test method has a sufficiently small measurement uncertainty (MU) for the specific test situation. Measurement uncertainty should be determined in accordance with ISO/IEC Guide 98-3.

Test methods designated as “open methods” in this clause can be used on any ALHS platform, while those designated as “closed methods” in this IWA are specific to the ALHS platform referenced within the respective method.

The following [Table 2](#) is intended to provide an overview of methods suitable for evaluating the volumetric performance of ALHS. For each method, this table provides the cross-reference to the method abstract in [6.2](#) and the full method description in [Annex B](#). It further describes the plate and liquid types, which can be used for testing ALHS performance with a given method, as well as the volumes, which can be measured. It also lists typical accuracy and precision values achievable if the method is conducted as described in detail in [Annex B](#). The suitability of a method may also be determined by the required equipment or environmental conditions under which it needs to be carried out. Published references on the measurement uncertainty and traceability chain are available for some methods as noted in [Table 2](#) and in the detailed method descriptions. References on measurement uncertainty and traceability are provided as information to the user of a given method and have not necessarily been harmonized between methods or independently verified by inter-lab comparisons.

Some methods described in this IWA are very similar to each other, yet each one has its own merits and is therefore described as an individual method. No analysis of overlap, correlation, or intercomparison between methods has been performed, and the user of this IWA shall determine the most suitable method for their test requirements.

Table 2 — Overview of methods suitable for determining ALHS volumetric performance

Method No.	Method type	Number of channels individually analysed	Plate type	Fluid type	Volume range [μl]	Typical accuracy of method (a,b) [%]	Typical precision of method (a,c) [%]	Environmental requirements of method	Published uncertainty of measurement of method (a,d)	Published traceability chain of method	Required equipment
Ratiometric photometry methods											
6.2.1 B.1	Dual dye ratiometric photometry	1 to 384	96-wells	Aqueous / DMSO	0,2 – 350 (e)	2	0,15 – 0,20	Temperature: aqueous: 15 °C to 30 °C DMSO: 19 °C to 30 °C	[10,11]	[12]	Artel MVS system
					0,1 – 0,2	3	0,20 – 0,25				
			384-wells	Aqueous / DMSO	0,05 – 55 (f)	2,5	0,35 – 0,40				
					0,02 – 0,05	3,5	0,40 – 0,46				
					0,01 – 0,02	5,5	0,46 – 0,50				
Gravimetric methods											
6.2.2. B.2	Gravimetry, single channel measurement	1 channel	n/a	Aqueous	1 – 10	≤ 1,4	≤ 0,6	Temperature: 15 °C to 30 °C RH: 40 % to 70 %	[13]	[13]	Balance; Air temperature sensor; Water temperature sensor; Relative humidity sensor; Absolute pressure sensor.
					> 10 - 100	≤ 0,9	≤ 0,3				
					> 100 – 1000	≤ 0,9	≤ 0,3				

Table 2 — (continued)

Method No.	Method type	Number of channels individually analysed	Plate type	Fluid type	Volume range [μl]	Typical accuracy of method (a,b) [%]	Typical precision of method (a,c) [%]	Environmental requirements of method	Published uncertainty of measurement of method (a,d)	Published traceability chain of method	Required equipment
6.2.3 B.3	Gravimetry, whole plate measurement	Entire plate	96-wells	Any liquid	200	0,032 (g)	n/a	Temperature: (20 ± 0,5) °C RH: ≥ 50 %	[14]		Specialized environment; First balance: Resolution of 0,01 mg and 0,02 mg repeatability; Second balance: Resolution of 0,001 mg and 0,002 mg repeatability; Density meter with six decimal places; Anti-electrostatic equipment; ALHS with 96-channel and 384-channel heads.
					0,1 - 199	0,058 (h)					
			384-wells	Any liquid	55	0,033 (g)					
					0,03 - 49	0,058 (h)					
6.2.4 B.4	Gravimetric regression method for low volumes (by non-contact dispensing)	1 channel	n/a	Any liquid	0,04 - 1,0	< 5	< 2	Temperature: 15 °C to 25 °C RH: ≥ 50 %	[15]	n/a	Micro balance with draft-shield and readability of at least 10 μg, 1 μg or 0,1 μg (depending on the volume range and uncertainty requirements); Vibration isolated table recommended.

