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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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Contents

Page

Foreword

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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
p.a.	*pro analysi* (purity grade of chemicals)
p-NP	*para*-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.

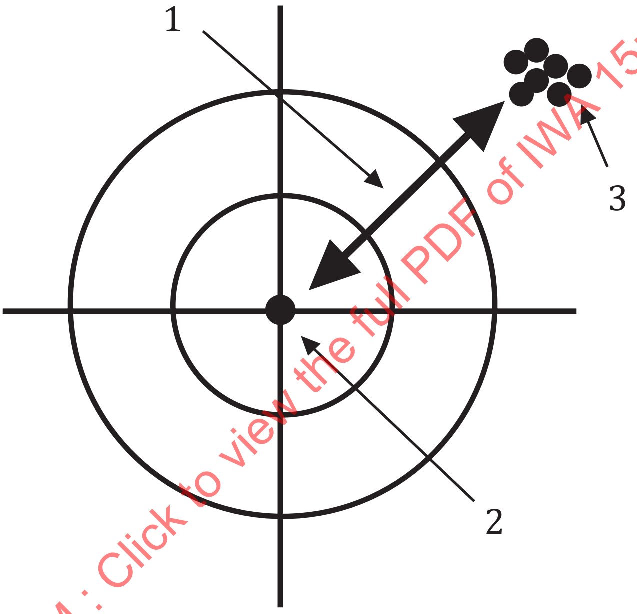
Automated liquid handling systems are designed to handle a variety of liquids of differing physical properties such as density, viscosity, surface tension and contact angle against solid surfaces. The volumetric performance of the ALHS can vary depending on these physical properties, so a description 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.

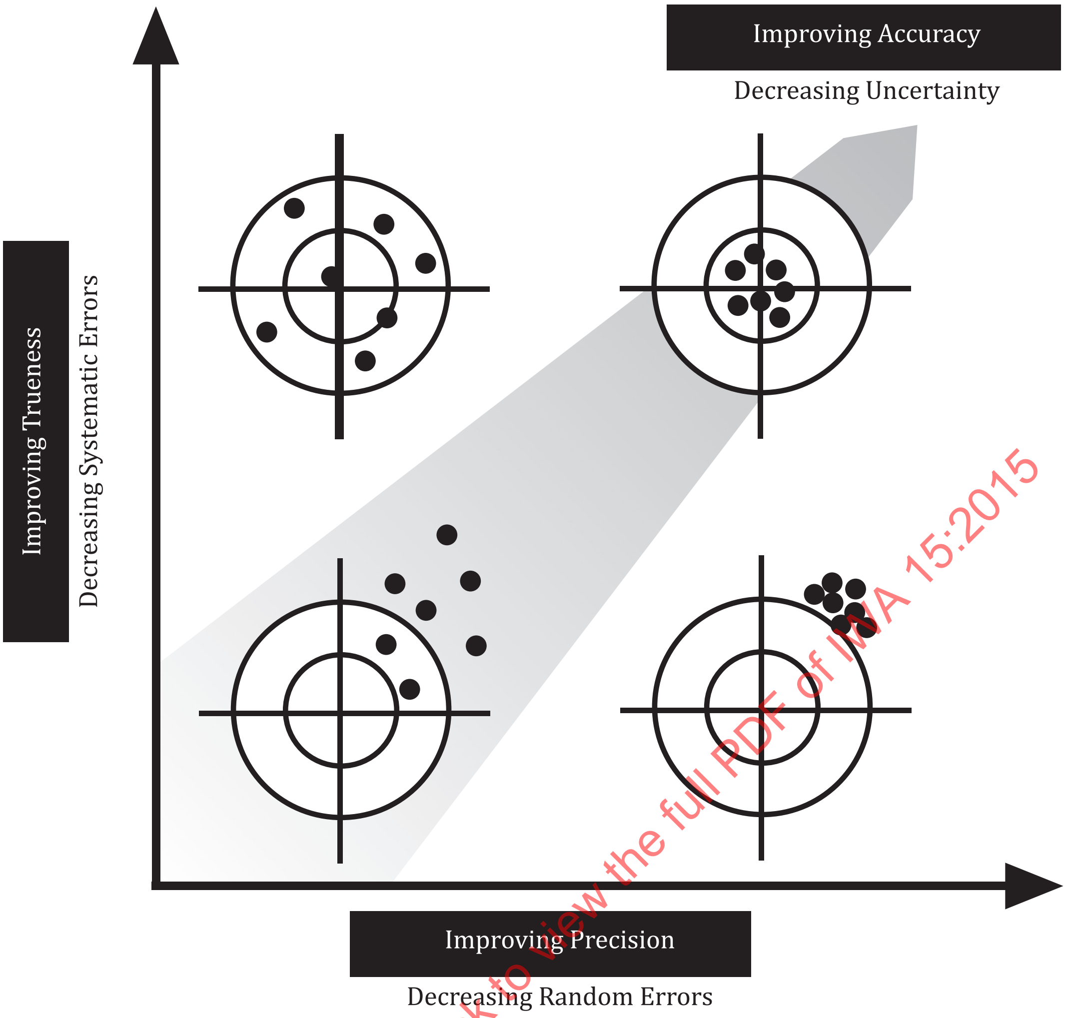


Figure 2 — Relationship between trueness, precision, and accuracy of an ALHS

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.

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

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 µl and the system delivers an actual volume of 97 µl, the systematic error is −3 µl (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.

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

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):

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

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).

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

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).

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

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).

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

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).

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

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.

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

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 programing 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).

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

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.

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

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

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

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).

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

where $\bar{V}_{\text{GA}}$ 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).

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

where $C_{V,\text{OA}}$ 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 ($C_{V,\text{C2C}}$), calculated as shown in Formula (14).

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

where $C_{V,\text{C2C}}$ 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]	[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 *p*-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) [%]	Environ-mental requirements of method	Published uncertainty of meas-urement 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	Aqueous	5 - 1000	≤ 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
			384-wells	Aqueous	2 - 50	≤ 1,0	≤ 1,0				

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) [%]	Environ-mental requirements of method	Published uncertainty of meas-ure-ment 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	0,5	3	Temperature: 15 °C to 30 °C RH: 40 % to 80 %	n/a	n/a	Integra calorimetric reader and measurement plates.
					10 - 300	0,5	1,5				
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

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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 (±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.

7 Specification of ALHS volumetric performance

7.1 Mandatory information to be supplied by manufacturer

The ALHS manufacturer shall provide information essential to the proper use of the apparatus and its accessories. This information shall be in the published specification, on which the purchase contract is based, or in instructions that accompany the automated liquid handling system, or in the certificate of conformity and shall be as follows.

a) The working volume range of the system shall be described.

b) The maximum permissible systematic and random errors shall be specified at individual volumes or across the specified volume range.

NOTE The working volume range of the ALHS can be wider than the specified volume range. Generally manufacturers' testing results only apply within the specified range.

c) The method(s) and environmental conditions for determining the trueness and precision shall be reported. When applicable, the description of the method shall include the tip type and instrument settings. Reference to methods described in this IWA is encouraged.

d) When applicable, the list of tips and their reference numbers, which the ALHS manufacturer recommends for use with the system shall be specified.

7.2 Optional information that can be supplied by manufacturer

a) Any recommendations to assist end-users for establishing a routine testing schedule and protocol.

b) An indication that volume variations may result from the measurement of liquids of different physical properties.

c) Any information regarding the care, cleaning and routine maintenance of the automated liquid handling system.

d) Upon request, information regarding the interaction of the materials of the automated liquid handling system with organic and inorganic solutions and solvents.

e) Information on possible liquid handling errors and recommended corrective measures to mitigate these.

8 Reporting

8.1 Reporting the results

8.1.1 General

The results of each test, calibration, or series of tests or calibrations carried out by the laboratory shall be reported accurately, clearly, unambiguously and objectively, and in accordance with any specific instructions in the test or calibration methods. The results shall be reported, usually in a test report or a calibration certificate (see NOTE 1), and shall include all the information requested by the customer and necessary for the interpretation of the test or calibration results and all information required by the method used. This information is normally that required by subclauses 8.1.2 and 8.1.3.

In the case of tests or calibrations performed for internal customers, or in the case of a written agreement with the customer, the results may be reported in a simplified way. Any information listed in 8.1.2 and 8.1.3, which is not reported to the customer shall be readily available in the laboratory which carried out the tests or calibrations.

In the case where the test or calibration report claims compliance with ISO/IEC 17025, the additional requirements of ISO/IEC 17025 are normative to this IWA 15.

NOTE 1 Test reports and calibration certificates are sometimes called test certificates and calibration reports, respectively.

NOTE 2 The test reports or calibration certificates may be issued as hard copy or by electronic data transfer provided that the requirements of this International Workshop Agreement are met.

8.1.2 Test reports and calibration certificates

Each test report or calibration certificate shall include at least the following information, unless the laboratory has valid reasons for not doing so:

a) a title (e.g. “Test Report” or “Calibration Certificate”);

b) the name and address of the laboratory, and the location where the tests and/or calibrations were carried out, if different from the address of the laboratory;

c) the name and address of the end-user, if known;

d) identification of the test process used; information on the test process may include reference to the respective method in this IWA;

e) a description of and unambiguous identification of the ALHS tested or calibrated;

f) the date(s) of performance of the test or calibration;

g) reference to the sampling plan and procedures used by the laboratory or other bodies where these are relevant to the validity or application of the results;

h) the test or calibration results with, where appropriate, the units of measurement;

i) the name(s), function(s) and signature(s) or equivalent identification of person(s) authorizing the test report or calibration certificate;

j) a record of measuring instruments, reagents, and supplies used in the testing process of the ALHS;

k) deviations from, additions to, or exclusions from the test method, and information on specific test conditions, such as environmental conditions;

l) where relevant, a statement of compliance/non-compliance with requirements or specifications, including acceptance criteria and units of measurement.

Test reports and calibration certificates should include the page number and total number of pages.

It is recommended that laboratories include a statement specifying that the test report or calibration certificate shall not be reproduced except in full, without written approval of the laboratory.

8.1.3 Calibration certificates

8.1.3.1 In addition to the requirements listed in 8.1.2, calibration certificates shall include the following, unless the laboratory has valid reasons for not doing so:

a) a unique identification of the calibration certificate (such as the serial number), and on each page an identification in order to ensure that the page is recognized as a part of the calibration certificate, and a clear identification of the end of the calibration certificate;

b) the conditions (e.g. environmental) under which the calibrations were made that have an influence on the measurement results;

c) the uncertainty of measurement and/or a statement of compliance with an identified metrological specification or clauses thereof;

d) evidence that the measurement results are traceable to the SI unit of volume, the litre.

NOTE Evidence of traceability includes documentation of the calibration status of the measuring instruments used in the testing process.

8.1.3.2 When an instrument for calibration has been adjusted or repaired, the calibration results before and after adjustment or repair, if available, shall be reported.

8.1.3.4 A calibration certificate (or calibration label) should contain a recommendation on the calibration interval, based upon the requirements of the ALHS end-user, if these are known. Legal regulations may supersede this recommendation.

9 Potential future work

A number of the participants in the IWA expressed an interest in continuing the technical work of standardization within the structure of ISO/TC 48. This clause presents a summary of possible subjects for additional work.

The following subjects were discussed, although there was insufficient time for inclusion in this workshop agreement:

- a classification as to the different types of ALHS.
- a table of maximum permissible errors based on type of ALHS.

NOTE 1 A number of liquid handling technologies have been developed that are not based on traditional mechanisms. These include acoustic liquid transfer, ink jet, pin tools, and other technologies which are not considered in detail in this IWA.

Workshop participants also propose the following suggestions to ISO/TC 48.

- Consideration should be given to harmonizing the work of this IWA with future revisions to the ISO 8655- series of standards.
- Technical work should begin to study the methods available, and reduce repetition and redundancy among these in accord with ISO/IEC Directives, Part 2 (2011), 6.3.5.6.

Consideration should be given to developing an ISO Technical Report on the estimation of uncertainty for liquid volume measurement methods for ALHS including:

- weighing in microplates;
- weighing by the gravimetric regression method;
- dual dye ratiometric photometry in microplates;
- single dye photometry in microplates;
- other methods such as calorimetric measurements and pressure sensing.

NOTE 2 Inter-comparisons between the measurement methods should be conducted as part of validating the uncertainty analysis in the proposed technical reports.

NOTE 3 Hybrid methods are considered as combinations of the uncertainty of the two parts - weighing and single dye photometry.

Consideration should be given to developing ISO standards for:

- terminology for ALHS;
- standard volume measurement methods for ALHS;
- address the subject of maximum permissible errors for ALHS with these goals:
  - give manufacturers guidance on format for presentation of performance claims;
  - give users guidance on appropriate volumetric performance for particular applications.

NOTE 4 A format for expressing volumetric performance specifications, and for interpolation of specifications between fixed points should be considered as an element in one of these standards.

NOTE 5 Figures 1 and 2 could be revised in the technical report on measurement uncertainty to show a more mathematically precise expression of these ideas, such as the use of bell curves or histograms in place of the targets.

ISO/TC 48 may wish to consider these recommendations within their overall consideration of laboratory equipment and piston operated volumetric apparatus.

In addition to these recommendations for future work within ISO/TC 48, the participants note that there is so far a lack of metrology support at national measurement institutions for volume measurements in the sub-microlitre range. Such support from national or regional metrology institutions would be a benefit to further progress in standardization of latest generation automated liquid handling systems.

Annex A
(informative)

Applications of descriptive statistics

A.1 General

This annex includes four examples showing how the descriptive statistics of this IWA can be applied to testing of ALHS. Each example includes a description of the experimental design including plate layouts, an arrangement of 'measurement results' in plate layout format, and calculations for various descriptive statistics using the formulae found in Clause 5.

Example 'measurement results' are not representative of the performance of any particular real ALHS. Instead, these results were generated using a random number generator. Nevertheless, they provide useful examples of how the descriptive statistics can be applied to data arranged in plate format and used to evaluate the volumetric performance of any ALHS configuration.

The following sections of this annex begin with a short discussion of experimental design before proceeding to four examples.

A.2 Experimental design

The examples in this annex illustrate the flexibility inherent in the indexing systems described in Clause 5. Every experimental design should include a decision about the elements shown in Table A.1. The first three elements (channels, runs and replicates) relate to the *l,m,n* indexing scheme described in Clause 5. For measurements made in microplates it is necessary to define the plate density (micro analytical 96- or 384-wells) along with the number of plates that will be used for the testing.

Table A.1 — Experimental design for each example

Element	Symbol	A.3	A.4	A.5	A.6
ALHS channels	L	8	8	96	8
Runs completed	M	1	3	1	1
Replicates/run	N	12	12	3	48
Wells	W	96	96	96	384
Number of microplates	P	1	3	3	1
Individual measurements	-	96	288	288	384

A.2.1 Plate layouts

In addition to the information in Table A.1, it is important to define the position and order that each channel delivers liquid into the microplate or set of microplates. Figures A.1 through A.3 show the order that will be used in the examples found in this annex.

Figure A.1 shows a common layout when delivering into a 96-well plate using an eight channel ALHS. In this figure, the eight channels proceed left to right, so that the first delivery from each channel is into the first column, then repeats in order across the entire plate, for a total of 12 deliveries per channel. This plate layout will be used in Examples 1 and 2.

		Column Number											
Channel	Row	1	2	3	4	5	6	7	8	9	10	11	12
$l = 1$	A	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
$l = 2$	B	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
$l = 3$	C	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
$l = 4$	D	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
$l = 5$	E	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
$l = 6$	F	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
$l = 7$	G	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
$l = 8$	H	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$

Figure A.1 — Plate layout for eight channels into a 96-well plate

Figure A.2 shows a seemingly trivial example where a 96-channel head is used to deliver into a 96-well plate. Here we see that each well in the plate contains replicate number 1. To collect additional replicates per channel, it is necessary to use additional plates. The lower part of Figure A.2 shows a recommended pattern for numbering channels when it is necessary to label channels by number rather than using a row and column address. This plate layout will be used in Example 3.

	Column Number											
Row	1	2	3	4	5	6	7	8	9	10	11	12
A	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$
B	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$
C	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$
D	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$
E	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$
F	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$
G	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$
H	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$

	Column Number											
Row	1	2	3	4	5	6	7	8	9	10	11	12
A	$l = 1$	$l = 2$	$l = 3$	$l = 4$	$l = 5$	$l = 6$	$l = 7$	$l = 8$	$l = 9$	$l = 10$	$l = 11$	$l = 12$
B	$l = 13$	$l = 14$	$l = 15$	$l = 16$	$l = 17$	$l = 18$	$l = 19$	$l = 20$	$l = 21$	$l = 22$	$l = 23$	$l = 24$
C	$l = 25$	$l = 26$	$l = 27$	$l = 28$	$l = 29$	$l = 30$	$l = 31$	$l = 32$	$l = 33$	$l = 34$	$l = 35$	$l = 36$
D	$l = 37$	$l = 38$	$l = 39$	$l = 40$	$l = 41$	$l = 42$	$l = 43$	$l = 44$	$l = 45$	$l = 46$	$l = 47$	$l = 48$
E	$l = 49$	$l = 50$	$l = 51$	$l = 52$	$l = 53$	$l = 54$	$l = 55$	$l = 56$	$l = 57$	$l = 58$	$l = 59$	$l = 60$
F	$l = 61$	$l = 62$	$l = 63$	$l = 64$	$l = 65$	$l = 66$	$l = 67$	$l = 68$	$l = 69$	$l = 70$	$l = 71$	$l = 72$
G	$l = 73$	$l = 74$	$l = 75$	$l = 76$	$l = 77$	$l = 78$	$l = 79$	$l = 80$	$l = 81$	$l = 82$	$l = 83$	$l = 84$
H	$l = 85$	$l = 86$	$l = 87$	$l = 88$	$l = 89$	$l = 90$	$l = 91$	$l = 92$	$l = 93$	$l = 94$	$l = 95$	$l = 96$

Figure A.2 — Plate layout for 96-channels into a 96-well plate

Figure A.3 shows a layout where an eight channel head delivers to a 384-well plate. In this layout, the eight channels make the first delivery ($n = 1$) into the first column of alternating rows (i.e. wells A1, C1, E1, O1). Then the channels proceed left to right across the entire plate until reaching the end of the row (micro analytical A24). Next, each channel moves downward, and continues delivering while travelling right to left along the second row to complete the operation. This plate layout will be used in Example 4.

