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

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Contents

Foreword

This Draft International Workshop Agreement was developed by Technical Committee ISO/TC 48 *"Laboratory equipment."*

ISO IWA 15 consists of one part under the general title *Specification and method for the determination of volumetric performance parameters of automated liquid handling systems.*

Annex A is an informative representation of descriptive statistics concepts discussed in clause 5.

Annex B is an informative description of testing methods, and is arranged in subparts for each method.

Annex C is normative and describes the conversion of weight to volume.

Annex D is a listing of workshop participants.

Introduction

This International Workshop Agreement (IWA) addresses the needs of:

- Suppliers of automated liquid handling systems (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.

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

1 Scope

This IWA specifies methods for testing the volumetric performance of air-displacement, system-liquid filled and positive displacement automated liquid handling systems, 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.

It 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 formulas to be used for summarizing test results.

This IWA also specifies the information to be provided to users of automated liquid handling systems (ALHS), including the display of summary results and performance claims.

This IWA is applicable to all automated liquid handling systems with complete, installed liquid handling gantries, 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.

2 References

The following document, in whole or in part, is normatively referenced in this document and is 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 Automated liquid handling system terminology

3.1.1
automated liquid handling system
ALHS

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

NOTE Examples of automated liquid handling systems include automated pipetting systems (APS), and automated dispensing systems (ADS).

3.1.2
pipetting system

system for aspirating and dispensing a specified volume of liquid

3.1.3
dispensing system

system for dispensing liquids which performs liquid handling without separate aspiration of liquid

3.1.4
system liquid

incompressible liquid used to transmit energy between a mechanical piston and the test liquid

NOTE 1 System liquids can reduce or completely eliminate system dead air volume.

NOTE 2 System liquid is usually water. For special applications organic solvents as DMSO or aqueous solutions as saline (e.g., 0.9 % NaCl) can be used.

NOTE 3 System liquid can be used for flushing and rinsing tips to prevent cross contamination.

3.1.5
individually controlled channel

liquid handling channel that can be operated independently of other channels

3.1.6
multichannel head

a group of liquid handling channels operated in common

NOTE 1 Common arrangements of multichannel heads include 8, 96, 384, and 1536 channel heads. Other arrangements are possible, e.g. 2 -1536 channel configurations.

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

3.1.7
labware

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

NOTE Labware includes disposable tips, reservoirs, receiving vessels, adapters and microplates.

[SOURCE: Toolpoint - MODIFIED]

3.1.8
disposable tip

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

NOTE 1 Disposable tips are usually made of plastic.

NOTE 2 Disposable tips are in contrast to fixed tips, which are described in 4.4.4.1.

NOTE 3 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).

3.1.9
liquid container
reservoir
cavity that contains the test liquid

[SOURCE: Toolpoint]

3.1.10
microplate
a flat plate with an array of wells

NOTE Some dimensions of microplates are defined in ANSI/SLAS standards.

3.1.11
positive displacement
liquid handling principle in which the piston is in direct contact with the liquid

[SOURCE: 8655-2]

3.1.12
air displacement
liquid handling principle in which a body of air is contained between the piston and the liquid

NOTE It is possible to have a large air gap (piston systems), or smaller air gap for liquid filled systems.

3.1.13
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 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 Commonly, an air gap can be adjusted through ALHS program parameters, while the dead air volume or captive air volume cannot be adjusted (see clause 4.2.1).

3.1.14
forward mode pipetting
direct mode pipetting
<air displacement pipetting> pipetting mode where the entire aspirated volume is delivered

3.1.15
reverse mode pipetting

<air displacement pipetting> pipetting mode in which excess volume is aspirated and remains in tip after delivery

3.1.16
wet contact dispensing
dispensing of the liquid while tip is in contact with a prefilled liquid in the target cavity

[SOURCE: Toolpoint]

3.1.17
dry contact dispensing
dispensing of liquid while tip is in contact with the dry target cavity

[SOURCE: Toolpoint]

3.1.18
contact-free dispensing
free-jet dispensing
non-contact dispensing
dispensing of the liquid while tip is in air and without contacting the target cavity or the liquid contained in the target cavity

[SOURCE: Toolpoint]

3.1.19
single dispense
individual dispense
<automated pipetting systems> single dispense per aspiration

NOTE It is recommended that test reports include disclosing the mode used in testing.