Table 2 — (continued)

Method No.	Method type	Number of channels individually analysed	Plate type	Fluid type	Volume range [μl]	Typical accuracy of method (a,b) [%]	Typical precision of method (a,c) [%]	Environmental requirements of method	Published uncertainty of measurement of method (a,d)	Published traceability chain of method	Required equipment
Photometric methods											
6.2.5 B.5	Orange G	96	96-wells	Aqueous	2,0 – 10,0	3	1,5	Temperature: 15 °C to 30 °C RH: 40 % to 70 %	n/a	n/a	Balance (0,001 mg resolution); Manual pipettes; Volumetric flasks; pH meter; Magnetic stirrer; Microplate shaker; Microplate reader with 492 nm and 620 nm filters.
					10,0 – 100,0						
					100,0 – 200,0						
		384	384-wells	Aqueous	0,5 – 2,0						
					2,0 – 20,0						
					25,0 – 110,0						
6.3.2 B.13	Tartrazine (closed method) (i)	96 or 384	96- or 384-wells	DMSO	≥ 2 % of tip volume	5 (j)	n/a	Temperature: 20 °C to 30 °C RH: 10 % to 70 %	n/a	n/a	Microplate reader with 405 nm filter.

Table 2 — (continued)

Method No.	Method type	Number of channels individually analysed	Plate type	Fluid type	Volume range [µl]	Typical accuracy of method (a,b) [%]	Typical precision of method (a,c) [%]	Environmental requirements of method	Published uncertainty of measurement of method (a,d)	Published traceability chain of method	Required equipment
Hybrid methods using gravimetry and photometry											
6.2.6 B.6	Gravimetry and photometry with Tartrazine	1 to 384	96-wells	Aqueous	1 – 5	0,8	1	Temperature 18 °C to 25 °C, Stability < ± 0,5 °C RH > 50 %	n/a	n/a	Balance < 0,01 mg; T, RH, P measuring equipment; Absorbance reader; Microplate shaker; Centrifuge tubes 1,5 ml; Flat bottom microplates; DI water; Tartrazine.
					5 – 20	0,6	0,7				
					20 – 300	0,2	0,5				
			384-wells	Aqueous	1 – 5	1	1,5				
					5 – 20	0,4	0,9				
6.2.7 B.7	Photometry with <i>p</i> -nitrophenol, plus gravimetry	1 to 384	96-wells	Aqueous, other liquids possible	0,1 – 200	0,1 - 2 µl: < 1 2 – 200 µl: < 0,2	< 0,5	Temperature variation: < ± 1 °C RH variation: < ± 10 %	[16]	[16]	Microplate reader with 405 nm and 620 nm filters; Analytical balance, readability 0,01 mg or better; Microplate shaker; Microplate Centrifuge; Clear flat bottom microplates; Sealing tape; Test reagents.
			384-wells		0,1 – 50						

Table 2 — (continued)

Method No.	Method type	Number of channels individually analysed	Plate type	Fluid type	Volume range [μl]	Typical accuracy of method (a,b) [%]	Typical precision of method (a,c) [%]	Environmental requirements of method	Published uncertainty of measurement of method (a,d)	Published traceability chain of method	Required equipment
6.2.8 B.8	Gravimetry and photometry with Ponceau S	96 or 384	96-wells (k)	Aqueous	2 – 100	0,5	0,4	Temperature variation: < ± 1 °C RH variation: < ± 10 %	[17]	n/a	Balance < 0,1 mg; T, RH, P measuring equipment; Microplate reader with 540 nm filter; Flat bottom microplates; Pipettes; Tips; DI Water; Ponceau S.

Table 2 — (continued)

Method No.	Method type	Number of channels individually analysed	Plate type	Fluid type	Volume range [μl]	Typical accuracy of method (a,b) [%]	Typical precision of method (a,c) [%]	Environmental requirements of method	Published uncertainty of measurement of method (a,d)	Published traceability chain of method	Required equipment
6.3.1 B.12	Gravimetry (closed method)	1-16 channels	n/a	Aqueous	10 - 1000	≤ 0,5	≤ 0,5	Temperature: 15 °C to 35 °C RH: 15 % to 85 % without condensation Pressure: up to 2,000 m above sea level	n/a	[18]	Hamilton FV2 Kit
6.3.1 B.12	Gravimetry and photometry (closed method)	1 to 384	96-wells 384-wells	Aqueous	5 - 1000 2 - 50	≤ 1,0 ≤ 1,0	≤ 1,0 ≤ 1,0	Temperature: 15 °C to 35 °C RH: 15 % to 85 % without condensation Pressure: up to 2,000 m above sea level	n/a	[18]	Hamilton FV2 Kit