		Column Number											
Channel	Row	1	2	3	4	5	6	7	8	9	10	11	12
$l = 1$	A	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
	B	$n = 48$	$n = 47$	$n = 46$	$n = 45$	$n = 44$	$n = 43$	$n = 42$	$n = 41$	$n = 40$	$n = 39$	$n = 38$	$n = 37$
$l = 2$	C	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
	D	$n = 48$	$n = 47$	$n = 46$	$n = 45$	$n = 44$	$n = 43$	$n = 42$	$n = 41$	$n = 40$	$n = 39$	$n = 38$	$n = 37$
$l = 3$	E	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
	F	$n = 48$	$n = 47$	$n = 46$	$n = 45$	$n = 44$	$n = 43$	$n = 42$	$n = 41$	$n = 40$	$n = 39$	$n = 38$	$n = 37$
$l = 4$	G	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
	H	$n = 48$	$n = 47$	$n = 46$	$n = 45$	$n = 44$	$n = 43$	$n = 42$	$n = 41$	$n = 40$	$n = 39$	$n = 38$	$n = 37$
$l = 5$	I	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
	J	$n = 48$	$n = 47$	$n = 46$	$n = 45$	$n = 44$	$n = 43$	$n = 42$	$n = 41$	$n = 40$	$n = 39$	$n = 38$	$n = 37$
$l = 6$	K	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
	L	$n = 48$	$n = 47$	$n = 46$	$n = 45$	$n = 44$	$n = 43$	$n = 42$	$n = 41$	$n = 40$	$n = 39$	$n = 38$	$n = 37$
$l = 7$	M	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
	N	$n = 48$	$n = 47$	$n = 46$	$n = 45$	$n = 44$	$n = 43$	$n = 42$	$n = 41$	$n = 40$	$n = 39$	$n = 38$	$n = 37$
$l = 8$	O	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$	$n = 9$	$n = 10$	$n = 11$	$n = 12$
	P	$n = 48$	$n = 47$	$n = 46$	$n = 45$	$n = 44$	$n = 43$	$n = 42$	$n = 41$	$n = 40$	$n = 39$	$n = 38$	$n = 37$

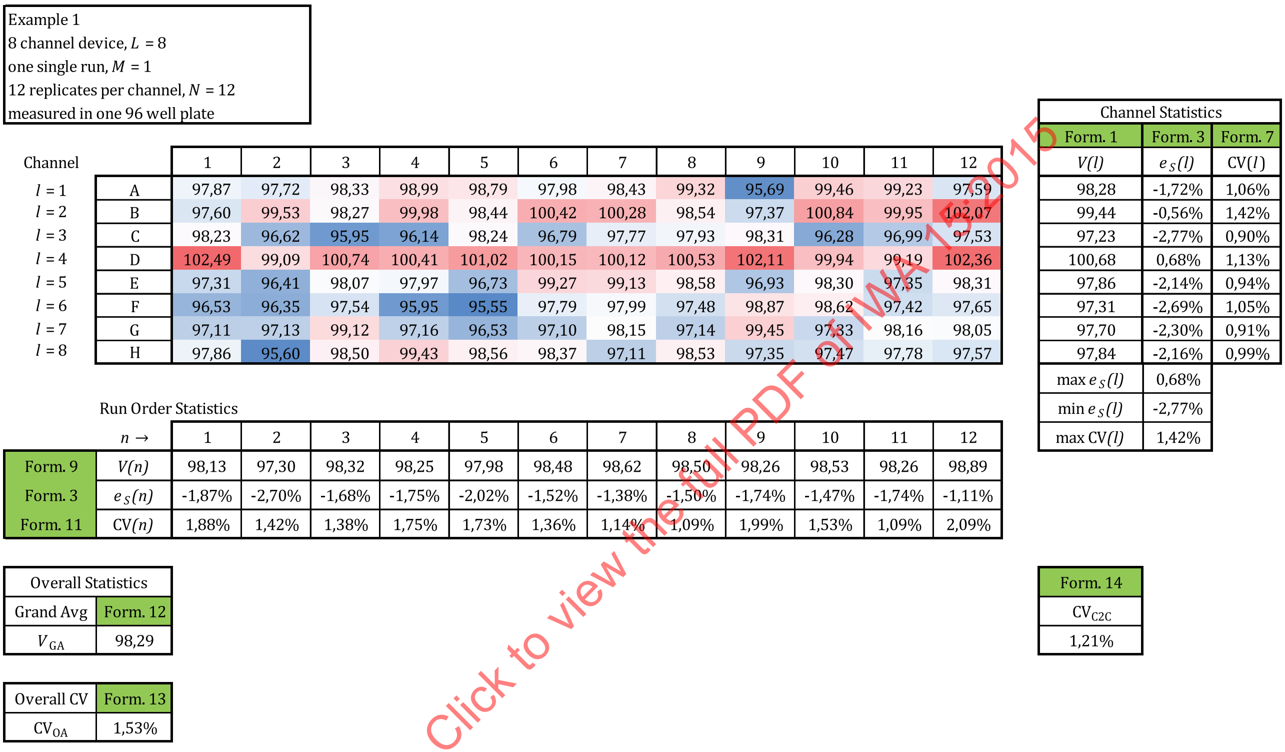
Column Number											
13	14	15	16	17	18	19	20	21	22	23	24
$n = 13$	$n = 14$	$n = 15$	$n = 16$	$n = 17$	$n = 18$	$n = 19$	$n = 20$	$n = 21$	$n = 22$	$n = 23$	$n = 24$
$n = 36$	$n = 35$	$n = 34$	$n = 33$	$n = 32$	$n = 31$	$n = 30$	$n = 29$	$n = 28$	$n = 27$	$n = 26$	$n = 25$
$n = 13$	$n = 14$	$n = 15$	$n = 16$	$n = 17$	$n = 18$	$n = 19$	$n = 20$	$n = 21$	$n = 22$	$n = 23$	$n = 24$
$n = 36$	$n = 35$	$n = 34$	$n = 33$	$n = 32$	$n = 31$	$n = 30$	$n = 29$	$n = 28$	$n = 27$	$n = 26$	$n = 25$
$n = 13$	$n = 14$	$n = 15$	$n = 16$	$n = 17$	$n = 18$	$n = 19$	$n = 20$	$n = 21$	$n = 22$	$n = 23$	$n = 24$
$n = 36$	$n = 35$	$n = 34$	$n = 33$	$n = 32$	$n = 31$	$n = 30$	$n = 29$	$n = 28$	$n = 27$	$n = 26$	$n = 25$
$n = 13$	$n = 14$	$n = 15$	$n = 16$	$n = 17$	$n = 18$	$n = 19$	$n = 20$	$n = 21$	$n = 22$	$n = 23$	$n = 24$
$n = 36$	$n = 35$	$n = 34$	$n = 33$	$n = 32$	$n = 31$	$n = 30$	$n = 29$	$n = 28$	$n = 27$	$n = 26$	$n = 25$
$n = 13$	$n = 14$	$n = 15$	$n = 16$	$n = 17$	$n = 18$	$n = 19$	$n = 20$	$n = 21$	$n = 22$	$n = 23$	$n = 24$
$n = 36$	$n = 35$	$n = 34$	$n = 33$	$n = 32$	$n = 31$	$n = 30$	$n = 29$	$n = 28$	$n = 27$	$n = 26$	$n = 25$
$n = 13$	$n = 14$	$n = 15$	$n = 16$	$n = 17$	$n = 18$	$n = 19$	$n = 20$	$n = 21$	$n = 22$	$n = 23$	$n = 24$
$n = 36$	$n = 35$	$n = 34$	$n = 33$	$n = 32$	$n = 31$	$n = 30$	$n = 29$	$n = 28$	$n = 27$	$n = 26$	$n = 25$
$n = 13$	$n = 14$	$n = 15$	$n = 16$	$n = 17$	$n = 18$	$n = 19$	$n = 20$	$n = 21$	$n = 22$	$n = 23$	$n = 24$
$n = 36$	$n = 35$	$n = 34$	$n = 33$	$n = 32$	$n = 31$	$n = 30$	$n = 29$	$n = 28$	$n = 27$	$n = 26$	$n = 25$
$n = 13$	$n = 14$	$n = 15$	$n = 16$	$n = 17$	$n = 18$	$n = 19$	$n = 20$	$n = 21$	$n = 22$	$n = 23$	$n = 24$
$n = 36$	$n = 35$	$n = 34$	$n = 33$	$n = 32$	$n = 31$	$n = 30$	$n = 29$	$n = 28$	$n = 27$	$n = 26$	$n = 25$

Figure A.3 — Plate layout for 8 channels into a 384-well plate

A.3 Example 1: Eight channels into a single 96-well plate

A.3.1 Experimental design and measurement results

The experimental design for this example is an eight channel ALHS delivering 12 replicates into a 96-well plate. The plate layout is as shown in Figure A.1, and the experiment is accomplished in a single run using only one plate. The measurement results and calculated descriptive statistics are shown in Figure A.4.



Example 1
8 channel device, L = 8
one single run, M = 1
12 replicates per channel, N = 12
measured in one 96 well plate

Channel		1	2	3	4	5	6	7	8	9	10	11	12
l = 1	A	97,87	97,72	98,33	98,99	98,79	97,98	98,43	99,32	95,69	99,46	99,23	97,59
l = 2	B	97,60	99,53	98,27	99,98	98,44	100,42	100,28	98,54	97,37	100,84	99,95	102,07
l = 3	C	98,23	96,62	95,95	96,14	98,24	96,79	97,77	97,93	98,31	96,28	96,99	97,53
l = 4	D	102,49	99,09	100,74	100,41	101,02	100,15	100,12	100,53	102,11	99,94	99,19	102,36
l = 5	E	97,31	96,41	98,07	97,97	96,73	99,27	99,13	98,58	96,93	98,30	97,35	98,31
l = 6	F	96,53	96,35	97,54	95,95	95,55	97,79	97,99	97,48	98,87	98,62	97,42	97,65
l = 7	G	97,11	97,13	99,12	97,16	96,53	97,10	98,15	97,14	99,45	97,33	98,16	98,05
l = 8	H	97,86	95,60	98,50	99,43	98,56	98,37	97,11	98,53	97,35	97,47	97,78	97,57

Channel Statistics		
Form. 1	Form. 3	Form. 7
$V(l)$	$e_S(l)$	CV(l)
98,28	-1,72%	1,06%
99,44	-0,56%	1,42%
97,23	-2,77%	0,90%
100,68	0,68%	1,13%
97,86	-2,14%	0,94%
97,31	-2,69%	1,05%
97,70	-2,30%	0,91%
97,84	-2,16%	0,99%
max $e_S(l)$	0,68%	
min $e_S(l)$	-2,77%	
max CV(l)	1,42%	

Run Order Statistics

	n →	1	2	3	4	5	6	7	8	9	10	11	12
Form. 9	$V(n)$	98,13	97,30	98,32	98,25	97,98	98,48	98,62	98,50	98,26	98,53	98,26	98,89
Form. 3	$e_S(n)$	-1,87%	-2,70%	-1,68%	-1,75%	-2,02%	-1,52%	-1,38%	-1,50%	-1,74%	-1,47%	-1,74%	-1,11%
Form. 11	CV(n)	1,88%	1,42%	1,38%	1,75%	1,73%	1,36%	1,14%	1,09%	1,99%	1,53%	1,09%	2,09%

Overall Statistics	
Grand Avg	Form. 12
V_{GA}	98,29

Overall CV	Form. 13
CV_{OA}	1,53%

Form. 14
CV_{C2C}
1,21%

Figure A.4 — Results and calculated statistics for Example 1

A.3.2 Statistics results

Channel statistics are shown to the right of the data. The eight channel averages are calculated by applying Formula (1) to each of the eight rows, and relative systematic error (e_S) is calculated by comparing each of these channel averages to the target volume using Formula (3). Channel CV is calculated for each row using Formula (7).

Run order statistics are shown below the data. The 12 run order average volumes are calculated using Formula (9) and the relative systematic error is calculated by using Formula (3). The run order CV is calculated by applying Formula (11) to the eight measurements in each column.

Overall statistics are shown at the bottom left part of Figure A.4. The grand average volume (V_{GA}) and overall CV (CV_{OA}) are calculated using Formulae (12) and (13), respectively.

The channel to channel CV (CV_{C2C}) is shown in the lower right and is calculated using the eight channel means in the column shown above the channel to channel CV box in Figure A.4. In this example, the overall CV result is 1,53 % which is somewhat larger than the channel to channel CV (1,21 %). Also, the maximum channel CV is 1,42 % [maximum of all the CVs calculated by Formula (7)]. In this example, we see that both channel to channel differences and channel CV contribute to overall CV.

A.4 Example 2: Eight channels into three 96-well plates

A.4.1 Experimental design and measurement results

The experimental design for this example is an extension of Example 1, again with an eight channel ALHS delivering 12 replicates into a 96-well plate. The plate layout is as shown in Figure A.1. However, in Example 2, the experiment consists of three separate runs using three plates. The measurement results and calculated descriptive statistics are shown in Figure A.5. This example shows how multiple runs may be combined for a more thorough testing.

A.4.2 Statistics results for Example 2

Channel statistics are shown to the right of each set of plate data. The eight channel averages are calculated by applying Formula (1) to each of the eight rows, and relative systematic error is calculated by comparing each of these channel averages to the target volume using Formula (3). Channel CV is calculated for each row using Formula (7).

NOTE Because the experiment was defined to be three different runs, we have calculated three different sets of channel statistics here. However, if the experiment had been defined as one run across three plates ($N = 24$), then only one set of channel statistics would have been calculated. It is important to precisely define the experiment in order to know how the data should be analysed.

Run order statistics are shown below the last plate of data. The 12 run order average volumes are calculated using Formula (9) which includes a summation over the $M = 3$ runs; 24 data points are averaged for each of these calculated means. The relative systematic error is calculated by using Formula (3). The run order CV is calculated by applying Formula (11) to the eight measurements in each column for each plate (24 volume measurement points for each run order CV result).

Overall statistics are shown at the bottom left part of Figure A.5. The grand average volume and overall CV are calculated using Formulae (12) and (13), respectively.

Because this experiment includes three runs, we have the opportunity to calculate channel statistics which are combined from all three runs. These are shown in the lower right and use Formulae (4) and (8).

Lastly, the channel to channel CV is shown in the extreme lower right and is calculated using the eight channel mean volumes calculated by Formula (4) and shown in the column above the channel to channel CV box. When multiple runs are included in a single experiment, Formula (14) shows that the combined channel statistics should be used, as is illustrated in this example.

The overall CV result in Example 2 is similar to the channel to channel CV. From this we see that channel to channel differences are the dominant contributor to overall CV in this example.