3.1.20
multi dispense
repeat dispense
sequential dispense
<automated pipetting systems> a collection of dispenses without intervening aspiration

NOTE 1 First dispense can be different, and is frequently wasted.
NOTE 2 Repeat dispenses usually dispense repeatedly the same volume, while sequential dispenses usually dispense different volumes.

3.1.21
immersion depth
depth of the tip below the liquid surface

NOTE Immersion depth can be applied to both aspiration and dispensing (wet contact).

[SOURCE: Toolpoint - MODIFIED]

3.1.22
dispensing height
height at which the test liquid is dispensed relative to the bottom of the cavity or the surface of the liquid

[SOURCE: Toolpoint]

3.2 Volumetric Performance Testing Terminology

3.2.1
test liquid
liquid used for the volume measurement

NOTE 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.

[SOURCE: Toolpoint MODIFIED]

3.2.2
maximum specified volume
the largest volume for which the manufacturer offers specifications

NOTE The maximum specified volume may vary depending on instrument configuration (e.g. disposable tip size, syringe size).

3.2.3
minimum specified volume
the smallest volume for which the manufacturer offers specifications

NOTE The minimum specified volume may vary depending on instrument configuration.

3.2.4
target volume
indicated volume
selected volume
volume which is intended to be delivered

3.2.5
delivered volume
the quantity delivered by a liquid handling system

NOTE Delivered voume is a conceptual term n and cannot be known with complete certainty due to measurement error

3.2.6
measured volume
the quantity reported by a volume measuring system

NOTE 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.

3.2.7

outlier

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

3.2.8

test result

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

NOTE 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 3534-1 MODIFIED]

3.2.9

accuracy

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

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

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

3.2.10

ALHS uncertainty

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

NOTE Uncertainty is inversely related to accuracy, and is a quantity value. This value should be expressed in accordance with the GUM.

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

3.2.11

trueness

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

NOTE Trueness is inversely related to systematic error, but is not related to random error.

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

3.2.12

systematic error

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

NOTE Systematic error is estimated by calculating the average volume of a series of deliveries and comparing 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, definition 2.17 - modified]

3.2.13

precision

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

NOTE 1	The measure of precision is usually expressed in terms of imprecision and computed as a standard deviation of the test results. Less precision is reflected by a larger standard deviation.
NOTE 2	Precision is conceptual and not a quantity value.
NOTE 3	Measurement precision is usually expressed numerically by measures of imprecision, such as standard deviation, variance, or coefficient of variation under the specified conditions of measurement.
NOTE 4	The 'specified 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 3534-1 modified]

3.2.14

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, definition 2.19 - modified]

3.2.15

factory acceptance testing

internal vendor testing to ensure verification with design intent

NOTE	This testing should be conducted under carefully monitored environmental conditions, which shall be recorded and reported with the test results.

3.2.16

site acceptance testing

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

NOTE	This testing should be conducted under the prevailing environmental conditions under which the instrument is operated. The environmental conditions should be recorded.

3.2.17

verification

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

NOTE 1	The term "verified" is used to designate the corresponding status.
NOTE 2	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, definition 2.44 - modified]

3.2.18

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 The term “validated” is used to designate the corresponding status.

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

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

[SOURCE: ISO/IEC Guide 99:2007, definition 2.45 - modified]

3.2.19

calibration

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

NOTE A calibration may be expressed by a statement, a calibration curve or 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, definition 2.39 - modified]

3.2.19

maximum permissible error

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

[SOURCE: ISO/IEC Guide 99:2007, definition 4.26 – modified]

3.2.20

measurement uncertainty

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

NOTE Uncertainty is inversely related to accuracy, and is a quantity value. This value should be expressed in accordance with the GUM.