Table 2 — (continued)

Method No.	Method type	Number of channels individually analysed	Plate type	Fluid type	Volume range [μl]	Typical accuracy of method (a,b) [%]	Typical precision of method (a,c) [%]	Environmental requirements of method	Published uncertainty of measurement of method (a,d)	Published traceability chain of method	Required equipment
Other measurement methods											
6.2.9 B.9	Pressure sensing	1 to 384	96- or 384-well plate	Any Liquid	5 - 500	< 150 μl: < 5 μl > 150 μl: < 3 %	< 100 μl: < 2 μl > 100 μl: < 2 %	Temperature: 18 °C to 32 °C RH: 10 % to 90 % Elevation: < 3000 m	n/a	n/a	Stratec Tholos system
6.2.10 B.10	Calorimetric measurement	1 to 384	96- or 384-well plate	Any Liquid	1 - 10 10 - 300	0,5 0,5	3 1,5	Temperature: 15 °C to 30 °C RH: 40 % to 80 %	n/a	n/a	Integra calorimetric reader and measurement plates.
6.2.11 B.11	Optical image analysis	1 - 16 (in a row)	n/a	Any Liquid	Free flying droplets < 0,5	< 5	< 2	Temperature: 15 °C to 25 °C RH: ≥ 50 %	[19]	[19]	Stroboscopic camera or high-speed camera; Automatic image detection software.

Table 2 — (continued)

(a)	The numerical values for accuracy and precision are provided by the method author, based on their knowledge of the measurement method. Where supporting documentation of an uncertainty analysis is available, it is referenced in the uncertainty column. Evaluation of the correctness of these numerical values is proposed as future work as described in Clause 9 .
(b)	These values should be equivalent to a 95 % confidence that the true value of measured volume lies within these bounds. Where an uncertainty analysis has been performed, this value is expressed as an expanded uncertainty of the measurement result ($k = 2$), and the reference is provided in the uncertainty column.
(c)	These values approximate the random error of the measurement method expressed as either a standard deviation with units of microlitres or as a percentage CV. Where supporting documentation of precision is available, it is referenced in the uncertainty column.
(d)	Traceability is defined in 3.1.46 . Where documentation of measurement traceability is available, it is referenced in this column.
(e)	Volume range for DMSO: 0,2 µl to 10 µl.
(f)	Volume range for DMSO: 0,05 µl to 2,5 µl.
(g)	This value applies only to the average across the entire plate, and does not apply to the volume in a single well which would have a larger uncertainty.
(h)	These values are achieved by pre-diluting the test solution before filling the plate with 200 µl or 55 µl of diluted solution. If the test solution were delivered directly to the plate the uncertainty would be greater. See B.3 and references for details.
(i)	This method is a representative example; performance of the actual method is dependent on tip volume and head configuration.
(j)	This value includes the measurement uncertainty of the ALHS unit under test.
(k)	Adaptable to 384-well plates.

6.2 Methods for use with any ALHS platform (open methods)

The following test methods can be used for the volumetric performance evaluation of ALHS without restriction to a particular model, platform, or technology.

6.2.1 Ratiometric photometry

This method is a photometric method using the absorbances of two different dye solutions, Ponceau S (red) and copper chloride (blue), to determine the dispensed volume in each well of a 96- or 384-well microplate. Both dye solutions are delivered into the wells, mixed, and the absorbance at 520 nm and 730 nm is read in a microplate reader, which allows the determination of the accuracy and precision of the volume delivered into each individual well. A commercially available kit based on this method is offered by Artel, Inc. as Multichannel Verification System (MVS®)¹⁾.[\[20-24\]](#)

This kit allows the determination of volumes of aqueous solutions 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 DMSO solutions can be determined from 0,11 µl to 10 µl in 96-well plates, and from 0,01 to 2,5 µl in 384-well plates.