Example 2
8 channel device, $L = 8$
three replicate runs, $M = 3$
12 replicates per channel, $N = 12$
measured in three 96 well plates

Run 1, $m = 1$

	1	2	3	4	5	6	7	8	9	10	11	12
A	101,72	101,17	100,87	102,38	101,44	100,92	101,17	102,21	100,79	103,27	101,44	102,19
B	97,03	96,15	98,14	97,67	98,51	98,14	97,41	99,15	99,01	96,82	97,95	97,36
C	99,55	101,91	97,83	100,25	99,72	99,60	100,04	97,47	98,78	99,35	99,91	101,99
D	99,44	100,09	100,54	100,23	100,01	100,02	101,32	100,99	100,10	99,45	101,89	98,76
E	103,21	102,69	105,47	102,15	102,57	103,80	103,99	103,90	105,39	105,27	103,35	104,53
F	102,33	103,78	101,74	102,14	102,83	101,94	103,73	103,37	100,97	103,74	102,09	102,37
G	103,61	101,71	101,20	102,11	102,39	102,31	101,00	102,13	104,14	102,92	101,29	102,54
H	100,78	100,78	100,53	100,99	100,58	99,10	99,27	98,20	100,73	99,84	100,67	99,58

Channel Statistics, Run 1		
Form. 1	Form. 3	Form. 7
$V(l,m)$	$e_S(l,m)$	CV(l,m)
101,63	1,63%	0,74%
97,78	-2,22%	0,91%
99,70	-0,30%	1,36%
100,24	0,24%	0,86%
103,86	3,86%	1,09%
102,59	2,59%	0,88%
102,28	2,28%	0,93%
100,09	0,09%	0,88%
max $e_S(l,m)$	3,86%	
min $e_S(l,m)$	-2,22%	
max CV(l,m)	1,36%	

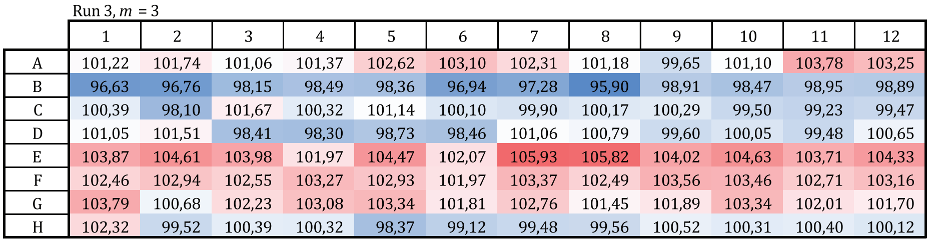
Run 2, $m = 2$

	1	2	3	4	5	6	7	8	9	10	11	12
A	102,75	101,86	100,11	102,28	100,49	101.37	102.17	101.09	102,54	101.81	101,16	102,37
B	95,86	96,51	99,81	99,12	97,14	97,94	99,26	98,25	96,94	98,89	96,30	95,05
C	101,22	99,44	99,59	99,29	99,59	97,86	98,92	98,19	98,01	99,91	99,79	99,53
D	99,22	101,46	99,09	100,01	100,33	99,22	100,39	101,03	100,30	99,63	99,18	99,35
E	103,49	102,06	101,60	103,17	104,01	103,54	104,55	104,50	105,74	103,99	103,25	101,88
F	102,86	105,07	102,15	102,51	100,86	101,60	104,07	104,36	102,79	103,89	102,55	102,54
G	102,09	101,11	100,88	101,52	103,30	101,82	100,84	101,15	101,63	103,00	103,13	102,76
H	100,27	100,29	101,33	99,52	96,28	98,66	100,27	101,69	99,92	100,46	99,21	101,61

Channel Statistics, Run 2		
Form. 1	Form. 3	Form. 7
$V(l,m)$	$e_S(l,m)$	CV(l,m)
101,67	1,67%	0,82%
97,59	-2,41%	1,55%
99,28	-0,72%	0,94%
99,93	-0,07%	0,78%
103,48	3,48%	1,17%
102,94	2,94%	1,17%
101,94	1,94%	0,89%
99,96	-0,04%	1,48%
max $e_S(l,m)$	3,48%	
min $e_S(l,m)$	-2,41%	
max CV(l,m)	1,55%	

Figure A.5 (*continued on the next page*)

Run 3, m = 3



	1	2	3	4	5	6	7	8	9	10	11	12
A	101,22	101,74	101,06	101,37	102,62	103,10	102,31	101,18	99,65	101,10	103,78	103,25
B	96,63	96,76	98,15	98,49	98,36	96,94	97,28	95,90	98,91	98,47	98,95	98,89
C	100,39	98,10	101,67	100,32	101,14	100,10	99,90	100,17	100,29	99,50	99,23	99,47
D	101,05	101,51	98,41	98,30	98,73	98,46	101,06	100,79	99,60	100,05	99,48	100,65
E	103,87	104,61	103,98	101,97	104,47	102,07	105,93	105,82	104,02	104,63	103,71	104,33
F	102,46	102,94	102,55	103,27	102,93	101,97	103,37	102,49	103,56	103,46	102,71	103,16
G	103,79	100,68	102,23	103,08	103,34	101,81	102,76	101,45	101,89	103,34	102,01	101,70
H	102,32	99,52	100,39	100,32	98,37	99,12	99,48	99,56	100,52	100,31	100,40	100,12

Channel Statistics, Run 3		
Form. 1	Form. 3	Form. 7
$V(l,m)$	$e_S(l,m)$	$CV(l,m)$
101,87	1,87%	1,15%
97,81	-2,19%	1,07%
100,02	0,02%	0,91%
99,84	-0,16%	1,17%
104,12	4,12%	1,15%
102,91	2,91%	0,47%
102,34	2,34%	0,90%
100,04	0,04%	0,97%
max $e_S(l,m)$	4,12%	
min $e_S(l,m)$	-2,19%	
max $CV(l,m)$	1,17%	

Run Order Statistics, all three runs combined

	n →	1	2	3	4	5	6	7	8	9	10	11	12
Form. 9	$V(n)$	101,13	100,91	100,80	100,94	100,83	100,48	101,27	101,04	101,09	101,38	100,98	101,08
Form. 3	$e_S(n)$	1,13%	0,91%	0,80%	0,94%	0,83%	0,48%	1,27%	1,04%	1,09%	1,38%	0,98%	1,08%
Form. 11	$CV(n)$	2,23%	2,30%	1,81%	1,62%	2,20%	1,95%	2,21%	2,39%	2,25%	2,25%	1,95%	2,23%

Overall Statistics	
Grand Avg	Form. 12
V_{GA}	101,00

← calculated as average of all 288 wells

Overall CV	Form. 13
CV_{OA}	2,10%

← calculated as CV of all 288 wells

Channel Statistics, All Runs		
Form. 4	Form. 3	Form. 8
$V(l)$	$e_S(l)$	$CV(l)$
101,72	1,72%	0,92%
97,73	-2,27%	1,21%
99,67	-0,33%	1,09%
100,00	0,00%	0,95%
103,82	3,82%	1,14%
102,81	2,81%	0,89%
102,19	2,19%	0,91%
100,03	0,03%	1,14%
max $eS(l)$	3,82%	
min $eS(l)$	-2,27%	
max $CV(l)$	1,21%	

Form. 14
CV_{C2C}
1,97%

Figure A.5 — Results and calculated statistics for Example 2

A.5 Example 3: 96-channels into three 96-well plates

A.5.1 Experimental design and measurement results for Example 3

The experimental design for Example 3 is a 96-channel head delivering 3 replicates into a series of three 96-well plates. The plate layout is as shown in Figure A.2, and the experiment consists of only one run. The measurement results and some calculated descriptive statistics are shown in Figure A.6. Additional calculated statistics are shown in Figure A.7.

A.5.2 Statistics results for Example 3

Run order statistics are shown to the right of each set of plate data. Because each plate contains only one delivery from each channel the mean and CV across each plate is a run order statistic. This example shows how the interpretation of the descriptive statistic depends on the channel arrangement and experimental design.

Calculation of channel statistics is shown in Figure A.7. The 96 different channel averages are calculated by applying Formula (1) to the same well in each of the three plates. Channel CV is calculated in the same manner using Formula (7). The channel CV results are heat mapped, green to yellow. The seemingly larger values in the yellow cells are a consequence of the experimental design and are explained in the following note.

NOTE Because the experiment design has $N = 3$ replicates the calculated CV has 2 degrees of freedom. This results in a wider range of values for the channel CV. In Figure A.7, the channel CV results range from (0,07 % up to 2,01 %). This range is not the result of different performance in channels as all results were obtained with random number generator modelling identical CV for each channel. This example illustrates the difficulty of estimating CV with a small number of replicates.

As with other examples, calculation of grand average volume and overall CV use Formulae (12) and (13), respectively, and the statistics are calculated over all 288 measurement results in the set. The channel to channel CV is calculated using all 96 of the channel averages shown at the top of Figure A.7.

Example 3
96 channel device, L = 96
one run, M = 1
3 replicates per channel, N = 3
measured in three 96 well plates

Replicate 1, n = 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	98,43	98,81	98,39	100,34	99,36	98,34	98,49	102,71	102,02	94,83	98,09	103,42
B	99,93	97,58	100,76	98,21	95,77	103,25	96,26	99,94	100,39	99,96	95,20	99,59
C	103,28	100,27	99,64	101,40	98,99	99,92	99,98	100,50	103,40	99,59	96,34	102,09
D	99,29	100,41	96,95	103,55	98,32	96,17	94,54	102,70	102,96	98,59	102,60	104,71
E	98,77	100,24	101,83	101,65	98,09	103,05	97,34	96,74	99,64	98,42	99,64	102,72
F	99,08	102,96	101,07	101,92	100,03	97,56	102,43	100,11	98,18	100,63	105,77	100,67
G	102,24	101,84	99,32	100,64	98,67	101,21	101,08	102,43	103,59	98,05	103,20	100,84
H	98,18	100,74	100,69	102,00	97,40	98,53	103,11	100,65	100,97	99,72	96,52	100,19

Run Order Statistics, n = 1		
Form. 9	Form. 3	Form. 11
$V(n)$	$e_S(n)$	CV(n)
100,07	0,07%	2,30%

Replicate 2, n = 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	98,74	100,27	98,38	99,08	96,88	97,69	97,66	101,24	101,25	95,56	98,31	102,92
B	98,07	96,85	99,27	98,34	97,83	101,93	95,50	98,34	100,16	100,36	96,69	100,44
C	100,23	100,86	102,30	102,29	99,95	99,66	99,18	100,65	101,31	98,14	95,73	101,52
D	98,46	99,00	97,37	101,94	98,90	96,56	94,66	99,11	101,99	97,68	101,32	102,14
E	98,61	101,55	100,17	98,77	98,43	102,56	97,64	99,05	99,78	99,23	98,46	101,27
F	99,92	104,86	100,28	100,17	101,65	99,98	99,76	100,00	98,22	99,46	105,33	98,49
G	102,98	103,50	98,75	100,50	98,52	101,65	102,28	101,89	103,09	98,44	103,18	101,08
H	97,85	102,29	100,85	105,04	98,56	99,92	103,30	98,73	100,87	99,92	98,04	102,42

Run Order Statistics, n = 2		
Form. 9	Form. 3	Form. 11
$V(n)$	$e_S(n)$	CV(n)
99,90	-0,10%	2,15%

Replicate 3, n = 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	98,61	99,50	98,70	99,55	97,34	98,56	96,87	102,61	101,70	93,64	101,05	102,18
B	99,67	100,46	100,85	98,23	97,97	102,70	95,10	100,39	103,25	98,80	95,70	99,84
C	101,01	101,21	101,07	100,88	101,05	101,17	100,55	99,85	102,85	98,79	97,43	101,11
D	98,36	99,07	97,92	102,76	99,15	96,18	94,55	100,12	101,17	96,76	104,88	100,63
E	101,15	99,80	100,06	100,23	97,20	102,26	98,66	95,37	97,76	99,08	98,09	100,23
F	99,46	103,70	98,74	103,38	99,93	99,23	101,56	99,23	98,05	99,30	103,24	100,00
G	104,09	102,95	98,91	101,70	98,41	102,47	101,35	101,29	103,35	98,92	101,70	101,53
H	97,15	102,38	100,38	103,49	98,00	97,35	102,77	101,20	101,22	101,35	96,20	102,31

Run Order Statistics, n = 3		
Form. 9	Form. 3	Form. 11
$V(n)$	$e_S(n)$	CV(n)
99,98	-0,02%	2,27%

Figure A.6 — Results and some calculated statistics for Example 3

Example 3 (continued)
96 channel device, L = 96
one run, M = 1
3 replicates per channel, N = 3
measured in three 96 well plates

Average Volume by Channel, $V(l)$

Form. 1	1	2	3	4	5	6	7	8	9	10	11	12
A	98,59	99,52	98,49	99,65	97,86	98,20	97,67	102,19	101,66	94,68	99,15	102,84
B	99,22	98,29	100,29	98,26	97,19	102,63	95,62	99,56	101,27	99,71	95,86	99,96
C	101,51	100,78	101,00	101,53	100,00	100,25	99,90	100,33	102,52	98,84	96,50	101,57
D	98,70	99,49	97,41	102,75	98,79	96,30	94,58	100,64	102,04	97,68	102,94	102,49
E	99,51	100,53	100,69	100,22	97,91	102,62	97,88	97,06	99,06	98,91	98,73	101,41
F	99,49	103,84	100,03	101,82	100,54	98,92	101,25	99,78	98,15	99,80	104,78	99,72
G	103,10	102,76	98,99	100,95	98,53	101,78	101,57	101,87	103,34	98,47	102,69	101,15
H	97,73	101,81	100,64	103,51	97,99	98,60	103,06	100,19	101,02	100,33	96,92	101,64

Form. 14
CV_{C2C}
2,10%

Systematic Error by Channel, $e_S(l)$

Form. 3	1	2	3	4	5	6	7	8	9	10	11	12
A	-1,41%	-0,48%	-1,51%	-0,35%	-2,14%	-1,80%	-2,33%	2,19%	1,66%	-5,32%	-0,85%	2,84%
B	-0,78%	-1,71%	0,29%	-1,74%	-2,81%	2,63%	-4,38%	-0,44%	1,27%	-0,29%	-4,14%	-0,04%
C	1,51%	0,78%	1,00%	1,53%	0,00%	0,25%	-0,10%	0,33%	2,52%	-1,16%	-3,50%	1,57%
D	-1,30%	-0,51%	-2,59%	2,75%	-1,21%	-3,70%	-5,42%	0,64%	2,04%	-2,32%	2,94%	2,49%
E	-0,49%	0,53%	0,69%	0,22%	-2,09%	2,62%	-2,12%	-2,94%	-0,94%	-1,09%	-1,27%	1,41%
F	-0,51%	3,84%	0,03%	1,82%	0,54%	-1,08%	1,25%	-0,22%	-1,85%	-0,20%	4,78%	-0,28%
G	3,10%	2,76%	-1,01%	0,95%	-1,47%	1,78%	1,57%	1,87%	3,34%	-1,53%	2,69%	1,15%
H	-2,27%	1,81%	0,64%	3,51%	-2,01%	-1,40%	3,06%	0,19%	1,02%	0,33%	-3,08%	1,64%

max $e_S(l)$	4,78%
min $e_S(l)$	-5,42%

Coefficient of Variation by Channel, CV(l)

Form. 7	1	2	3	4	5	6	7	8	9	10	11	12
A	0,16%	0,73%	0,19%	0,64%	1,35%	0,46%	0,83%	0,81%	0,38%	1,03%	1,66%	0,61%
B	1,02%	1,94%	0,88%	0,07%	1,26%	0,64%	0,61%	1,08%	1,70%	0,81%	0,79%	0,44%
C	1,56%	0,47%	1,32%	0,70%	1,03%	0,80%	0,69%	0,42%	1,06%	0,74%	0,89%	0,48%
D	0,51%	0,80%	0,50%	0,78%	0,43%	0,23%	0,07%	1,84%	0,88%	0,94%	1,75%	2,01%
E	1,43%	0,91%	0,99%	1,44%	0,65%	0,39%	0,71%	1,92%	1,14%	0,43%	0,82%	1,23%
F	0,42%	0,92%	1,18%	1,58%	0,96%	1,25%	1,34%	0,48%	0,09%	0,73%	1,29%	1,12%
G	0,91%	0,82%	0,30%	0,65%	0,13%	0,62%	0,62%	0,56%	0,24%	0,44%	0,84%	0,35%
H	0,54%	0,91%	0,24%	1,47%	0,59%	1,30%	0,26%	1,29%	0,18%	0,88%	1,01%	1,24%
min CV(l)	0,07%											
max CV(l)	2,01%	← note the broad range that results from CV values with only n = 3 replicates										

max CV(l)	2,01%

Overall Statistics	
Grand Avg	Form. 12
V_{GA}	99,98

Overall CV	Form. 13
CV_{OA}	2,23%

Figure A.7 — Additional calculated statistics for Example 3

A.6 Example 4 – Eight channels into one 384-well plate

A.6.1 Experimental design and measurement results for Example 4

The experimental design for Example 4 is an eight channel head delivering 48 replicates into one 384-well plate. The plate layout is as shown in Figure A.3 with the pattern shown as travelling left to right down the first row, then moving downward and travelling back in the reverse direction on the second row to complete the operation. The experiment consists of one run. The measurement results and calculated descriptive statistics are shown in Figure A.8.

A.6.2 Statistics results for Example 4

Channel statistics for each of the eight channels is shown on the right hand side of Figure A.8 using the indicated formulae. In this example, each channel delivered into two rows, so the calculations must span the proper rows.

Run order statistics are shown below the plate results. There are $N = 48$ replicates per channel, which results in 48 run order statistics.

Calculation of grand average volume and overall CV use Formulae (12) and (13), respectively, and the statistics are calculated over all 384 measurement results. The channel to channel CV is calculated using the results of the eight channel averages.