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

3.2.21

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 This IWA can be referenced as an applicable industry standard.

3.2.22
test report
document reporting the result of the testing

NOTE Details on information contained in test reports is discussed in clause 8 of this IWA.

3.3 Acronyms and Symbols

Table 1. Acronyms and symbol used in this document.

Acronym	**Explanation**
ALHS	Automated Liquid Handling System
APS	Automated Pipetting System
ADS	Automated Dispensing System
IWA	International Workshop Agreement
VIM	International Vocabulary of Metrology
GUM	Guide to the Expression of Uncertainty in Measurement
IEC	International Electrotechnical Commission
MU	Measurement Uncertainty
RH	Relative Humidity [%]
RSE	Relative Systematic Error
C2C	Channel-to-Channel
GA	Grand Average
OA	Over All

4 Operational parameters of piston-operated ALHS

4.1 Types of piston operated automated liquid handling systems

Automated liquid handling systems can be designed 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 volumes~~.~~

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 liquid working fluid (liquid filled system).

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.

4.2 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. Pipetting parameters may need to be adjusted in the following cases:

- accommodate liquid-specific properties, or
- following the replacement of system components, or
- 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 pipetting parameters such as the aspiration and dispense speeds, immersion depth of the tip, dispense height, air gaps, and others.

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

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 volumentric performance of the ALHS shall report the test liquid, instrument settings, and environmental basis for each tested liquid class.

NOTE 1 An aqueous liquid class can be established by testing the ALHS with pure water (conforming to ISO 3696), or water containing minerals, surfactants, or other additives.

NOTE 2 Optimizing the volumetric performance of an ALHS for non-aqueous liquid classes usually requires adjustment of operational settings. Test reports should include the type of test liquid and instrument settings for the test.

4.2.3 Adjustment of ALHS settings

Some automated liquid handling systems have 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 Environmental conditions

4.3.1 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.

4.3.2 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 hours prior to testing in an environment as defined by the ALHS manufacturer. Sensitivity to temperature stability and humidity of the environment is dependent on the test method employed.The environmental conditions (temperature, RH, barometric pressure), including their maximum and minimum values during the time of testing shall be recorded. At a minimum, the temperature or temperature range during the time of testing shall be reported with the factory acceptance test results for all equipment used during this testing. 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.

4.3.3 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 minimal and maximal 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.

4.4 Tips

4.4.1 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 wetability.

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

4.4.2 Air-displacement tips

4.4.2.1 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.

4.4.2.2 Tips made of plastic with air interface shall be fitted in accordance with the automated liquid handling system supplier's instructions to form a good seal between the tip and the ALHS dispensing head, and are designed for single use (see clause 3.1.8).

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.

4.4.2.3 The form of the tips to be used with a multi-channel dispensing heads on automated liquid handling systems shall be so straight that all tips fitted are positioned with parallel axes in the same plane in order to allow for even liquid dispensing in the target vessels, e.g. the adjacent wells of a microplate.

4.4.3 Positive displacement tips

4.4.3.1 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 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).

4.4.3.2 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.

4.4.4 Fixed tips

4.4.4.1 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.

4.4.4.2 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.

4.4.4.3 Maintenance and Exchange of Fixed Tips

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

5 Volumetric performance

5.1 Introductory discussion

Liquid handling systems are designed to deliver aliquots of liquid at a target volume. Target volume is typically set using software or other digital control. Volumetric performance is assessed by measuring the volume of each delivered aliquot and evaluating the data.

Volumetric performance is typically assessed by suppliers as part of the manufacturing process quality control or a suppliers 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 may vary depending on these physical properties, so a description of the test liquid should be included when reports of volumetric performance are made. This description of the test liquid can 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. Users and others 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 aliquots. The measured volume is a quantity which consists of a numerical value and units. The recommended units are microliters (µL) though related units such as milliliters and nanoliters are sometimes used and are also acceptable. The measured volume is an estimate of the delivered volume and departs slightly from true delivered volume due to measurement uncertainty. 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 center 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 center and this distance of each mark from the center 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 center of the target.