This method is suitable to determine the performance of ALHS dispensing heads with 1, 2, 4, 6, 8, 12, 16, 24, 96, or 384 channels. The operating environment for this method is 15 °C to 30 °C (19 °C to 30 °C for DMSO solutions), and it is not dependent on the prevailing relative humidity and barometric pressure at the test location.

Measurements performed with the Artel MVS kit are fully traceable to SI Units through primary reference standards maintained by NIST (National Institute of Standards and Technology, USA) and the NPL (National Physical Laboratory, UK), and through the calibrators provided with the kit.[\[12\]](#)

A detailed description of this method is provided in [B.1](#).

6.2.2 Gravimetry, single channel measurement

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.

This method can be utilized to evaluate the volumetric performance of an ALHS, provided accommodations are made for:

- the placement of the balance and the weighing vessel;
- the environmental conditions affecting the mass to volume conversion of the measurement;
- manufacturers' recommendations regarding good practices for ALHS liquid delivery and performance specifications are followed.

A detailed description of this method is provided in [B.2](#).

6.2.3 Gravimetry, full-plate measurement for correlation with photometry

This method allows a direct determination of the total liquid volume delivered to a microplate by a 96- or 384-channel liquid handling system. This method is suitable for the comparison of gravimetric and photometric results. It may be used directly on sample solutions when a large volume is delivered, and can also be combined with sample preparation by pre-dilution in order to accurately weigh small amounts of dye samples.

Immediately following the gravimetric measurement, volume in the filled microplate can be measured using a photometric method. Comparison between the gravimetric and photometric results can be

1) Multichannel Verification System (MVS®) is the trade name of a product supplied by Artel, Inc. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

used to measure systematic errors. For this application, the method must be performed with careful attention to detail.

This method uses a calibrated analytical balance with a resolution of 5 decimal places located on a vibration-free support and in a climate controlled environment ($\pm 0,5$ °C stability during equilibration and test period). Electrostatic forces are minimized, and evaporation is compensated by timed measurements of the dispense and measurement cycles.

Traceability to SI units is achieved through calibration of the balance and accounting for density and air buoyancy as described in [Annex C](#).

A detailed description of this method can be found in [B.3](#).

6.2.4 Gravimetric regression method for low volumes

The gravimetric regression method (GRM) is suitable for the measurement of very small liquid volumes, between 0,04 µl and 1 µl, where the evaporation of the test liquid during the measurement is significant. The method is based on a gravimetric balance as primary measurement device (similar to those described in ASTM E542, ISO 4787, and ISO 8655-6).

This method is intended to be used for non-contact dispensing devices (e.g. dispensing valves, acoustic dispensing or PipeJet-dispensing) that deliver the liquid volume as free flying droplet or jet to the balance receptacle. The method was developed, tested and validated using such non-contact dispensing technologies as devices under test.

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 dispensed 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 for compensation 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 accounted for in the estimation of the measurement uncertainty.

Typical test liquid volumes between 0,04 µl to 1,0 µl can be measured with an accuracy of 2 nl to 12 nl, and a precision of 2,8 % to 0,4 % CV.

Traceability of the results to SI standards is achieved through the calibrated balance.

A detailed description of the method is provided in [B.4](#).

6.2.5 Photometry using Orange G

This method describes the volumetric performance test of ALHS, using an aqueous liquid with dispensing heads with 1 to 384 channels. The measurement results are traceable to SI units through the use of a calibrated balance, calibrated pipettes, a calibrated microplate reader, and volumetric flasks.

A detailed description of this method is provided in [B.5](#).

6.2.6 Hybrid method: gravimetry and photometry with Tartrazine

The hybrid photo-gravimetric method allows the evaluation of volumetric performance of single- and multichannel ALHS by a combination of a gravimetric reference measurement with subsequent photometric measurements to characterize the other channels of the instrument.

This method can be used to calculate the accuracy and precision of the ALHS' volumetric performance. A detailed description of this method is provided in [B.6](#).