Example 4
8 channel device, L = 8
one run, M = 1
48 replicates per channel, N = 48
measured in one 384 well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	52,44	52,31	52,22	51,76	52,37	52,91	51,90	52,65	52,46	52,97	52,03	51,38
B	51,83	52,40	51,80	51,95	52,28	52,16	52,20	52,90	51,24	51,46	52,04	52,98
C	50,31	49,40	50,99	50,47	50,23	51,11	50,28	49,50	50,66	50,72	50,04	50,40
D	50,44	50,15	50,39	50,08	50,06	49,59	50,33	50,48	50,85	49,91	49,89	50,19
E	49,57	48,81	49,43	49,84	49,60	50,44	49,93	49,47	50,35	49,51	49,14	48,70
F	49,64	49,59	49,83	49,74	48,60	49,78	49,52	50,34	49,79	49,67	49,93	49,13
G	52,09	51,77	51,09	51,57	52,84	51,38	51,79	51,33	51,20	51,98	51,41	51,02
H	52,73	50,82	51,41	50,67	51,13	52,16	50,95	52,63	51,70	50,91	51,69	51,29
I	50,35	50,04	50,31	51,50	49,81	50,32	50,48	49,98	50,83	50,21	49,75	50,91
J	49,72	50,30	50,80	49,74	50,28	50,32	49,95	49,60	50,16	49,74	50,13	50,03
K	49,38	49,48	49,03	48,33	48,98	48,79	49,01	49,31	49,12	49,34	48,87	49,91
L	48,27	48,88	48,81	48,94	48,93	48,99	49,08	48,78	49,09	48,19	48,97	49,17
M	51,02	50,27	50,78	50,59	51,49	50,84	50,29	51,41	50,59	50,88	50,46	50,71
N	50,71	49,59	51,13	51,50	50,96	51,76	50,54	51,09	50,53	49,83	51,13	51,37
O	49,38	49,38	48,48	48,88	48,83	48,68	47,95	48,57	48,69	48,78	49,58	48,23
P	49,94	49,57	48,60	48,90	48,55	48,73	49,45	49,30	48,81	49,62	49,35	48,05

13	14	15	16	17	18	19	20	21	22	23	24
52,59	51,68	52,69	53,02	51,88	52,76	52,54	52,95	52,07	52,27	52,19	51,94
52,38	52,11	52,53	52,15	51,29	52,62	52,82	51,74	52,67	51,94	51,66	51,70
50,29	50,18	49,51	50,29	50,70	50,07	49,34	50,27	49,55	51,05	50,70	49,76
50,26	50,25	49,19	50,58	50,20	49,84	50,20	49,99	50,27	49,82	50,08	49,32
48,76	50,16	49,83	49,52	50,39	49,85	49,95	50,07	50,14	50,81	49,88	49,83
50,06	49,80	49,46	50,65	50,04	48,47	50,25	50,42	50,02	49,29	49,90	49,60
51,24	51,65	52,50	50,79	52,09	51,88	51,60	50,63	51,60	51,66	52,09	50,88
51,22	51,24	51,94	51,46	51,43	51,31	50,93	51,33	51,27	52,25	50,78	51,01
50,55	50,51	49,93	50,00	50,01	50,75	50,58	49,61	49,59	50,99	49,48	50,72
50,42	50,76	50,52	50,02	49,82	49,47	50,65	49,89	50,56	49,75	50,49	50,74
49,45	47,93	49,13	49,59	48,81	47,98	48,72	49,15	48,42	48,64	49,56	49,42
49,08	49,19	48,33	49,37	48,80	48,49	48,89	48,73	48,73	49,09	49,42	48,55
51,65	50,26	50,15	51,78	51,53	50,12	51,62	51,26	50,52	50,50	49,97	52,04
50,63	51,35	50,81	51,33	50,80	51,24	50,68	50,23	50,89	50,58	51,21	51,86
48,71	49,00	49,54	49,03	49,38	48,78	49,08	49,52	49,23	49,57	49,18	48,76
49,39	49,81	48,97	48,90	48,82	48,18	49,76	49,29	48,90	50,30	48,75	48,57

	Channel statistics N = 48		
	Form. 1	Form. 3	Form. 7
Channel	$V(l)$	$e_S(l)$	CV(l)
1	52,23	4,45%	0,91%
2	50,34	0,68%	0,91%
3	49,56	-0,87%	1,04%
4	51,62	3,24%	1,06%
5	50,37	0,75%	0,90%
6	49,13	-1,74%	0,86%
7	50,78	1,56%	1,12%
8	48,79	-2,43%	1,03%

Run order statistics

Form. 9	n = 1	n = 2	n = 3	n = 4	n = 5	n = 6	n = 7	n = 8	n = 9	n = 10	n = 11	n = 12
$V(n)$	50,57	50,18	50,29	50,37	50,52	50,56	50,20	50,28	50,49	50,55	50,16	50,16
	n = 48	n = 47	n = 46	n = 45	n = 44	n = 43	n = 42	n = 41	n = 40	n = 39	n = 38	n = 37
	50,41	50,16	50,35	50,19	50,10	50,44	50,25	50,64	50,27	49,91	50,39	50,28
	n = 13	n = 14	n = 15	n = 16	n = 17	n = 18	n = 19	n = 20	n = 21	n = 22	n = 23	n = 24
	50,41	50,17	50,41	50,50	50,60	50,27	50,43	50,43	50,14	50,69	50,38	50,42
	n = 36	n = 35	n = 34	n = 33	n = 32	n = 31	n = 30	n = 29	n = 28	n = 27	n = 26	n = 25
	50,43	50,56	50,22	50,56	50,15	49,95	50,52	50,20	50,41	50,38	50,29	50,17

Form. 14
CV_{C2C}
2,36%

Overall Statistics	
Grand Avg	Form. 12
V_{GA}	50,34

Overall CV	Form. 13
CV_{OA}	2,35%

Figure A.8 — Results and calculated statistics for Example 4

Annex B
(informative)

Test methods for ALHS performance

The following methods are described in this annex.

Open methods:

B.1	Ratiometric photometry
B.2	Gravimetry, single channel measurement
B.3	Gravimetry, full plate measurement
B.4	Gravimetric regression method
B.5	Photometry with Orange G
B.6	Hybrid method: gravimetry and photometry with Tartrazine
B.7	Hybrid method: photometry and gravimetry with *p*-nitrophenol
B.8	Hybrid method: gravimetry and photometry with Ponceau S
B.9	Pressure sensing
B.10	Calorimetric measurement
B.11	Optical image analysis

Closed methods:

B.12	Gravimetry and hybrid method: gravimetry and photometry
B.13	Photometry with Tartrazine

B.1 Ratiometric photometry[2)]

A dual-dye ratiometric photometry method and system for performance verification of multichannel liquid delivery devices

Outline of the method:

B.1.1 Introduction

The ratiometric photometry method and system described herein is performed through a patented[20] technology developed and offered for sale by Artel, Inc. This commercially available system is known as the Multichannel Verification System, or MVS®[3)]. The core principles of operation are briefly discussed herein; more detailed descriptions can be found elsewhere.[21-24]

This document provides an operational overview of the MVS, its capabilities, and the traceability of measurements[12] made through this system.

B.1.2 Principle of operation

The MVS determines volumes delivered to microplates using the following components:

1. Sample solutions: These solutions contain two dyes with distinct absorbance maxima at 520 nm (red) and 730 nm (blue). The red dye concentration varies from one type of sample solution to the next, which allows for measuring different volume ranges (e.g. more concentrated dye is used to measure lower volumes). The blue dye concentration is fixed and uniform between different sample solutions. Sample solutions are stable for more than 1 year when stored at room temperature and when protected from light (amber bottles).
2. Diluent: The diluent contains only the blue dye, but at the same concentration as in the sample solutions. The diluent solution is stable for more than 1 year when stored at room temperature and when protected from light (amber bottles).
3. Microplates: ANSI/SLAS standard 96-well or 384-well microplates are characterized on a lot basis to determine the bottom diameter or width (depending on plate type), and side-wall taper angle. Additionally, wells are characterized to correct for any non-uniformity in well dimensions. This characterization is used to generate a well-specific correction, which is then applied to every well throughout that lot of plates.
4. Microplate reader/Calibrator plate: A plate reader fitted with tailored interference filters optimized for absorbance measurements of the red (520 nm) and blue (730 nm) dyes is used. As with any spectrometer, the measured results are dependent on instrument stability and on the characteristics of the individual interference filters used for wavelength selection. To account for these two variables, a calibrator plate is used which consists of sealed precision cuvettes containing

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2) Contributed by Artel, Inc., Westbrook, Maine, USA

3) 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.

solutions of the same dyes used in the diluent and sample solutions. The calibrator plate provides an optical standard for calibrating the daily performance of the plate reader. The calibrator plate is stable for one year when stored at room temperature and when protected from light (storage case).

The above system components are used to measure the volume of sample solution dispensed into a well(s) of a microplate. As shown in Figure B.1, the liquid dispenser under test is used to dispense the target volume of sample solution into the microplate wells. Diluent is also added, either before or after the sample dispense. The amount of diluent added is used only to create a measurable volume of solution. Once the diluent and sample are in the wells, the solutions are thoroughly mixed, and the plate reader measures the absorbance at 520 nm and 730 nm for each solution-filled well in the microplate. The measured absorbance values, the known concentrations of red and blue dyes, and the known dimensions of the microplate wells are all used to calculate the volume of sample in each well.

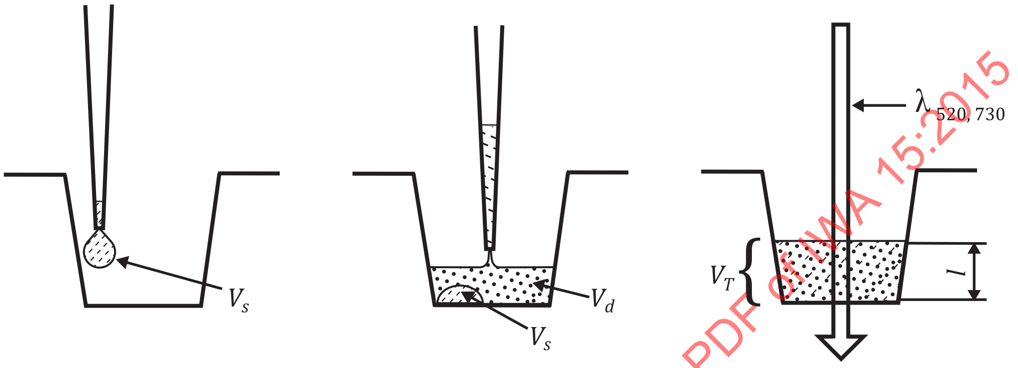

Figure B.1 — Liquid dispenser used to dispense the target volume into the microplate wells

Figure B.1 explanation: For small test volumes (V_S), a dispensed droplet of sample solution will adhere to the well sidewall and will not spread across the plate bottom. To allow for accurate photometric measurements at 520 nm and 730 nm ($\lambda_{520,730,}$), the well is backfilled with diluent (V_d) to a total working volume (V_T). The two solutions are mixed prior to making any photometric measurements. Because the sample solution contains the same concentration of blue dye as the diluent, the total working volume is determined from a photometric measurement at 730 nm of the beam path (l) through the solutions. Although this figure depicts the sample solution being dispensed first, the order of solution dispense can be altered in practice.

B.1.3 Procedure

1. Ensure proper environmental conditions for operation. Operating temperature range is 15 °C to 30 °C. All system components should be in thermal equilibrium (±2 °C) with the liquid delivery device being tested.

2. Calibrate the plate reader using the calibrator plate. This ensures that normal daily fluctuations of the plate reader are removed from the measurement. This calibration measurement is good for 10 h, or until the temperature changes by more than ± 2 °C, at which point a new measurement of the calibrator plate should be collected.

3. Fill a microplate with baseline solution (a clear buffer solution). For 96-well microplates, the proper fill volume is 200 µl. For 384-well microplates, the volume is 55 µl. The baseline plate should be mixed on the orbital mixer to evenly spread the solution and provide a uniform surface meniscus for all wells. Measure the prepared baseline plate in the plate reader. This provides a baseline reading at 520 nm and at 730 nm which will be used for the next 10 h, or until the plate reader is calibrated again.

4. Fill a solution reservoir appropriate to the liquid dispenser under test with the desired sample solution (see Table B.1). Keep the reservoir covered when not in use to protect from evaporation. Solutions should be left exposed for no longer than 20 min to 60 min, depending on exposed surface area, reservoir fill volume and environmental conditions. Each sample solution contains a known

concentration of Ponceau S dye with a quantified peak absorbance at 520 nm. This known peak absorbance is reported as the absorbance per pathlength (a_r, see Formula (B.3) below).

Table B.1 — Sample solution concentration and operating volume range

	Range HV	Range A	Range B	Range C	Range D	Range E	Diluent
Nominal absorbance per pathlength of red dye (a_r)	1,88	3,71	14,58	72,53	185,21	738,50	0
Nominal absorbance per pathlength of blue dye (a_b)	0,610	0,610	0,610	0,610	0[a]	0[a]	0,610
Testable volume range, 96-well (µl)	200,1–350,0	50,00–200,0	10,00–49,99	2,000–9,999	1,000 – 1,999	> 0,1000	NA
Testable volume range, 384-well (µl)	NA	10,00–55,0	2,500–9,999	0,500–2,499	0,3000–0,4999	> 0,3000	NA

[a] Concentration of blue dye for these solutions is irrelevant because the final test volume is composed mostly of diluent.

5. Fill a separate reservoir with diluent. Keep the reservoir covered when not in use to protect from evaporation. Solutions should be left exposed for no longer than 20 min to 60 min, depending on exposed surface area, reservoir fill volume and environmental conditions. Note: the diluent does not have to be dispensed by the same liquid dispenser under test. It can be dispensed from any device that is capable of delivering the approximate required volume. The diluent solution contains a controlled, known concentration of a copper-EDTA complex with a quantified peak absorbance at 730 nm. This known peak absorbance is reported as the absorbance per pathlength [a_b, see Formula (B.1) below].

6. Using the liquid delivery device under test, dispense the target volume of sample solution into the microplate. This dispense can either be a “dry contact dispense” into empty wells, or a “wet contact dispense” into wells pre-filled with the diluent.

7. Pre-fill or post-fill diluent into the microplate. The amount of diluent required is only approximate (±10 %).

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

9. Mix the total volume of solution (sample plus diluent) on an orbital mixer with a rotary orbit of 1 mm, at a speed of 1 300 rpm to 1 500 rpm for 60 s for 96-well microplates. For 384-well microplates, mix at a speed of 2 600 rpm to 2 700 rpm for 120 s.

NOTE Improper mixing may result in non-homogeneous solutions which will incorrectly skew the final results.

10. Insert the fully mixed microplate into the plate reader and measure the absorbance at 520 nm and 730 nm.

B.1.4 Volume calculations

The amount of sample volume delivered is calculated in three steps:

1. Because the concentration of the blue dye is the same in the sample solution and in the diluent, Formula (B.1) is used to determine the liquid depth (l) in each well, which is equivalent to the pathlength of light passed through the sample (see Figure B.1). By measuring the absorbance of the blue dye at 730 nm (A_{730}) and incorporating the known absorbance per unit pathlength of the blue dye at 730 nm (a_b), the liquid depth is determined by:

$$l=\frac{A_{730}}{a_b} \qquad \text{(B.1)}$$

where

l is the pathlength;

A_{730} is the absorbance at 730 nm;

a_b is the absorbance per unit pathlength.

2. Once the liquid depth is known, the geometrical equation for the volume of a truncated cone can be used to determine the total volume of liquid in the well. (NOTE: for 384-well plates, the geometry is a truncated, square-based pyramid.) At this point, the well-specific geometrical correction is applied to reduce well-to-well variability. The calculation of the total volume (V_T) is based on the liquid depth (l) as determined from Formula (B.1), and the known quantities of taper angle (θ) and diameter (D) of the microplate wells, as shown in Formula (B.2):

$$V_T=\pi l\frac{D^2}{4}+\pi Dl^2\frac{\tan\theta}{2}+\pi l^3\frac{\tan^2\theta}{3} \qquad \text{(B.2)}$$

where:

V_T is the total volume;

D is the diameter of the well bottom;

θ is the side wall taper angle.

3. The calculation of the sample volume (V_S) is based on the total volume (V_T), the measured absorbance of the blue dye at 730 nm (A_{730}), the measured absorbance of the red dye at 520 nm (A_{520}), and the known quantities for the absorbance per unit path length of the blue dye at 730 nm (a_b) and the absorbance per unit path length of the red dye at 520 nm (a_r), as given by Formula (B.3).

$$V_s=V_T\left(\frac{a_b}{a_r}\right)\left(\frac{A_{520}}{A_{730}}\right) \qquad \text{(B.3)}$$

where

V_S is the sample volume;

a_r is the absorbance per unit pathlength at 520 nm;

A_{520} is the absorbance at 520 nm.

B.1.5 Traceability

Traceability of this ratiometric photometry system to the International System of Units (SI) occurs through three main measurement paths:

1) characterized microplates,

2) characterized solutions,

3) characterized plate reader absorbance measurements.

Every volume tested with this method produces a final result that is calculated based upon all three traceable paths.[12]

Characterized microplates

Microplates are characterized using traceable dimensional measurements of each well. Well-by-well variability is further characterized using absorbance photometry, and the final accuracy of measurements is confirmed using the gravimetric dilution method described in B.3.

Plate reader

The calibration plate provides the traceability path to the measured absorbance values from the plate reader. The solution filled cuvettes in the plate provide optical standards which are used to correlate the daily performance of the plate reader. The absorbance values of these cuvettes are determined using a reference spectrophotometer which is traceable to neutral density standards.

Sample solutions

The absorbance per unit pathlength of all solutions is measured directly, or through the gravimetric dilution method described in B.3. Traceable absorbance measurements are made using a reference spectrophotometer whose performance is maintained against calibrated ND glass, and calibrated wavelength standards. A cuvette of calibrated internal pathlength is also used to provide a known pathlength.

B.2 Gravimetry, single channel measurement[4]

Outline of the method:

B.2.1 Introduction

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.

B.2.2 Terminology

Refer to the terms and definitions in Clause 3.

B.2.3 Apparatus

B.2.3.1 Balance.

The gravimetric method requires the use of a balance of appropriate capacity for the test volume. The resolution of the balance used should be determined by the nominal volume of the apparatus under test, as shown in Table B.2.

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[4] Contributed by: Gilson, Inc., Middleton, Wisconsin, USA

Table B.2 — Recommended balance performance depending on measured volume

Nominal volume of apparatus under test (V)	Recommended balance resolution	Recommended balance repeatability	Recommended balance linearity
µl	mg	mg	mg
1 to 10	0,001	0,002	0,005
> 10 to 100	0,01	0,02	0,05
> 100 to 1 000	0,1	0,2	0,5

B.2.3.2 Liquid reservoir.

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

B.2.3.3 Weighing vessel.

Mass lost 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 Method B.4 for further details.

B.2.3.4 Environmental conditions monitoring

The environmental conditions of the test room shall be monitored using equipment with appropriate resolution and uncertainty of measurement, as shown in Table B.3.

Table B.3 — Equipment specifications for monitoring the test room environment

Equipment	Resolution	Accuracy
Thermometer for liquid	0,1 °C	0,5 °C
Thermometer for air	0,1 °C	1 °C
Hygrometer	1 % RH	5 % RH
Barometer	0,1 kPa	1 kPa

Provided that instrumentation is chosen in accordance with the table above, the instrumental measurement uncertainty due to environment monitoring equipment will not have a significant effect on the mass to volume conversion for the volume ranges covered by this method. Refer to ISO/TR 20461 for more information.

B.2.4 Test liquid

The reference liquid class for the gravimetric method is distilled or deionized water conforming to grade 3 as specified in ISO 3696, degassed or air-equilibrated. The water shall be at room temperature.