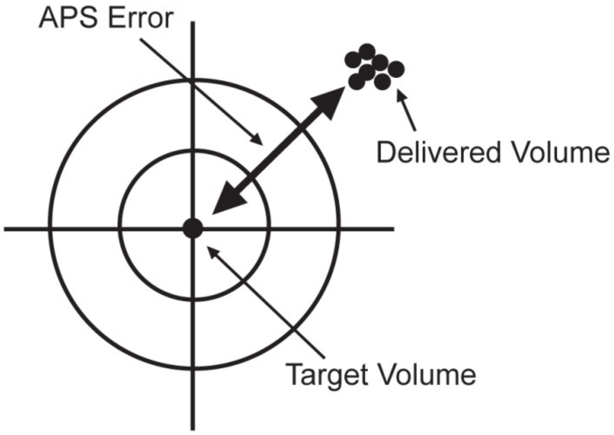


Figure 1. Relationships between target volume, deliverered volume and ALHS error.

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 center of the cluster is closer to the center 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.

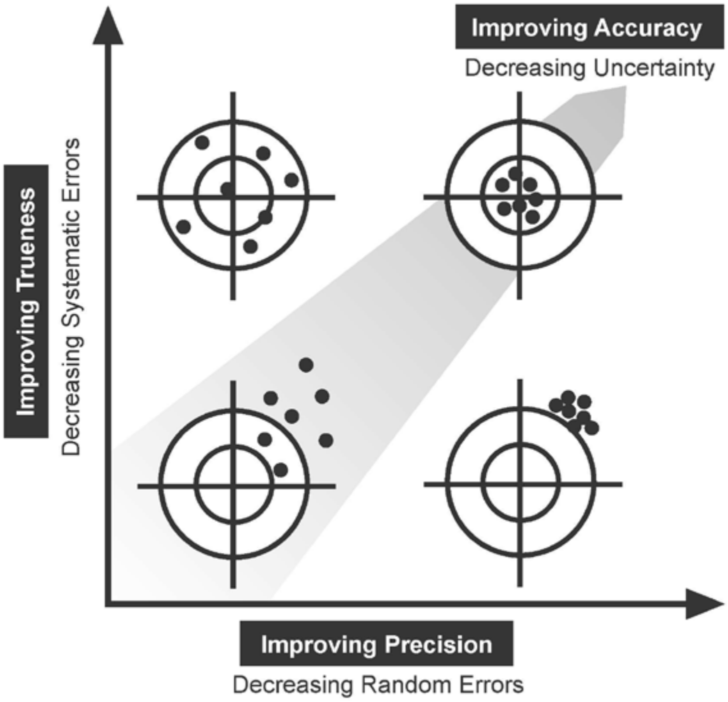


Figure 2. Relationship between trueness, precision, and accuracy of an ALHS.

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 aliquot delivered can be measured, to determine the measured volume V. Volume measurement methods are described in clause 6 of this IWA.

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, e.g. in [LINK TO TWO REFERENCES in BIBLIOGRAPHY]

5.3 Indexing to track data

Volumetric performance is assessed in a first step by making multiple volume deliveries and measuring the volume of each delivered aliquot, and then by evaluating the measurement results. 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 aliquot 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 aliquot 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 aliquots 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 is used to calculate averages or standard deviations as the reliability of these descriptive statistics depends on the number of replicates.

In this way, and measured volume of the n'th aliquot, delivered 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 clause 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 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 or 1 through 24). 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 Annex A.

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 aliquots 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 formulas 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 a particular channel during a particular run;

N is the number of replicate deliveries in the run;

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

Systematic error is estimated by the deviation of the measured mean volume from the target volume. If for example 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.

$$RSE_{l,m} = \frac{\bar{V}_{l,m} - V_T}{V_T} \tag{2}$$

where

$RSE_{l,m}$ is the relative systematic error;

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

Estimates of systematic error can be improved by increasing the number of measurements in the data set, either by increasing N, or 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 3.

$$\bar{V}_l = \frac{1}{MN} \sum_{n=1}^{M} \sum_{n=1}^{N} V_{l,m,n} \tag{3}$$

where

$\bar{V}_l$ is the mean measured volume from channel l;

M is the number of runs included in the average.