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

6.2.7 Hybrid method: photometry and gravimetry with *p*-nitrophenol

The method describes the performance evaluation of ALHS based on photometry, followed by gravimetry. In a first step, the random error (precision) is determined by an absorbance measurement in microplates using *p*-nitrophenol (synonym: 4-nitrophenol, abbr. *p*-NP). This dye is stable at room temperature and soluble in water (11,6 mg/ml at 20 °C), chloroform, methanol, DMSO, and ethanol (100 mg/ml at 20 °C). It has the absorption peak at 405 nm and at a pH > 9,2 which is realized by using 0,1 N NaOH as well as standard solvent and diluent. The coefficient of variation (CV, in %) is calculated from the absorbance measurement signals of individual microplate wells. Smaller test volumes are transferred in wells prefilled with 0,1, 1 N NaOH, where they have to be dispersed homogeneously before measurement. The dye concentrations of the different test solutions are specifically adapted to the test volumes to give always a microplate type specific constant final volume and a constant final dye concentration of 120 µl in all wells of the measurement plate, which is within the optimal dynamic range of the absorption reader. The random error (precision) is always determined first in the evaluation of an automated multichannel pipetting system, followed by a gravimetric determination of the systematic error. The microplate absorption reader and the analytical balance have to be calibrated at regular intervals and the test conditions have to be considered strictly.

A detailed description of this method is provided in [B.7](#).

6.2.8 Hybrid method: gravimetry and photometry with Ponceau S

This method uses gravimetry and photometry to test the volumetric performance of an ALHS, and can be used to determine whether it fulfils desired performance specifications. The test used to verify the accuracy is based on gravimetric measurements and the test used to verify the precision is based on relative absorbance measurements. The method requires an analytical balance with a resolution of 0,1 mg and a microplate photometer with a measurement range of 0 Abs to 2 Abs and a resolution of 0,001 Abs capable of measurement at 540 nm wavelength.

A detailed description of this method is provided in [B.8](#).

6.2.9 Pressure sensing

This method can be used to determine the volume of liquids contained in the wells of a 96- or 384-well microplate. A patented, pressure-based technology measures accurately and repeatedly the contents of each well, independent of the physical properties of the microplate or material measured.^[25]

A detailed description of this method is provided in [B.9](#).

6.2.10 Calorimetric measurement

The calorimetric method allows to measure liquid volume transferred to individual cavities of a measurement plate. A short heat pulse of defined energy induces a temperature change in the liquid. The volume can then be calculated from the measured temperature increase of the liquid.^[26,27] This method can be used to characterize up to at least 384 liquid handling channels in parallel. Volumes of 1 µl to 300 µl can be measured with this method.

A detailed description of this method is provided in [B.10](#).

6.2.11 Optical image analysis

This method measures the volume of delivered liquids by analysing images acquired by a high-speed camera and stroboscopic illumination during the dispense cycle. It is suitable for ALHS, which deliver liquid volumes as sequence of discreet micro droplets.^[19]

A reference to this method is provided in [B.11](#).

6.3 Methods specific to an ALHS model or accessory (closed methods)

The following methods describe the volumetric performance evaluation of specific ALHS models or accessories as defined in the respective method. These methods may be modified to be used with ALHS platforms other than those described.

6.3.1 Gravimetry and hybrid method: gravimetry and photometry

This method describes the volumetric performance evaluation of Hamilton ALHS, using either gravimetry by itself, or gravimetry combined with photometry (hybrid method). Both approaches are described within this method, which is provided in [B.12](#).

The gravimetric method uses a high precision balance and 8 pipetting cycles per single channel at the specified volume. A new disposable tip is used for each pipetting cycle (aspiration/dispensation). For volumes > 20 µl, the applied dispense mode is jet dispense. Volumes ≤ 20 µl are dispensed in (liquid) surface mode.

The hybrid method, gravimetric and photometric, uses a high precision balance and a photometer. Eight pipetting cycles per single channel at the specified volume are performed. For each pipetting (aspiration/ dispensation) a new disposable tip is used. For volumes > 20 µl the applied dispense mode is jet dispense. Volumes ≤ 20 µl are dispensed in (liquid) surface mode.

6.3.2 Photometry with Tartrazine

This photometric method describes the volumetric performance evaluation of an Agilent Bravo 384-channel dispense head. It uses a 0,25 % (w/v) solution of tartrazine in DMSO, which is dispensed into the dry wells of a microplate (dry contact dispense). The tartrazine/DMSO solution is prepared by gravimetric and volumetric measurement of the constituents.

A detailed description of this method can be found in [B.13](#).