Other liquid classes may be used provided the sensitivity of the given liquid class to environmental conditions is known and accounted for where significant. The recommended values within Tables B.3 and B.4 may not be applicable.

B.2.5 Test environment conditions

In order to control the variability of the results, the test environment should be kept as stable as possible. Table B.4 below contains recommended ranges for environmental conditions. The change in recorded value over the duration of the session should remain within the recommended value.

Table B.4 — Environmental conditions during the test

Environment condition	Range	Change during the test
Water temperature	15 to 30 °C	≤ 1 °C
Air temperature	15 to 30 °C	≤ 3 °C
Relative humidity	40 to 70 %	≤ 10 %

Provided that test environment conditions remain in accordance with the table above, the instrumental measurement uncertainty due to the environment will not have a significant effect on the mass to volume conversion for the volume ranges covered by this method. Refer to ISO/TR 20461 for more information.

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

B.2.6 Procedure

B.2.6.1 Test setup

B.2.6.1.1 Testing volume

The test volume or volumes selected should be representative of typical use. Manufacturers will typically recommend three volumes:

- nominal volume,
- approximately 50 % of the nominal volume,
- the lower limit of the useful volume range or 10 % of the nominal volume (whichever is greater).

These test volumes conform to the test volume statement in ISO 8655-6.

B.2.6.1.2 Number of measurements per volume to be tested

For the establishment or re-establishment of conformity to manufacturer's specifications, 10 measurements shall be taken at each test volume.

If the gravimetric method for the determination of measurement error is used for other purposes, such as supplier's quality control or supplier's after-sales service:

- the number of tested volumes,
- the number of measurements per volume, and
- where applicable the number of tested channels

may be changed to an appropriate number. If correlation to the reference method is desired, it shall be based on empirical evidence.

B.2.6.1.3 Test environment conditions

The 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 B.4.

The ALHS and balance shall be powered on and allowed to equilibrate with the test environment before testing begins.

B.2.6.2 Test procedure

B.2.6.2.1 Preparation of 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.

B.2.6.2.2 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 preparing the ALHS to perform liquid delivery, 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 position and remove the lid if a lid is used. When positioning the weighing vessel, the following conditions shall be met:

   1) the test liquid shall not be delivered to the weighing vessel;

   2) the ALHS liquid delivery apparatus shall not come into physical contact with the weighing vessel.

c) If liquid must be delivered or the liquid delivery apparatus must come into contact 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.

d) Perform a blank delivery.

e) Record the weight of the weighing vessel as b_i. A single measurement of mass loss due to evaporation is calculated as shown in Formula (B.4):

$$b'_i = b_i - b_0 \tag{B.4}$$

where

b'_i is the mass loss due to evaporation;

b_i is the weight of the weighing vessel;

b_0 is the tare weight of the weighing vessel.

f) Find the average mass loss due to evaporation by taking the average of all blank measurements Formula (B.5).

$$\overline{b'} = \sqrt{\left(\frac{\sum_{i=1}^{n} b'_i}{n}\right)^2} \qquad \text{(B.5)}$$

where

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

n is the number of blank measurements.

B.2.6.2.3 Preparation of ALHS

If the ALHS under test uses a system-liquid, prepare the system-liquid according the to manufacturer’s recommendations.

The ALHS shall be prepared by attaching a disposable tip or tips (if applicable) and performing a pre-rinse or equilibration cycle. This will ensure the consistency of the delivered volume across all measurements.

a) A pre-rinse is typically performed when the ALHS is dispensing a single volume for every aspiration.
    1) Fill the tip with the test volume and expel to waste five times.
b) An equilibration cycle is typically performed when the ALHS is performing multi-dispenses.
    1) Fill the tip with test liquid sufficient for multiple dispenses and an additional volume to discard.
    2) Discard the additional test liquid to waste.

After the ALHS is prepared, place the weighing vessel on the balance weigh pan and tare or zero the balance.

B.2.6.2.4 Making a measurement

a) Record the test room environmental conditions (temperature, relative humidity, and barometric pressure) at the beginning of testing.
b) If the weighing vessel must be removed from the balance weigh 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 weigh pan in order to take a measurement, move the weighing vessel to the balance weigh 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 environment conditions at the end of testing.

B.2.7 Evaluation

B.2.7.1 Calculation of mass loss due to 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 (B.6):

$$m'_i = m_i + \overline{b'} \tag{B.6}$$

where

m'_i is the corrected mass with evaporation;

m_i is the balance measurement.

B.2.7.2 Conversion of corrected mass to volume

The values m_i obtained in accordance with B.2.6.2.4 are balance readings. A correction taking into account water density and air buoyancy is necessary for the conversion of the balance readings m_i to volumes V_i. The Z correction factors specified in Table C.1 (Annex C) can be used.

Convert each mass m_i obtained from B.2.6 by applying the Z correction factors at the mean temperature and barometric pressure measured in B.2.6 and the following Formula (B.7).

$$V_i = m'_i \cdot Z \tag{B.7}$$

where

V_i is the calculated volume from the mass m'_i;

Z is the Z correction factor.

If the test temperature is different from the temperature of adjustment and if the thermal expansion correction factor γ of the piston-operated volumetric apparatus is known, use the following Formula (B.8) instead of the previous Formula (B.7):

$$V_i = m'_i \cdot Z \cdot \gamma \tag{B.8}$$

where γ is the thermal expansion correction factor of the piston-operated volumetric apparatus.

See ISO/TR 20461 for further details regarding the calculation of the Z correction factor and the uncertainty factors associated with mass to volume conversion.

After all measurements have been collected for each test volume, refer to the volumetric performance section of this document for information on statistical interpretation and presentation of the results (see Clause 5).

B.2.8 Traceability

The traceability of the gravimetric method is dependent upon the following criteria.

- The calibration and adjustment of the balance shall be traceable to the relevant international or regional reference standards.
- The calibration and adjustment of the environmental monitoring equipment shall be traceable to the relevant international or regional reference standards.

B.3 Gravimetry, full plate measurement[5)]

Weighing method for determining total dispensed volume in a whole plate

B.3.1 General

This method may be used to directly determine the total liquid volume dispensed to a microplate by a liquid handling system with a 96-channel or 384-channel dispensing head.

This method may also be used for comparing photometric methods to gravimetric methods where volume measurements are made by both methods. Comparison of the two results can be used for purposes of method validation (cross checking) or for calibration of the photometric method.

This method can also be used to determine sample volume in cases where the sample is diluted to form a mixture prior to dispensing. This pre-dilution method is a particularly accurate way for assessing lack of bias of photometric methods at small volumes.

B.3.2 Materials/equipment

— Microplates (96-well or 384-well).

— Semi-micro analytical balance with 0,000 01 g readability, 200 g capacity and a draft shield.

— Plate weighing adapter for balance pan.

— Static removal device.

— Personal protection equipment (PPE).

— Liquid to be weighed. NOTE: in case of dye solutions or custom liquids, ensure complete and homogeneous mixing of the solution.

— Automated liquid handling system with multichannel heads of either 96-channels set to deliver 200 µl, or 384 channels set to deliver 55 µl.

— Test room environment according to ISO 8655-6.

— 60 ml amber screw-cap bottles, for preparing dilutions.

— Diluent liquid, for cases where smaller sample volumes are desired.

— Micro analytical balance of 0,000 001 g readability, 5 g capacity with an evaporation trap.

B.3.3 Discussion

1. This test measures volume by gravimetric weighing of sample solution which is dispensed into microplates with a single dispense cycle by a 96-channel or 384-channel dispensing head.

2. Gloves need to be worn while performing testing. Gloves must be kept clean to prevent weight changes due to deposit of material during handling of the microplate.

3. If the plates will be read by the photometric method, they must be inspected to ensure that they are free of scratches or imperfections. Reject plates with visible imperfections. Use filtered air to blow off every well of each plate to remove dust.

4. Before each weighing, remove static from each plate and plate cover by holding in front of an ionizing static eliminator.

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5) Contributed by: Artel, Inc., Westbrook, Maine, USA

5. Use a cover to protect plates from dust until ready for use, and also to minimize evaporation during the weighing process.

6. The density of all solutions must be known. Measure the density of sample solution, diluent, and any prepared dilutions

B.3.4 Procedure — Sample preparation by dilution

1. Ensure that all material samples have equilibrated within the laboratory for 2 h.

2. Mix each sample by gentle inversion.

3. Calculate the desired dilution volumes using Formulae (B.9) and (B.10).

$$V_{ls} = \frac{V_t V_b}{V_w} \tag{B.9}$$

where

V_{ls} is the amount of sample solution used to make the dilution;

V_t is the target amount of sample desired for each well;

V_b is the total volume of diluted sample in the bottle (typically 60 000 µl);

V_w is the working volume per well (e.g. 200 µl for 96-well plates and 55 µl for 384-well plates).

$$V_{ld} = V_b - V_{ls} \tag{B.10}$$

where V_{ld} is the amount of diluent to be used in preparing the diluted sample.

4. If the amount of sample is greater than 1 000 µl it is permissible to dispense sample directly into the bottle and weigh as described in steps 6 through 8. Smaller volumes of sample must be measured as described in steps 9 through 13.

5. Label each empty amber bottle to clearly identify the contents. Set a pipette or volumetric dispenser to the appropriate volume.

6. Place the amber bottle on the semi-micro analytical balance pan with cap on (loosely) and press tare.

7. Remove cap and dispense the appropriate amount of sample solution into the bottle, place the cap back on tightly and record the weight after balance has stabilized.

8. Remove cap and carefully dispense the appropriate amount of diluent into the bottle, using caution not to contaminate the tip of the dispenser. Tighten the cap, place bottle on centre of balance pan and record the weight after balance has stabilized. Continue with step 14.

9. To weigh samples smaller than 1 000 µl, place an empty vial on the micro analytical balance pan and ensure appropriate evaporation trap has been placed on balance and equilibrated. Add approximately 2 000 µl of sample solution to the vial.

10. Perform an evaporation measurement. Record initial weight. Open the trap door and insert the pipette tip. Without touching the vial on the balance, mimic aspiration of sample solution by aspirating air. Carefully withdraw the pipette, and close the trap door. When balance has stabilized record the final weight.

11. Place a 60 ml amber bottle on the semi-micro analytical balance pan and press tare.

12. Dispense the appropriate volume of diluent solution into the amber bottle. Record the net weight of diluent.

13. Record the initial weight from micro-analytical balance. Using a new pipette tip for each dilution, aspirate the appropriate volume of sample solution and dispense into the bottle containing dispensed diluent. Rinse the tip in the solution by aspirating and dispensing down the side of the bottle. Record the net weight of sample solution removed from the micro balance, corrected for evaporation.
14. Mix each dilution carefully by gentle inversion at least 20 times.

B.3.5 Procedure – Delivery to plate and weighing

1. Ensure that each solution is mixed, if necessary mix by gentle inversion.
2. Thermally equilibrate the liquid handling system, labware and test solution for at least 2 h in a stable test room (±0,5 °C) prior to conducting this test. Use a continuously recording environmental monitoring system to confirm stable temperature during the equilibration and testing period.
3. Place the weighing adapter centred on the balance.

NOTE The weighing adapter is designed to help centre the microplate, and also to ensure that the microplate is not supported by balance structures other than the weighing pan.

4. Exercise the balance and perform calibration checks. Note the density of the mass standards used for this calibration. This density of the metal mass standards is required for the conversion equations.
5. Use the static elimination device to remove static electricity from the microplate and the cover. Place the empty microplate with cover onto the balance pan, keeping the weight of the plate centred on the pan.
6. After balance is stabilized, press the tare button. Balance should display a reading of zero. Record this initial indicated weight as weight W_0. Move the plate to the deck of the liquid handling system.
7. Use a 96 tip multichannel head to dispense 200 µl of test solution into a 96-well plate. Start a stop watch at the moment that liquid is dispensed. Cover the plate as soon as possible after dispensing and record the elapsed time since filling. Alternatively, when testing in a 384-well plate, use a 384 tip multichannel head to dispense 50 µl of test solution.
8. Remove static charge, then place the filled, covered plate on the balance pan. Record or print weight after stabilization as W_1.
9. Measure evaporation by returning the microplate to the instrument deck, then removing the cover for the amount of time recorded in step 7 above. Place the cover onto the microplate and repeat the process described in step 8. Record the weight after this evaporation trial as W_2. Evaporation is equal to W_1 minus W_2.

B.3.6 Calculation of total volume delivered to the plate

1. Calculate the corrected weight change, W_L, following Formula (B.11).

NOTE This assumes that the amount of evaporation per weighing cycle is approximately constant.

$$W_L = (W_1 - W_0) + (W_1 - W_2) \tag{B.11}$$

where

W_L is the weight change;

W_0 is the initial indicated weight;

W_1 is the weight after liquid delivery, once it has stabilized;

W_2 is the weight after the evaporation trial.

2. Calculate the *Z* correction factor, which will be used to convert weight of liquid to volume, using the correct densities according to Annex C, Formula (C.2). Do not use the *Z* factor values in Table C.1.

3. Convert the weight of the liquid (W_L) to volume (V_L) by using Formula (B.12):

$$V_L = Z \cdot W_L \tag{B.12}$$

where

V_L is the volume of the liquid;

Z is the Z correction factor, per Annex C.

The result is the measurement of the total volume (V_L) delivered to the plate.

4. When the liquid volume (V_L) is for a dilute mixture, it is possible to calculate a dilution factor (F_{dil}) which is used to calculate the amount of sample solution delivered to the plate. The dilution factor (F_{dil}) is calculated in three steps.

First calculate the mass of liquid sample (m_{ls}) used to prepare the mixture with Formula (B.13):

$$m_{ls} = W_{ls} \frac{[1 - \rho_a / \rho_s]}{[1 - \rho_a / \rho_{ls}]} \tag{B.13}$$

where

m_{ls} is the mass of liquid sample used to prepare the mixture;

W_{ls} is the weight of sample solution used to prepare the mixture, as calculated from balance display indications;

ρ_{a} is the density of air as calculated in Annex C;

ρ_{s} is the density of the mass standards used to calibrate the balance;

ρ_{ls} is the density of the liquid sample solution.

Next, calculate the mass of liquid diluent, m_{ld}

$$m_{ld} = W_{ld} \frac{[1 - \rho_a / \rho_s]}{[1 - \rho_a / \rho_{ld}]} \tag{B.14}$$

where

m_{ld} is the mass of the liquid diluent;

W_{ld} is the weight of diluent solution used to prepare the mixture, as calculated from balance display indications;

ρ_{ld} is the density of the liquid diluent solution.

Then calculate the dilution factor, F_{dil}

$$F_{\mathrm{dil}} = \frac{\rho_{lm}}{\rho_{ls}} \frac{m_{ls}}{[m_{ld} + m_{ls}]} \tag{B.15}$$

where ρ_{lm} is the density of the liquid mixture.

Finally, the sample volume (V_S) is calculated as shown in Formula (B.16), by multiplying the total measured volume calculated with Formula (B.12) by the dilution factor F_{dil};

$$V_S = F_{\mathrm{dil}} \cdot V_L \tag{B.16}$$

where V_S is the sample volume.

This quantity (V_S) is the total volume of sample solution delivered to the plate. The total sample volume can be divided by the number of wells in the plate to calculate the average amount of sample solution per well.

B.3.7 Reporting

The test report for this method shall comply with the reporting requirements of Clause 8.

B.4 Gravimetric regression method (GRM)[6)]

B.4.1 Method description

In order to determine the volume of a liquid delivery in the sub-µl range based on the gravimetrically measured mass change caused by this liquid volume on a high precision balance, similar equations as provided by the ASTM E542, ISO 4787, or ISO 8655-6 standards are used. The required formulae and the basic measurement procedure are discussed in publications for large volume calibrations in detail. [28-33] For the GRM method described here, the formula for determination of the liquid volume V_{20} based on a gravimetrically measured mass m is considered as follows [Formulae (B.17) and (B.18)]:

$$V(T) = m \cdot \frac{1}{\rho_w(T) - \rho_a} \cdot \left(1 - \frac{\rho_a}{\rho_c}\right) \quad \text{(B.17)}$$

where

$V(T)$ is the volume at temperature T;

m is the gravimetrically measured mass of the sample;

ρ_w is the density of the measured liquid at temperature T;

ρ_a is the density of air at temperature T;

ρ_c is the density of the weights used to calibrate the balance.

$$V_{20} = V(T) \cdot \left[1 - \gamma(T - 20)\right] \quad \text{(B.18)}$$

where

V_{20} is the volume at the reference temperature of 20 °C;

T is the temperature in units of °C;

γ is the thermal coefficient of expansion of the measured liquid.