Formula 3 can be re-arranged as shown in formula 4, and the identical result can be obtained by either formula 3, or by taking the M replicated results of formula 1, and averaging them together.

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

Random error of a channel is usually assessed by calculating the standard deviation of a series of N measured volumes under repeatability conditions, dividing by the average volume, and multiplying by 100 to convert to a percentage. The recommended descriptive statistic for random error is the coefficient of variation (CV) as shown in Formula 5.

$$CV_{l,m} = \frac{\sqrt{1/(N-1) \sum_{n=1}^{N} (V_{l,m,n} - \bar{V}_{l,m})^2}}{\bar{V}_{l,m}} \tag{5}$$

where

$CV_{l,m}$ the coefficient of variation of a particular channel from a particular run.

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 5, while summing over multiple experiments using a root-mean-squares approach is shown in Formula 6 below. This formula is permissible when N is identical in each experiment.

$$CV_l = \sqrt{1/\mathrm{M}\sum_{m=1}^{M} \mathrm{C}V_{l,m}{}^2} \qquad (6)$$

where

CV_l is the coefficient of variation of a particular channel combing data from multiple runs.

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

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 deliver 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 ($\overline{V}_n$) can be calculated by Formula 7.

$$\overline{V}_n = \frac{1}{LM}\sum_{l=1}^{L} \sum_{m=1}^{M} V_{l,m,n} \qquad (7)$$

where

$\overline{V}_n$ is the mean measured volume from all channels and all runs.

In addition to run-order volume, a run order CV can also be calculated using Formula 8. This CV can be useful in determining whether 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.

$$CV_n = \frac{\sqrt{1/(LM-1)\sum_{l=1}^{L}\sum_{m=1}^{M}(\bar{V}_{l,m,n}-\bar{V}_n)^2}}{\bar{V}_n} \tag{8}$$

where

CV_n is the coefficient of variation of a particular n'th dispense across all channels, and combing 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 9. The grand average volume can be converted to a relative systematic error by analogy to formula 2.

$$\bar{V}_{\mathrm{GA}} = \frac{1}{LMN}\sum_{l=1}^{L}\sum_{n=1}^{M}\sum_{n=1}^{N} V_{l,m,n} \tag{9}$$

where

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

Overall CV 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 10.

$$CV_{\mathrm{OA}} = \frac{\sqrt{1/(LMN-1)\sum_{l=1}^{L}\sum_{m=1}^{M}\sum_{n=1}^{N}(\bar{V}_{l,m,n}-\bar{V}_{\mathrm{GA}})^2}}{\bar{V}_{\mathrm{GA}}} \tag{10}$$

where

CV_{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.

The influence on the volumetric precision of aliquots dispensed with different dispensing channels can also be evaluated by a channel-to-channel CV, calculated as in formula 11.

$$CV_{\mathrm{C2C}} = \frac{\sqrt{1/(L-1)\sum_{l=1}^{L} (\bar{V}_l - \bar{V}_{\mathrm{GA}})^2}}{\bar{V}_{\mathrm{GA}}} \qquad (11)$$

where

CV_{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-Aliquots

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

6 Measurement Methods

6.1 Overview of methods suitable for measuring ALHS performance

The following Table 2 provides an overview of methods for evaluating the volumetric performance of ALHS. Before choosing a test method, the user should evaluate its suitability for the specific test situation,which may include requirements for measurement traceability and estimation of measurement uncertainty. Open methods can be used on any ALHS platform, while closed methods reported here are specific to the reported ALHS platform.

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

Method No.	**Method type**	**Number of channels individually analyzed**	**Plate type**	**Fluid type**	**Volume range [µL]**	**Accuracy of method [%]**	**Precision of method [%]**	**Traceability**	**Environmental requirements**	**Statistical analyses as described in this IWA**				
										Individual Channel	Dispense Order	Row or Column	Cluster, Pattern	Whole Plate
Ratiometric photometry methods														
B.1	**Dual dye ratiometric photometry**	1 to 384	96 wells	Aqueous		Guaranteed Accuracy [%]	Typical Precision [%]	SI units: Documented traceability chain to NIST and NPL standards	Temperature 15-30 ºC					
					0.2 – 350	2	0.15 – 0.20			X	X	X	X	X
					0.1 – 0.2	3	0.20 – 0.25			X	X	X	X	X
				DMSO	0.2 – 10	2	0.15 – 0.20			X	X	X	X	X
					0.1 - 0.2	3	0.20 – 0.25			X	X	X	X	X
			384 wells	Aqueous	0.05 – 55	2.5	0.35 – 0.40			X	X	X	X	X
					0.02 – 0.05	3.5	0.40 – 0.46			X	X	X	X	X
					0.01 – 0.02	5.5	0.46 – 0.50			X	X	X	X	X
				DMSO	0.05 – 2.5	2.5	0.35 – 0.40			X	X	X	X	X
					0.02 - 0.05	3.5	0.40 – 0.46			X	X	X	X	X
					0.01 - 0.02	5.5	0.46 – 0.50			X	X	X	X	X
Gravimetric methods														
B.2	**Gravimetry, single channel measurement**					Typical Value @ MIN	Typical Value @ MIN							
		1 channel	n/a	Aqueous	1 – 10	≤ 0.2	≤ 0.6	SI units	20 ± 0.5 °C, ≥50% RH	X	X			X

					>10 - 100	≤ 0.2	≤ 0.2			X	X			X
					>100 - 1000	≤ 0.2	≤ 0.2			X	X			X
B.3	**Gravimetry, whole plate measurement**	Entire plate	96 or 384 well plate											X
B.4	**Gravimetric regression method for low volumes** (by non-contact dispensing)	1 channel	n/a	any liquid	0.04 – 1.0	10 nL- 2 nL	2.8 – 0.4	SI units		X				
Photometry methods														
B.5	**Orange G (492 nm and 620 nm)**	96	96 wells	Aque-ous	2.0 – 10.0									
					10.0 - 100.0									
					100.0 – 200.0									
		384	384 wells	Aque-ous	0.5 - 2.0									
					2.0 – 20.0									
					25.0 – 110.0									
B.13	**Tartrazine** (closed method)													
Hybrid methods using gravimetry and photometry														
B.6	**Gravimetry and photometry with Tartrazine**	1 to 384	96 wells	Aque-ous	1-5	1.8%	1%	SI units		X	X	X	X	X
					5-20	1.3%	0.7%							

					20-300	0.7%	0.5%							
			384 wells	Aqueous	1-5	2.5%	1.5%	SI units		X	X	X	X	X
					5-20	1.3%	0.9%							
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 µl: <0,2	<0,5	SI units	Temperature variation: < ±1°C RH variation: < ±10%	X	X	X	X	X
			384 wells		0,1 - 50									
B.8	**Gravimetry and photometry with Ponceau S**													
B.12	**Gravimetry and photometry** (closed method)	1 to 384	96 wells	Aqueous	5-1000			SI units: Documented traceability chain to NIST, UKAS and PTB standards		X				X
			384 wells	Aqueous	2-50					X				X
Other measurement methods														
B.9	**Pressure sensing**	1 to 384		Any Liquid	5-500 µl	<150µl: <5µL >150µl: <3%	<100µl: <2µl >100µl: <2%	SI units	18-32°C 10-90% RH <3000 m AMSL	X	X	X	X	X
B.10	**Calorimetric measurement**	1 to 384		Any Liquid	1-10 µl 10-300 µl	<3.5% <2%	<3% 1.5%	SI units (through calibrated plates)	15-30°C 40-80% RH	X	X	X	X	X
B.11	**Optical image analysis**	1 – 16 (in a row)		Any Liquid	Free flying droplets < 1µl			SI units (through calibrated camera)		X	X	X	X	X

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 plate 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®).[REF-MVS patents]

This kit allows the determination of volumes of aqueous solutions from 0.1 uL to 350 uL in 96-well plates, and from 0.01 uL to 55 uL in 384-well plates. Volumes of DMSO solutions can be determined from 0.11 uL to 10 uL in 96-well plates, and 0.01 to 2.5 uL 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 (19-30 °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.