The measured primary quantity is the total mass m on the weighing dish of the balance, which is conventionally weighed by the used balance. The mass change caused by the addition of a liquid volume ΔV is denoted Δm. The mass of the additional liquid is supposed to be the difference of mass before (m_{before}) and after (m_{after}) the dispensing event as given in Formula (B.19):

$$\Delta m = m_{\text{after}} - m_{\text{before}} \quad \text{(B.19)}$$

where

Δm is the mass of the added liquid;

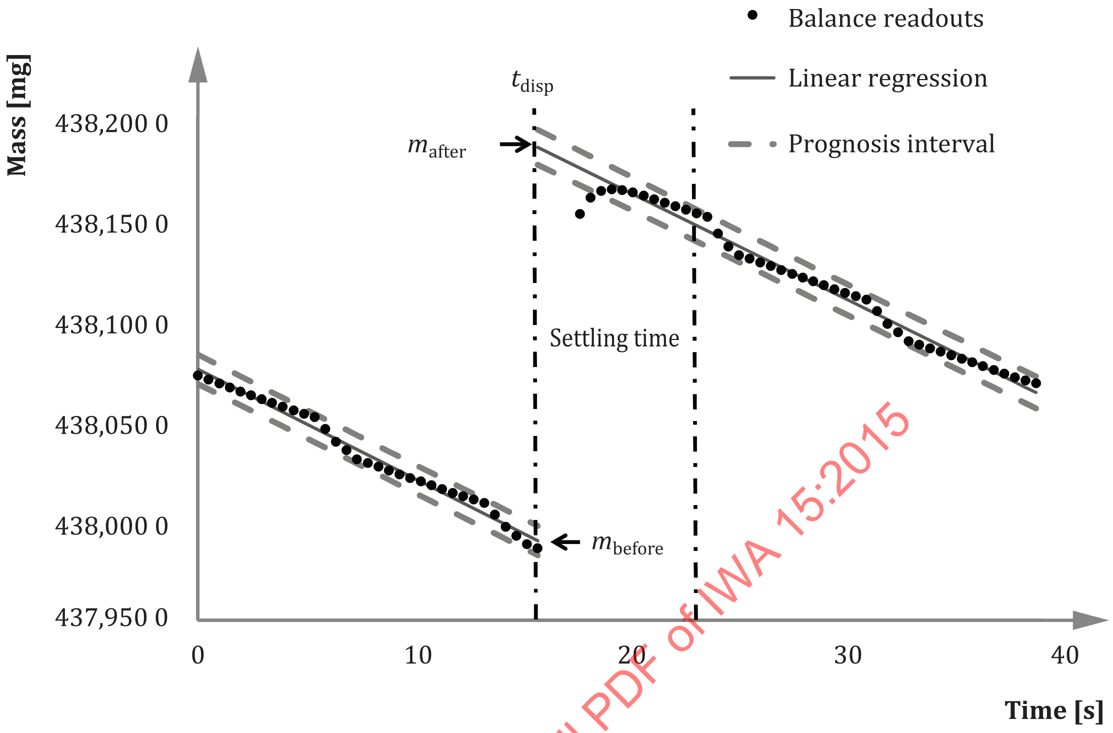
m_{before} is the mass before the dispense;

m_{after} is the mass after the dispense.

[6)] Contributed by: IMTEK, University of Freiburg, Freiburg im Breisgau, Germany

The mass values m_{before} and m_{after} are of course subject to a measurement error resulting from the error of the weighing process especially from the error of the used gravimetric balance.

Simply speaking the mass of the dispensed liquid volume is mainly determined by the difference of the mass before and after the dispensing event. Usually, the average values of a series of weighing results that are acquired before the dispensing event and after a settling time of a few seconds are considered as appropriate measurement results for m_{before} and m_{after}. However, due to evaporation the mass of the liquid loaded on the weighing dish of the balance changes over time. After the settling time, which is required to equilibrate the balance tray, the mass detected is no longer corresponding exactly to the mass directly after the dispensing event and it is continuously decreasing further due to evaporation. It is therefore not admissible to consider simple average values of balance readouts for determining m_{before} and m_{after} if the liquid volumes are so small that evaporation becomes significant during the measurement. Therefore, within the GRM method the values m_{before} and m_{after} are determined by a linear regression based on several balance readouts acquired before and after the dispensing event (see Figure B.2 below) instead of using an average value.



NOTE Balance readouts (dots) before and after dispensing are displayed as a function of time. Readouts acquired between the two vertical lines are not used for calculation (settling time of the balance).

Figure B.2 — Plot of a typical measurement

A detailed description of the measurement procedure and formulae for performing the linear regression are given in Reference [15] along with a detailed uncertainty analysis and a benchmark to the Artel MVS method (Method B.1 in this document) in the volume range from 40 nl to 400 nl.

B.4.2 Liquid classes

The GRM method can be executed with any liquid. The lower the vapor pressure of the liquid the lower the evaporation and the better the measurement accuracy. Liquids with high vapor pressure can be used, but will cause larger measurement errors. Therefore, and for consistency with existing standards, it is recommended to use water as test liquid.

B.4.3 Environmental conditions

The environmental conditions (temperature, humidity, pressure) have to be recorded in order to be considered in the volume calculations similar to other gravimetric methods. Though environmental conditions do influence the evaporation rate there is no requirement for specific or constant environmental conditions. As long as they are recorded the measurement algorithm accounts for the appropriate corrections. Still, conditions with low evaporation and vibration isolated tables for hosting the balance are favourable to achieve highest measurement accuracy.

B.4.4 Traceability

Traceability for the GRM can be established through the calibrated balance that is used for measuring the mass of the liquid or through comparison of measurements to the Artel MVS method (Method B.1), which is traceable in itself. In most practical cases use of a calibrated balance will be sufficient and no further verification and validation is needed. To calculate the final measurement result, a regression analysis of the measured balance readings before and after the addition of the liquid is required as well as the use of Formulae (B.17) and (B.18). The method is described in full detail in Reference [15].

B.4.5 Measurement uncertainty

The measurement uncertainty has been analysed according to ISO/IEC Guide 98-3 in the volume range between 40 nl and 1 000 nl.[15] In this work a combined standard uncertainty of typically 6 nl and an expanded uncertainty of typically 12 nl have been determined for volumes in the range from 40 nl to 1 µl. The uncertainty is dependent on the volume to be measured as well as environmental conditions. In particular, the uncertainty analysis of the GRM method does take random errors of the individual balance readouts before and after the addition of the liquid into account. Therefore, it is possible to assign each individual data set an individual measurement error. Thus, it is possible to distinguish results with high performance of the device under test but poor measurement accuracy (e.g. large weighing errors due to vibrations or unsteady evaporation by convection in the measurement cell) from result with poor performance of the device under test but high measurement accuracy. This is not possible by gravimetric methods that consider only the average mass value, but not the statistical distribution of individual balance readings.

According to the benchmark experiments presented in Reference [15], the results of the GRM method have been shown to be largely consistent with the Artel MVS photometric method that was used as reference (Method B.1). Systematic deviations (bias) between the methods were observed in the range of −26 nl to 6 nl (−10,7 % to 5,4 % relative bias). Over the whole range from 40 nl to 1 000 nl the results of the GRM method and the Artel MVS method were estimated to be consistent with a linearity of $R^2 = 1$.

B.5 Photometry with Orange G7)

Orange G colourimetric method for pipetting heads with 1 to 384-channel

Outline of the method:

B.5.1 Principle of the method

- The method is suitable for 96 and 384 multichannel heads and for ALHS with individual channels as well.
- The method is traceable to SI by calibrated balance, volumetric flasks and pipettes.
- The manual pipettes must be regularly serviced and calibrated according the manufacturers guide lines.
- The balance must have at least 0,001 mg resolution. It must be regularly serviced and calibrated according the manufacturers guide lines.
- The microplate reader must be regularly serviced and calibrated according the manufacturers guide lines.

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7) Contributed by: Tecan Schweiz AG, Männedorf, Switzerland.

- The pH meter must be regularly serviced and calibrated according the manufacturers guide lines.
- The method is used for aqueous liquids.
- The method can easily be adapted to DMSO and water/DMSO mixtures. The solubility of Orange G in DMSO and DMSO/water is similar to the solubility in aqueous liquids.
- The diluent is buffered at pH = 7 because Orange G is also a pH indicator shifting the absorbance maximum.
- The dye solution is pipetted in a microplate and the complementary amount of buffer solution is added to fill the well to a certain volume.
- Based on the measured ODs, the CVs are determined.
- Based on the calibration curve, the volumes pipetted and the accuracy are calculated.
- Four stock solutions at different concentrations are prepared.
- For every stock solution, a dilution series for coverage of the volume range intended is prepared by manual pipetting using a calibrated pipette.
- From every dilution, five replicates are manually pipetted in a microplate.
- The microplate is read out at 492 nm with 620 nm as reference wavelength.
- The measured ODs shall not exceed OD = 2.
- A calibration curve is calculated based on the data points measured.
- Store the Orange G solutions in amber glass bottles.
- The shelf life time of Orange G solutions and buffer solutions is generally 2 months at ambient temperature.
- Solutions that have developed streaks shall be disposed.

B.5.1.1 Applicable volume ranges

Table B.5 — Applicable volume ranges

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

B.5.2 Preparation of solutions

B.5.2.1 Chemical substances used

Table B.6 — Chemical substances used

Substances/ solutions	Degree of purity	Formula	Molecular weight	Safety data
Orange G	—	$C_{16}H_{10}N_2Na_2O_7S_2$	452,36 g/mol	—
Disodium hydrogen-phos-phate dihydrate	purum	$Na_2HPO_4 \bullet 2(H_2O)$	177,99 g/mol	—
Sodium hydroxide, 1 M	—	NaOH	40,0 g/mol	caustic
Deionised water	—	H_2O	18,0 g/mol	—

Operational Safety Note: Sodium hydroxide is caustic and may cause severe corneal damage if eye contact occurs. Wear chemical safety goggles and gloves when operating with sodium hydroxide. In case of contact with skin or eyes, immediately flush eyes with plenty of water and get medical aid.

B.5.2.2 Buffer solution 0,1 M

- Weighted sample: 35,6 g ± 0,1 g disodium hydrogen phosphate dehydrate.
- Add the weighted sample into a 2 000 ml beaker glass and fill it right up under the mark with deionized water.
- Add 2 000 µl (e.g. 2 × 1 000 µl with the hand pipette) of sodium hydroxide 1 M in into the volumetric flask.
- Stir the disodium hydrogen phosphate dehydrate until it has completely dissolved (approximately 30 min).
- Fill the solution into a plastic bottle for storage.
- Check the pH with a pH-meter (pH = 7). Record measured values.
- Check the pH before use for preparation of Orange G solutions. Record measured values.

B.5.2.3 Orange G 8,0 g/l

- Original weight: 4,0 g ± 0,05 g Orange G (analytical balance).
- Add the original weight into a 500 ml volumetric flask and fill it right up under the score with buffer solution.
- Put a magnet into the volumetric flask.
- Place the flask with magnet onto the magnetic stirrer.
- Stir the Orange G until it has completely dissolved.
- Remove the magnet and fill up the flask to the score with buffer solution.
- Fill the Orange G into an amber glass bottle for storage.

B.5.2.4 Orange G 2, 0 g/l

- Original weight: 2,0 g ± 0,01 g Orange G
- Add the original weight into a 1 000 ml volumetric flask and fill it right up under the score with buffer solution.
- Put a magnet into the volumetric flask.
- Place the flask with magnet onto the magnetic stirrer.
- Stir the Orange G until it has completely dissolved.
- Remove the magnet and fill up the flask to the score with buffer solution.
- Fill the Orange G into an amber glass bottle for storage.

B.5.2.5 Orange G 1, 6 g/l

- Admeasure 100 ml of the 8 g/l Orange G solution into a 100 ml volumetric flask.
- Transfer the 100 ml into a 500 ml volumetric flask.
- Rinse the 100 ml volumetric flask with buffer solution and add the rinse to the buffer solution into the 500 ml volumetric flask.
- Fill up the flask to the score with buffer solution.
- Close the 500 ml volumetric flask with the designated stub.
- Flip the 500 ml volumetric flask several times for good mixing.
- Fill the Orange G into an amber glass bottle for storage.

B.5.2.6 Orange G 0,2 g/l

- Admeasure 50 ml of the 2 g/l Orange G solution into a 50 ml volumetric flask.
- Transfer the 50 ml in 500 ml volumetric flask.
- Rinse the 50 ml volumetric flask with buffer solution and add the rinse the buffer solution into the 500 ml volumetric flask.
- Fill up the flask to the score with buffer solution.
- Close the 500 ml volumetric flask with the designated stub.
- Flip the 500 ml volumetric flask several times for good mixing.
- Fill the Orange G into an amber glass bottle for storage.

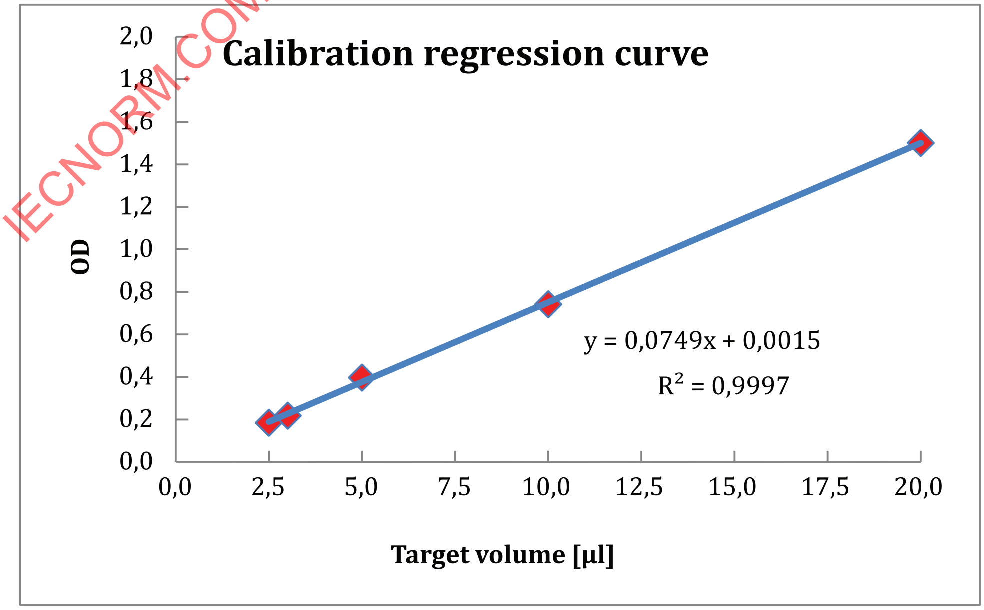
B.5.3 Calibration curves

B.5.3.1 Orange G 0,2 g/l in 384-well plate

Table B.7 — Calibration points for test volumes 2,5 µl to 20,0 µl in 384-well microplates

Orange G 0,2 g/l	Buffer solution	Combined volume	Equals to test volume
50 µl	1 ml + 450 µl	1,5 ml	2,5 µl
60 µl	1 ml + 440 µl	1,5 ml	3,0 µl
100 µl	1 ml + 400 µl	1,5 ml	5,0 µl
200 µl	1 ml + 300 µl	1,5 ml	10,0 µl
400 µl	1 ml + 100 µl	1,5 ml	20,0 µl

- Pipette volume of Orange G solution (according to the table above) in test tubes using a calibrated manual pipette.
- Add the complementary amount of buffer solution (according to the table above) to the test tubes.
- Mix the solutions by gently agitating the tubes.
- Pipette 5 replicates of 75 µl of each solution in a row of a 384 microplate using a calibrated manual pipette.
- Measure the microplate at a wavelength of 492 nm (Orange G) and 620 nm (Reference).
- Calculate the average of 5 replicates of the same solution. Reject outlier if appropriate.
- Plot test volumes versus OD values.
- Calculate the regression curve.



NOTE Each point represents the average of 5 replicates manually pipetted.

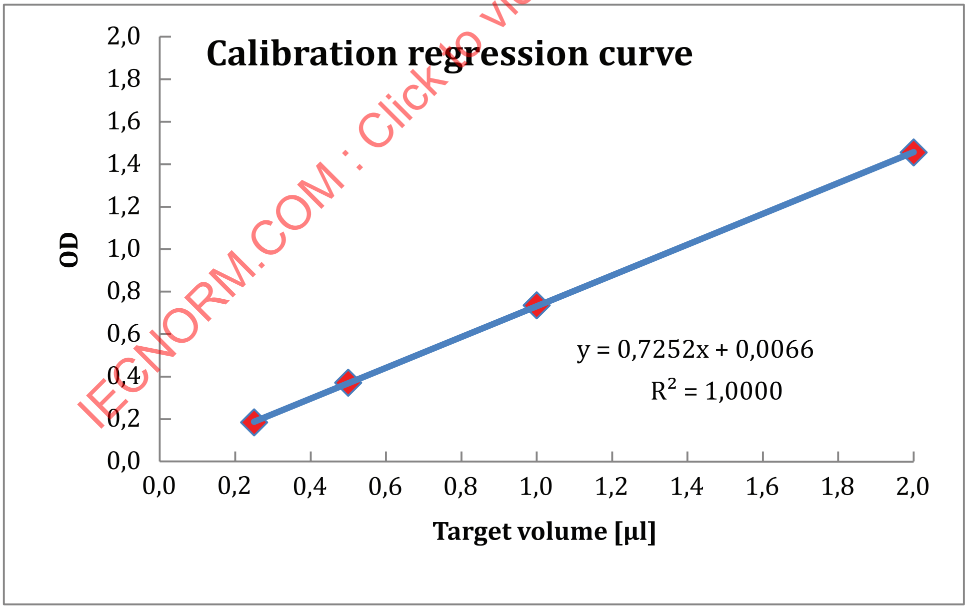
Figure B.3 — Calibration curve for test volumes 2,5 µl to 20,0 µl in 384-well microplates

B.5.3.2 Orange G 2, 0 g/L in 384-well plate

Table B.8 — Calibration points for test volumes 0,25 µl to 2,0 µl in 384-well microplates

Orange G 2,0 g/l	Buffer solution	Combined volume	Equals to target volume
10 µl	2 ml + 990 µl	3,0 ml	0,25 µl
20 µl	2 ml + 980 µl	3,0 ml	0,50 µl
40 µl	2 ml + 960 µl	3,0 ml	1,00 µl
80 µl	2 ml + 920 µl	3,0 ml	2,00 µl

- Pipette volume of Orange G solution (according to the table above) in test tubes using a calibrated manual pipette.
- Add the complementary amount of buffer solution (according to the table above) to the test tubes.
- Mix the solutions by gently agitating the tubes.
- Pipette 5 replicates of 75 µl of each solution in a row of a 384 microplate using a calibrated manual pipette.
- Measure the microplate at a wavelength of 492 nm (Orange G) and 620 nm (Reference).
- Calculate the average of 5 replicates of the same solution. Reject outlier if appropriate.
- Plot test volumes versus OD values.
- Calculate the regression curve.



NOTE Each point represents the average of 5 replicates manually pipetted.

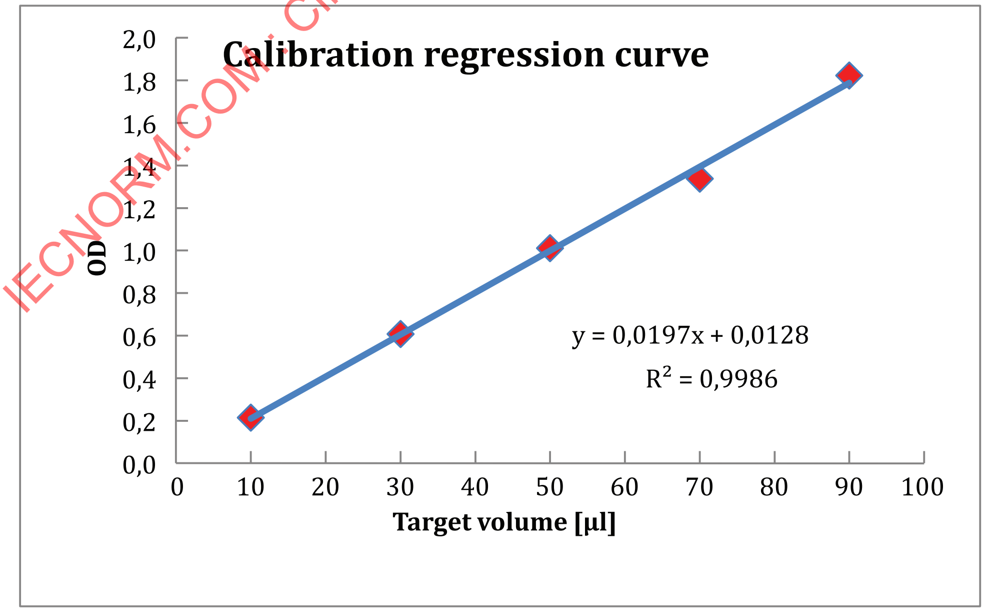
Figure B.4 — Calibration curve for test volumes 0,25 µl to 2,0 µl in 384-well microplates

B.5.3.3 Orange G 0,2 g/l in 96-well plate

Table B.9 — Calibration points for test volumes 10,0 µl to 100,0 µl in 96-well microplates

Orange G 0,2 g/l	Buffer solution	Combined volume	Equals to target volume
100 µl	1 ml + 900 µl	2,0 ml	10,0 µl
300 µl	1 ml + 700 µl	2,0 ml	30,0 µl
500 µl	1 ml + 500 µl	2,0 ml	50,0 µl
700 µl	1 ml + 300 µl	2,0 ml	70,0 µl
1 000 µl	1 ml	2,0 ml	100,0 µl

- Pipette volume of Orange G solution (according to the table above) in test tubes using a calibrated manual pipette.
- Add the complementary amount of buffer solution (according to the table above) to the test tubes.
- Mix the solutions by gently agitating the tubes.
- Pipette 5 replicates of 200 µl of each solution in a row of a 96 microplate using a calibrated manual pipette.
- Measure the microplate at a wavelength of 492 nm (Orange G) and 620 nm (Reference).
- Calculate the average of 5 replicates of the same solution. Reject outlier if appropriate.
- Plot test volumes versus OD values.
- Calculate the regression curve.



NOTE Each point represents the average of 5 replicates manually pipetted.

Figure B.5 — Calibration curve for test volumes 10,0 µl to 100,0 µl in 96-well microplates

B.5.3.4 Orange G 1, 6 g/l in 96-well plate

Table B.10 — Calibration points for test volumes 2, 0 µl to 10,0 µl in 96-well microplates

Orange G 1,6 g/l	Buffer solution	Combined Volume	Equals to target volume
20 µl	1 ml + 980 µl	2,0 ml	2,0 µl
30 µl	1 ml + 970 µl	2,0 ml	3,0 µl
50 µl	1 ml + 950 µl	2,0 ml	5,0 µl
70 µl	1 ml + 930 µl	2,0 ml	7,0 µl
100 µl	1 ml + 100 µl	2,0 ml	10,0 µl

— Pipette volume of Orange G solution (according to the table above) in test tubes using a calibrated manual pipette.

— Add the complementary amount of buffer solution (according to the table above) to the test tubes.

— Mix the solutions by gently agitating the tubes.

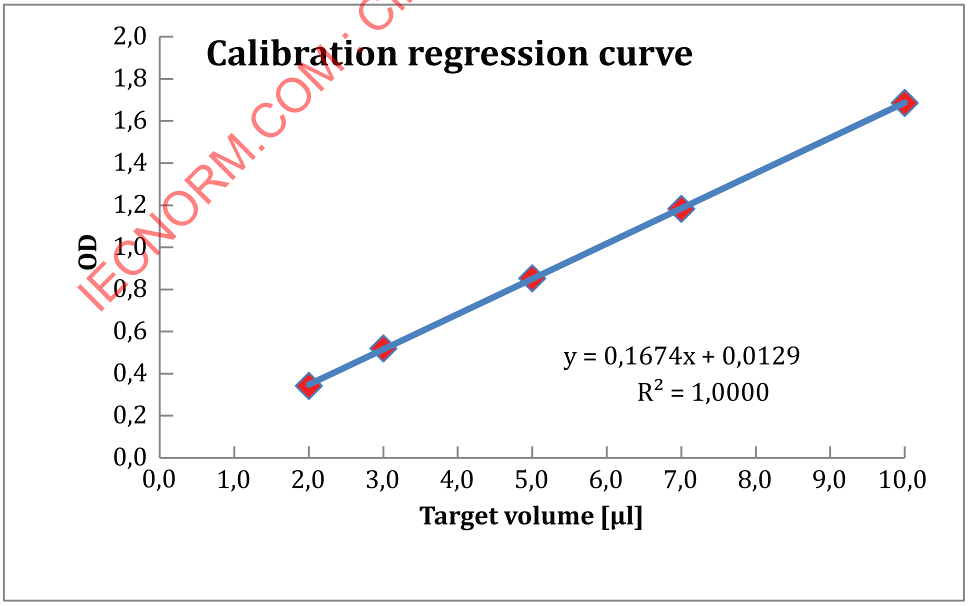
— Pipette 5 replicates of 200 µl of each solution in a row of a 96 microplate using a calibrated manual pipette.

— Measure the microplate at a wavelength of 492 nm (Orange G) and 620 nm (Reference).

— Calculate the average of 5 replicates of the same solution. Reject outlier if appropriate.

— Plot test volumes versus OD values.

— Calculate the regression curve.



NOTE Each point represents the average of 5 replicates manually pipetted.

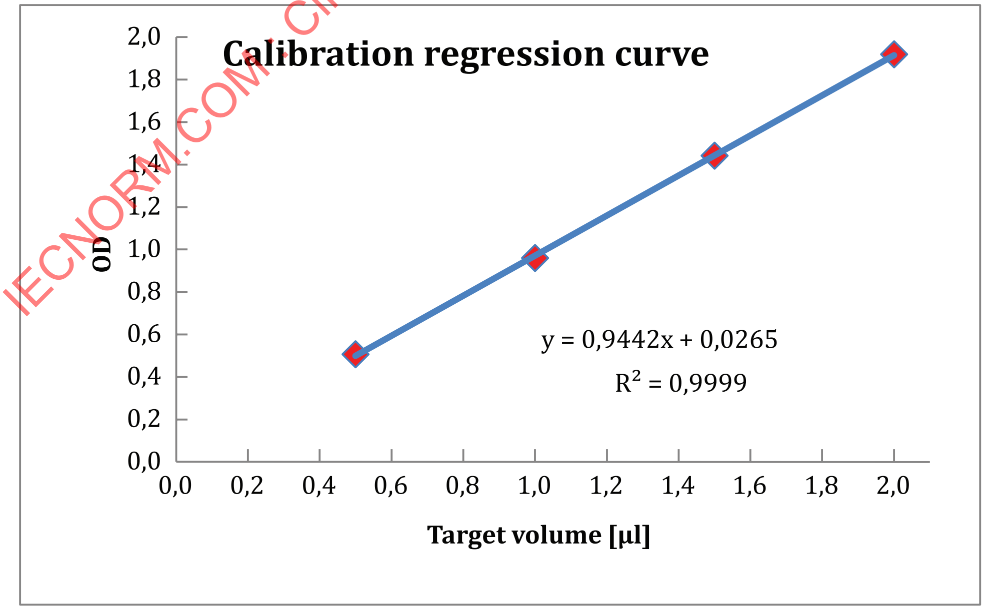
Figure B.6 — Calibration curve for test volumes 2,0 µl to 10,0 µl in 96-well microplates

B.5.3.5 Orange G 8, 0 g/l in 96-well plate

Table B.11 — Calibration points for test volumes 0,5 µl to 2,5 µl in 96-well microplates.

Orange G 8,0 g/l	Buffer solution	Combined Volume	Equals to target volume
5 µl	1 ml + 995 µl	2,0 ml	0,5 µl
10 µl	1 ml + 990 µl	2,0 ml	1,0 µl
15 µl	1 ml + 985 µl	2,0 ml	1,5 µl
20 µl	1 ml + 980 µl	2,0 ml	2,0 µl
25 µl	1 ml + 975 µl	2,0 ml	2,5 µl

- Pipette volume of Orange G solution (according to the table above) in test tubes using a calibrated manual pipette.
- Add the complementary amount of buffer solution (according to the table above) to the test tubes.
- Mix the solutions by gently agitating the tubes.
- Pipette 5 replicates of 200 µl of each solution in a row of a 96 microplate using a calibrated manual pipette.
- Measure the microplate at a wavelength of 492 nm (Orange G) and 620 nm (Reference).
- Calculate the average of 5 replicates of the same solution. Reject outlier if appropriate.
- Plot test volumes versus OD values.
- Calculate the regression curve.



NOTE Each point represents the average of 5 replicates manually pipetted.

Figure B.7 — Calibration curve for test volumes 0,5 µl to 2,5 µl in 96-well microplates

B.5.4 Pipetting procedure

B.5.4.1 96-well microplates

- The target volume of the appropriate Orange G concentration is pipetted to the wells of a 96-well clear flat bottom microplate.
- The complementary amount of buffer solution is added to fill the well to the end volume of 200 µl.
- The plate is shaken on a microplate shaker for mixing for 30 s at 1 000 rpm (rotations per minute).
- The microplate is centrifuged for 60 s with 164 rcf (relative centrifugal force) for removing air bubbles and establishing a defined meniscus.
- The microplate is measured at 492 nm with 620 nm as reference wavelength (10 flashes per measurement).

B.5.4.2 384-well microplates

- The target volume of the appropriate Orange G concentration is pipetted to the wells of a 384-well clear flat bottom microplate.
- The complementary amount of buffer solution is added to fill the well to an end volume of 75 µl.
- The plate is shaken on a microplate shaker for mixing for 15 s at 2 000 rpm (rotations per minute).
- The microplate is centrifuged for 60 s with 164 rcf (relative centrifugal force) for removing air bubbles and a defined meniscus.
- The microplate is measured at 492 nm with 620 nm as reference wavelength (10 flashes per measurement).

B.6 Hybrid method: Gravimetry and photometry with Tartrazine[8]

Outline of the method

B.6.1 Hybrid photo-gravimetric method

B.6.1.1 Method name and summary description

The hybrid photo-gravimetric method allows multichannel liquid handling instruments to be characterized by a combination of a gravimetric reference measurement with subsequent photometric measurements to characterize the other channels of the instrument.

B.6.1.2 Method description

B.6.1.2.1 Preparation

Materials

Precision balance with 0,01 mg readability or better;

Measurement equipment for temperature, humidity and atmospheric pressure;

Absorbance Reader;

Microplate Shaker;

Micro centrifuge tubes 1,5 ml;

Flat-bottom 96-well plates, non-treated, Polystyrene (PS) OR Flat-bottom 384-well plates, non-treated, Polystyrene (PS);

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[8] Contributed by: Integra Biosciences AG, Zizers, Switzerland

Reagent reservoirs 100 ml and 300 ml;

Distilled water for pre-wetting and dilution;

Test solution (see Fluid Properties B.6.1.3).

Tips need to be pre-wetted to coat the inside of the liquid contacting parts and to equilibrate humidity of the air space inside tip and the pipette. This is achieved by aspirating from the reservoir and dispensing to waste the maximum test volume 3 times. This is required every time a tip is changed.

The precision balance should be checked with ASTM Class 2 or OIML E2 test weights before measurements.

B.6.1.2.2 Gravimetric measurement

The grid of a 96- and 384-well plate is used to identify channels of a pipetting head.

The gravimetric measurement is performed on a corner channel (e.g. H1 for a 96-channel pipetting head, P1 on a 384-channel head). This will be the reference channel.

Five measurements of the reference channel are taken at the nominal volume. When working with tips, do not touch them by hand to avoid any thermal transfer.

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

2) Pre-wet the reference channel three times at the high test volume and dispense to the micro centrifuge tube that has been used to tare the balance. Lid needs to be closed immediately after dispense to avoid evaporation and place it on the balance. This first result is not recorded.

3) With the same tip aspirate the test volume again by immersing the tip only as much as necessary (2 mm to 3 mm).

4) Dispense to the test tube, touching the tip against the inside wall, close the tube immediately.

5) Record the weight in mg.

6) Repeat steps 3 to 5 four times to get five readings.

7) Repeat steps 2 to 6 for the medium (50 %) and low (10 %) test volume 5 times each.

The values obtained by balance readings are in milligrams. These values need to be converted to micro litres using the Z correction factors. They take into account the water density and air buoyancy during weighing at the corresponding test temperature.

Convert each weight obtained in step 5 to volume in µl by applying the correct Z correction factor as described in Annex C. Add together the delivered volumes V_i and divide the sum by 5 to provide the mean volume $\overline{V}$ delivered at test temperature, as shown in Formula (B.20).

$$\overline{V} = \frac{1}{5} \times \sum_{i=1}^{5} V_i \tag{B.20}$$

where

$\overline{V}$ is the mean volume of 5 delivered volumes;

V_i is the delivered volume.

B.6.1.2.3 Photometric measurement

B.6.1.2.3.1 For Tartrazine as dye, the measurement wavelength is 450 nm. A reference measurement is performed at 620 nm for each plate. Bandwidth of the measurement is 10 nm. The readings from the reference wavelength (620 nm) are subtracted from the readings of the absorbed wavelength (450 nm) to eliminate the background absorbance and to compensate for reader and microplate tolerances.

A conversion plate needs to be created to correlate the optical density (OD) as measured by the photometer to microlitres:

1) For test volumes < 100 µl: Aspirate 100 µl of distilled water from reservoir by immersing the tip(s) 2 to 3 mm below the surface of the water and dispense to one row of wells in a plate. Be sure to keep plate covered to prevent evaporation.

2) Aspirate the low test volume of the selected range of test solution from the reservoir by immersing the tip 2 to 3 mm below the surface of the solution and dispense to one column of a microplate.

3) Read partial plate with reader to obtain the optical density readings.

4) Subtract 620 nm readings from 420 nm absorbance readings.

B.6.1.2.3.2 Calculate the conversion factor to correlate optical density to microlitres as follows:

1) Calculate the mean of the OD according to Formula (B.21), where e.g. n = 8 for a 96-well plate, n = 16 for a 384-well plate:

$$\overline{OD} = \frac{1}{n} \times \sum_{i=1}^{n} OD_i \tag{B.21}$$

where

$\overline{OD}$ is the mean optical density;

OD_i is the optical density per well.

2) Calculate the low actual mean weight of the reference channel of the gravimetric measurement according to Formula (B.22):

$$\bar{M}_{\text{ref}} = \frac{1}{n} \times \sum_{i=1}^{5} M_i \tag{B.22}$$

where

$\bar{M}_{\text{ref}}$ is the low actual mean weight of the reference channel;

M_i is the weight measured for each channel.

3) Convert mass to volume by applying the appropriate Z correction factor (see Annex C) with Formula (B.23):

$$\bar{V}_{\text{ref}} = \bar{M}_{\text{ref}} \times Z \tag{B.23}$$

where $\bar{V}_{\text{ref}}$ is the volume delivered by the reference channel.

4) Calculate the conversion factor C according to Formula (B.24):

$$C = \frac{\overline{V}_{\mathrm{ref}}}{OD} \qquad \text{(B.24)}$$

where C is the conversion factor correlating optical density to volume.

Dispense all plates to be measured in a row:

1) For test volumes < 100 µl and 96-well plates: Aspirate distilled water from reservoir by immersing the tip(s) 2 mm to 3 mm below the surface of the water and dispense to all wells of the plate: use 100 µl for 96-well plates and 60 µl for 384-well plates. Be sure to keep the plate covered to prevent evaporation.
2) If working with disposable tips: Load new tips to the pipetting head and pre-wet the tips.
3) For volumes < 10 µl: Aspirate 10 µl distilled water and an air gap of 0,5 µl before aspirating the test volume.
4) Aspirate the low test volume (10 %) of test solution from the reservoir by immersing the tip 2 mm to 3 mm below the surface of the solution and dispense to all wells of the plate.
5) Place plate on shaker and shake at medium speed for 2 min.
6) Read the optical density of entire plate.
7) Subtract reference readings from high absorbance readings.
8) Using the conversion factor C, convert the OD readings to volume, following Formula (B.25):

$$V_i = C \times OD_i \qquad \text{(B.25)}$$

B.6.1.3 Fluid properties

B.6.1.3.1 Introduction

Dye: Tartrazine, CAS 1934-21-0, Supplier: Alfa Aesar GmbH and Co KG, ensure homogenous dilution by appropriate mixing.