A detailed description of this method is provided in Annex 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 volumentric 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 Annex B.2.

6.2.3 Gravimetry, full-plate measurement

This method allows a direct determination of the total liquid volume delivered to a microplate by a 96- or 384-channel liquid handling system. 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 used to confirm the absence of 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). Static electic 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 Annex 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 aliquot 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 uL – 1.0 uL can be measured with an accuracy of 2.0 nL – 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 Annex B.4.

6.2.5 Photometry using Orange G

This method describes the volumetric performance test of ALHS, using an aqueous liquid with single channel, 96- or 384-channel dispensing heads. 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 Annex 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 Annex B.6.

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 (160 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 forwarded 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 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 Annex 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 and ALHS, and can be used to determine whether it fulfills 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 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 Annex B.8.

6.2.9 Pressure sensing

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

A detailed description of this method is provided in Annex 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. This method can be used to characterize up to at least 384 liquid handling channels in parallel. Volumes of 1-300 µl can be measured with this method.

A detailed description of this methodis provided in Annex 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 microdroplets.

A reference to this method is provided in Annex 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 repective method. These methods may be modified to be used with ALHS platforms other than those describned.

6.3.1 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 Annex 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 ≤ 20µl are dispensed in (liquid) surface mode.

The hybrid method, gravimetric and photometric, uses a high precision balance and a photometer. 8 pipetting cycles per single channel at the specified volume are performed. For each pipetting (aspiration/ dispensation) a new disposable tip is used. For volumes > 20µl the applied dispense mode is jet dispense. Volumes ≤ 20µl 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. The tartrazine/DMSO solution is prepared by gravimetric and volumetric measurement of the constituents.

A detailed description of this method can be found in Annex B.13.

7 Specification of ALHS volumetric performance

7.1 Automated liquid handling systems

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 trueness and precision shall be specified at individual volumes or across the specified volume range.

NOTE The working volume range of the ALHS can be wider thatn the specified volume range.

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

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

7.2 Other information that can be supplied

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 8.1.2, and 8.1.3 or 8.1.4.

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 to 8.1.4 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 17025, the additional requirements of ISO 17025 are normative to this IWA.

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) A unique identification of the test report or 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 test report or calibration certificate, and a clear identification of the end of the test report or calibration certificate.

d) The name and address of the end-user, if known.

e) Identification of the test process used; information on the test process may include reference to the respective method in this IWA.

f) A description of and unambiguous identification of the ALHS tested or calibrated.

g) The date(s) of performance of the test or calibration.

h) 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.

i) The test or calibration results with, where appropriate, the units of measurement.

j) The name(s), function(s) and signature(s) or equivalent identification of person(s) authorizing the test report or calibration certificate.

k) A record of measuring instruments, reagents, and supplies used in the testing process of the ALHS.

NOTE 1 Test reports and calibration certificates should include the page number and total number of pages.

NOTE 2 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 Test reports

8.1.3.1 In addition to the requirements listed in 8.1.2, test reports shall, where necessary for the interpretation of the test results, include the following:

a) Deviations from, additions to, or exclusions from the test method, and information on specific test conditions, such as environmental conditions.

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

8.1.4 Calibration certificates

8.1.4.1 In addition to the requirements listed in 8.1.2, calibration certificates shall include the following, where necessary for the interpretation of calibration results:

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

b) The uncertainty of measurement and/or a statement of compliance with an identified metrological specification or clauses thereof.

c) Evidence that the measurement results are traceable to the SI unit of volume, the liter.