Diluent: Distilled water, filtered with 0,5 µm syringe filter.

The concentration should be adjusted so that the measured optical density lies in the optimal reading range of the plate reader (usually an OD < 2).

The following two sections are recommended stock solutions for measuring volumes in 96- and 384-well plates. Shelf life of these solutions is 2 weeks when stored in a closed container at 20 °C and protected from direct light.

B.6.1.3.2 Recommended stock solutions for measuring 96-well plates

Table B.12 — Recommended stock solutions for measuring 96-well plates

Volume measurement range [µl]	1,25 to 12,5	12,5 to 125	30 to 300	125 to 1 250
Working conc. [mg/ml]	10	1	0,4	0,1
Tartrazine [mg]	50	20	20	20
Distilled water [ml]	5	20	50	200

B.6.1.3.3 Recommended stock solutions for measuring 384-well plates

Table B.13 — Recommended stock solutions for measuring 384-well plates

Volume measurement range [µl]	1,25 to 12,5	12,5 to 125
Working conc. [mg/ml]	2,5	0,25
Tartrazine [mg]	12,5	5
Distilled water [ml]	5	20

B.6.1.4 Calibration

To calibrate the subsequent photometric measurement one channel is measured gravimetrically. The accuracy and precision of this channel is determined and is then used as a reference for the rest of the channels. All other channels are measured by photometry and are then relatively compared to the reference channel.

B.6.1.5 Environmental conditions

Measurements should be performed under conditions of the ISO 8655-6 standard.[32]

- Draft free environment;
- Ambient temperature 18 °C to 25 °C, constant (±0,5 °C) throughout the measurements;
- Ambient relative humidity > 50 %;
- Liquid handling equipment and consumables must be stored in test environment for at least 2 h prior to measurements to reach temperature equilibrium with ambient.

B.6.1.6 Traceability

Precision balance and measurement equipment for temperature, humidity and atmospheric pressure must be calibrated by an accredited test lab. Calibration history needs to be documented for reference.

B.7 Hybrid method: Photometry and gravimetry with *p*-nitrophenol[9)]

Photometric and gravimetric liquid handling check procedure to determine the random error (precision) and the systematic error (accuracy) of ALHS

Outline of the method

B.7.1 Summary of the method principle

The method describes a reliable liquid handling check procedure for automated multichannel liquid handling systems.[16,34-36] In a first step the random error (precision) is determined by an absorption 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 maximum at 405 nm at $pH > 9{,}2$ which is realized by using 0,1 N NaOH as standard solvent and diluent. The coefficient of variation (CV in %) is calculated from the absorption measurement signals of individual microplate wells. Smaller test volumes are transferred in wells pre-filled with 0,1 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 µM in all wells of the microplate, which is within the optimal dynamic range of the absorption reader. The random error (precision) is determined always at first in the evaluation of an automated multichannel liquid handling system followed by a gravimetric determination of the systematic error (accuracy). The microplate absorption reader and the analytical balance have to be calibrated at regular intervals and the test conditions have to be considered strictly.

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9) Contributed by: Analytik Jena AG, Jena, Germany.

B.7.2 Method details

B.7.2.1 Test equipment[10)]

- Photometer for 96- and 384-well microplates with 405 nm and 620 nm option, e.g. Tecan GENios Plus (resolution 0,0001 OD, linearity 0 OD to 3,0 OD: ± 1,5 % and 0,005 OD, precision 0 OD to 3,0 OD: ± 1,0 % and 0,005 OD, accuracy 0 OD to 2,0 OD: ± 1,0 % and 0,01 OD), scheduled service and manufacturer calibration is needed.
- Calibrated analytical balance, e.g. Mettler Toledo AG245 (Capacity 41 g to 210 g, readability 0,1 mg to 0,01 mg, repeatability 0,1 mg to 0,02 mg, linearity ± 0,2 mg), scheduled service and authorized certified calibration is needed.
- Calibrated manual pipettes to prepare the test solutions.
- High quality clear flat bottom polystyrol 96- and 384-well microplates suitable for absorbance measurements, e.g. Greiner Bio One # 655 010 and # 781 101, respectively, vacuum packed microplates have to be unpacked 1 week before the measurement.
- High quality microplate sealing tape, e.g. Nunc # 236 269.
- Microplate shaker, e.g. BioShake iQ.
- Microplate centrifuge, e.g. SIGMA 6K 15.

B.7.2.2 Test reagents

- *p*-nitrophenol, MW 139,11 g/mol, spectrophotometric grade.
- Sodium hydroxide (NaOH) pellets, MW 40,00 g/mol, *p.a.*, for preparation of 0,1 N NaOH as solvent and diluent, density = 1,004 g/cm^3 at 20 °C.
- DI water (purity type II, conductivity < 1 µS/cm), density = 0,998 g/cm^3 at 20 °C.
- Optional DMSO dried, purity ≥ 99,9 %, density = 1,10 g/cm^3 at 20 °C.

The final *p*-NP concentration in the wells of the microplate should be 120 µM to result in an OD value of about 1, which is within the optimal dynamic range. In Tables B.14 and B.15, the corresponding optimal *p*-NP concentrations for the different test volumes in both, 96- and 384-well microplate formats are summarized.

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10) The examples given include trade names of products. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the products named.

Table B.14 — Optimal *p*-NP test concentrations for different test volumes in 96-well microplate format with 200 µl final volume per well

Test volume	Prefilled volume	Test concentration
	0,1 N NaOH	*p*-NP
µl	µl/well	mM
200,00	0	0,12
100,00	100,00	0,24
50,00	150,00	0,48
25,00	175,00	0,96
10,00	190,00	2,40
5,00	195,00	4,80
2,00	198,00	12,00
1,00	199,00	24,00
0,50	199,50	48,00
0,25	199,75	96,00
0,20	199,80	120,00
0,10	199,90	240,00

Table B.15 — Optimal *p*-NP test concentrations for different test volumes in 384-well microplate format with 50 µl final volume per well

Test volume	Prefilled volume	Test concentration
	0,1 N NaOH	*p*-NP
µl	µl/well	mM
50,00	0,00	0,12
25,00	25,00	0,24
10,00	40,00	0,60
5,00	45,00	1,20
2,00	48,00	3,00
1,00	49,00	6,00
0,50	49,50	12,00
0,25	49,75	24,00
0,20	49,80	30,00
0,10	49,90	60,00

The different *p*-NP test concentrations are prepared from a stock solution. All solutions have to be filtered before use and should be stored in the dark at room temperature no longer than 3 months. While 0,1 N NaOH is used as diluent as well as standard solvent to prepare the test solutions for the liquid handling check procedure, other solvents to prepare the test solutions are possible. According to all of our experiences the liquid handling performance for higher test volumes than described in the Table B.14 is comparable or becomes better.

B.7.2.3 Test conditions

The tests have to be performed in an air conditioned room or at room temperature at constant environmental conditions (temperature variation less than ± 1 °C, air humidity variation less than ± 10 %). All test equipment, disposables and all reagents have to be in equilibrium with these environmental conditions for at least 1 h. Temperature and air humidity at the beginning and the end of the tests have to be documented in the test report. The analytical balance has to be placed nearby the liquid handling device under evaluation to limit evaporation.

B.7.2.4 Test execution

The test results have to be calculated from at least 3 parallel measurements per test volume. The tests have to be performed at least with the smallest specified volume and one, or even more, higher volumes.

B.7.2.4.1 Photometric determination of the random error (precision)

The precision as described here defines the variation of the transferred test volume between the different wells of a microplate. The microplates have to be prefilled with the appropriate volume (see Tables B.14 and B.15) considering the test volume and the microplate format. The prefilled volume is always in the uncritical high volume range where the random error is very small. The absorbance measurement signal is mainly caused by the test solution and even a potential systematic error of the prefilled volume transfer would not influence the random error calculation of the test volume in a significant way.

The pipetting methods have to be set-up in the software of the liquid handling device with the following rules and system parameters:

- One set of new tips per volume and pipetting mode;
- Reduce piston speed to default speed/3;
- Reduce vertical speed for moving tips out of the liquid to default speed/3;
- Prime tips at least 5 times with the maximum tip volume;
- Implement a break of 1 s after every aspiration and dispensing step;
- Immersion depth of the tips in the liquid should be 1 mm to 2 mm;
- Reverse mode pipetting of the test volume with at least 5 µl additional aspiration volume;
- Pipetting back the first test volume in the test solution reservoir followed by pipetting of the test volume in the microplate;
- Ejecting the residual volume with maximum blow-out volume back into the source reservoir or waste, move the tips out of the liquid and set pistons back to start position (zero);
- Immediate sealing of the microplate;
- Shaking of the plates for at least 10 min (with careful acceleration and a final speed adapted to the microplate format used to avoid splashing);
- Centrifugation of the microplates to remove bubbles and to flatten meniscus (e.g. 2 min at 2 000 rpm);
- Read-out not earlier than 1 h after finishing the pipetting procedure;
- Daily quick cross-check of the absorption reader performance by turning a test plate by 180° and comparison of the results (in addition to scheduled service and manufacturer calibration) to exclude trending or patterns caused by the reader.

B.7.2.4.2 Gravimetric determination of the systematic error (accuracy)

The accuracy as described here defines the agreement between the mean transferred volume in the corresponding destination wells of a microplate and the volume setting per well in the pipetting method. The variation of the accuracy from well to well is defined by the random error of the liquid handling device, which should be checked at first and has to be in the specified range.

Immediately after the prefilling of the microplates with the prefilled volume (see Tables B.14 and B.15) the tare weight has to be read after the stabilization of the balance. Then the test solution has to be transferred as described in B.7.2.4.1 and the microplate weight has to be read again immediately. The time interval from weighing before and after the test solution transfer should not be greater than 15 s to limit the evaporation to be less than 1 ‰ of the test volume, otherwise the evaporation has to be measured at the specific environmental conditions and the calculation has to be corrected accordingly.

It is possible to combine the determination of the systematic error with the determination of the random error *via* weighing the microplates before and after the transfer of the test solution. Here the specific density of the test solution (see B.7.2.2) has to be considered in the calculation of the accuracy (see B.7.2.5). The results are only acceptable if the random error of the automated liquid handling system under evaluation is in the specified range for the tested volume.

B.7.2.5 Test evaluation

The random error (precision) is calculated as relative coefficient of variation (CV in %). It describes the relative standard deviation between the OD signals of all single wells of a microplate in relation to the mean OD signal over this microplate.

The systematic error (accuracy) describes the proximity of the measurement results to the target values. The real volume per well is measured by weighing considering the specific density of the test liquid and the number of wells. The difference between the measured volume and the target volume is calculated as accuracy (in %) in relation to the target volume.

Please refer to Clause 5 of this document for further details on these calculations.

B.7.2.6 Test report

The test report shall conform to Clause 8 of this document, and include the following additional details:

- test volumes and test solutions (*p*-NP concentration dissolved in 0,1 N NaOH or another specified solvent);
- test method with system parameters (e.g. pre-wetting of tips, forward or reverse mode pipetting, single- or multi-dispensing, prefilled volume, additional aspiration volume, piston speed, breaks, vertical speed, method references, etc.).

B.7.2.7 Possible error sources

- Bubbles or uneven liquid surfaces in the wells;
- Splashing due to heavy shaking or inattentive removal of the sealing tape;
- Inhomogeneous distribution of the test solution in the diluent, e.g. caused by insufficient shaking or waiting time;
- Incomplete or incorrect system parameter settings;
- Irregularities in the microplates;
- Evaporation;
- Incorrect or instable environmental conditions.

B.7.2.8 Method traceability

The traceability of the gravimetric method to SI units is achieved through frequent calibration of the balance with certified standards by authorized institutions. The date of the last calibration shall be documented in the test report.

B.8 Hybrid method: Gravimetry and photometry with Ponceau S11)

Outline of the method

B.8.1 Combined gravimetric and photometric test method

B.8.1.1 Method name and summary

The combined gravimetric and photometric test method provides a simple procedure for the end users to determine the performance of multichannel liquid handling instruments at customer premises. The method comprises a gravimetric whole plate accuracy measurement and photometric measurement to determine the precision of liquid dispensing.

B.8.1.2 Method description

This method is used to verify that the microplate dispensing instrument fulfills given 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 to 2 Abs and a resolution of 0,001 Abs, capable of measurement at 540 nm wavelength. The measurement uncertainty of this method is described in a supplemental document.[17]

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11) Contributed by: Thermo Fisher Scientific Oy, Vantaa, Finland

B.8.1.2.1 Dispense accuracy

B.8.1.2.1.1 Items needed for the dispense accuracy test

Dispense accuracy is determined using gravimetric measurements.

- Microplate dispensing instrument with the appropriate dispensing head;
- Calibrated analytical balance (resolution of 0,1 mg);
- Calibrated thermometer (readable to 0,5 °C);
- At least three 96-well plates with lids for each tested volume;
- Disposable gloves;
- Deionized or distilled water according to ISO 3696 grade 3;
- Volume conversion factor (*Z* correction factor) table for water (see Annex C).

Ensure that the deionized or distilled water does not contain particles > 50 µm.

It is important to equilibrate the deionized or distilled water to room temperature before performing this procedure.

B.8.1.2.1.2 Gravimetric measurement

Dispense accuracy is determined using gravimetric measurements.

Method

Test is performed for the volumes of interest above 2 µl and not exceeding the maximum usable well volume.

Three plates are dispensed for each test volume.

1. Set the system parameters for the appropriate liquid and volume.
2. Use high-purity distilled water and fill the liquid system as instructed by the manufacturer.
3. Tare the empty balance, weigh and record the empty test plate with lid in grams.
4. Place the empty test plate without lid on the instrument.
5. Dispense on the empty test plate, place the lid on the test plate, tare the empty balance and immediately weigh the filled plate to avoid evaporation. Time interval between the dispensing and weighing the test plate should not exceed 15 s.
6. Record the gross weight for the test plate in grams.
7. Calculate the **mean weight** dispensed **per well** ($\bar{M}$) for the test plate as follows:

$$\bar{M} = \frac{M_G - M_E}{N} \tag{B.26}$$

where

$\bar{M}$ is the mean dispensed weight per well;

M_G is the gross weight of the plate with the lid;

M_E is the weight of the empty plate with the lid;

N is the number of wells in the plate, e.g. $N = 96$.

NOTE This method relies on the assumption that the variation of individual channels and doses are under control. The variation is controlled through photometric precision measurement.

8. Calculate the **mean volume** dispensed **per channel** ($\bar{V}$) in microlitres for the test plate as follows:

$$\bar{V} = \bar{M} \times Z \times 1000 \tag{B.27}$$

where

$\bar{V}$ is the mean dispensed volume per channel;

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

9. Calculate the **relative systematic error** (accuracy) per channel as follows:

$$e_S = \frac{\bar{V} - V_T}{V_T} \times 100\% \tag{B.28}$$

where

e_S is the relative systematic error (accuracy) per channel;

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

10. Repeat steps 3 to 9 for the second and third test plates.

11. Compare the result of each plate to the accuracy specification set by the manufacturer. Results of each test plate should be within manufacturer's specification.

B.8.1.2.2 Dispense precision

Precision is determined using photometric measurement.

The concentration of the used colour dye should be adjusted so that the measured optical density lies in the optimal reading range of the plate reader (normally an OD ~1 Abs).

B.8.1.2.2.1 Items needed for the dispense precision test

Dispensing precision is determined using photometric measurements.

- Microplate photometer set to measure absorbance at 540 nm (measurement range 0 – 2 Abs, resolution 0,001 Abs), e.g. Thermo Scientific™ Multiskan™ FC.[12)]
- At least three clear, flat bottom 96-well plates for each tested volume, e.g. Thermo Scientific™ 96F Untreated 260836.
- Ponceau S reagent (e.g. BDH product # 341362T) with suitable concentration to read absorbance approximately 1 Abs at 540 nm. For example, 0,25 % (2 µl), 0,05 % (10 µl), 0,025 % (20 µl) and 0,005 % (100 µl) stock solutions are needed for different target volumes. See Table B.16. Use filtered and preferably fresh reagent. Refer to B.8.1.3 for details on the preparation of the Ponceau S stock solutions.
- With small orifice dosing nozzles, ensure that the dispensed liquid does not contain particles > 50 µm.
- A 0,02 % w/v solution of Tween (e.g. Fluka or equivalent) in deionized distilled water (see: B.8.1.3).
- Deionized or distilled water according to ISO 3696 grade 3.
- Hand-held pipette, 0,5 ml to 5 ml, e.g. Thermo Scientific™ Finnpipette™ F1.
- Pipette tips, e.g. Thermo Scientific™ Finntip™ 5 ml.

B.8.1.2.2.2 Photometric measurement

Method

Test is performed for the volumes above 2 µl and not exceeding the maximum usable well volume, and

Three plates are dispensed for each volume.

1. Adjust the system parameters for the appropriate liquid and volume.
2. Use appropriate Ponceau S solution for the test volume (Table B.16) and fill the liquid system as instructed by the manufacturer.
3. Place the test plate on the instrument.
4. Dispense the Ponceau S stock solution volume into the plate.
5. Dispense the same volume of Ponceau S stock solution into the two other test plates.
6. Flush the liquid system with distilled water until no colour is visible.
7. Set the instrument to dispense Tween solution and fill the liquid system as instructed by the manufacturer.

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12) Thermo Scientific™ Multiskan™ FC, Thermo Scientific™96F Untreated 260836, Thermo Scientific™ Finnpipette™ F1, Fluka and Thermo Scientific™ Finntip™ are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.