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

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

8.1.4.4 A calibration certificate (or calibration label) shall not contain any recommendation on the calibration interval except where this has been agreed with the customer. This requirement may be superseded by legal regulations.

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 formulas found in clause 5 of this IWA.

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 parameters (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 (e.g., 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.

	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

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 proceeds sequentially 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*=12
B	*l*=13	…										
C												
D												
E												
F												
G												
H												*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 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 sequentially across the entire plate until reaching the end of the row (e.g., A24). Next each channel moves downward, and continues delivering while traveling right to left along the second row to complete the operation. This plate layout will be used in example 4.

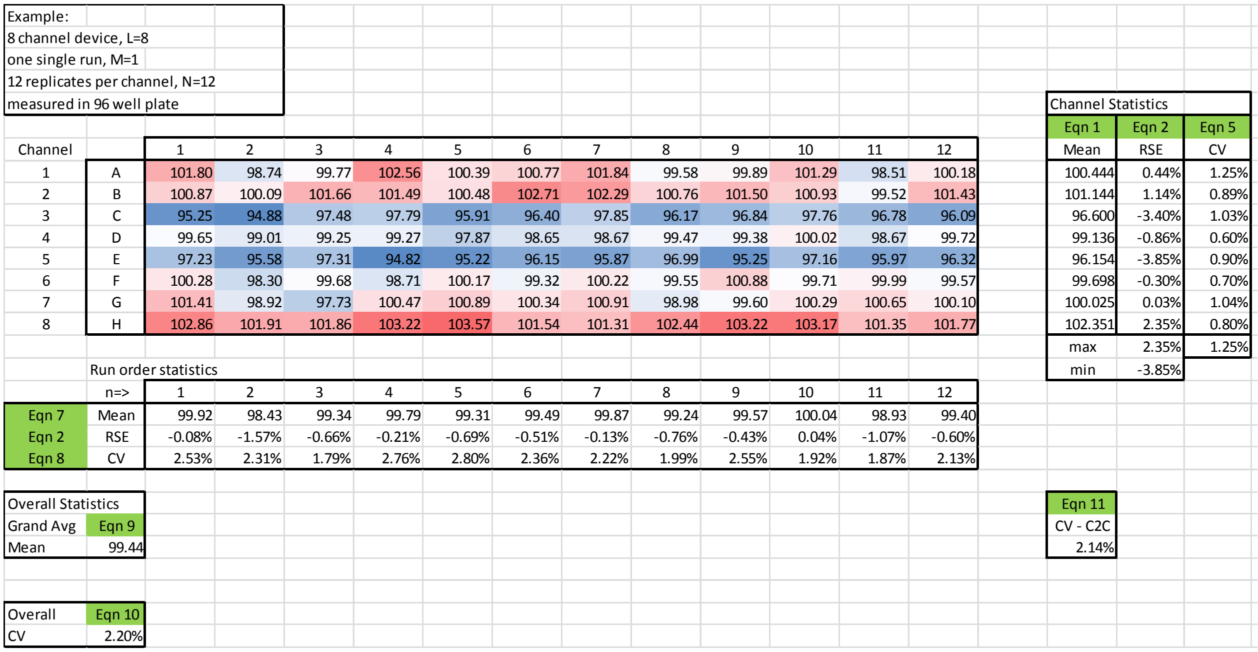
		Column number																							
Channel	Row	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	A	n=1	n=2	n=3	n=4	n=5	n=6	n=7	n=8	n=9	n=10	n=11	n=12	n=13	n=14	n=15	n=16	n=17	n=18	n=19	n=20	n=21	n=22	n=23	n=24
"	B	n=48	n=47	n=46	n=45	n=44	n=43	n=42	n=41	n=40	n=39	n=38	n=37	n=36	n=35	n=34	n=33	n=32	n=31	n=30	n=29	n=28	n=27	n=26	n=25
2	C	n=1	...																						
"	D																								
3	E	n=1	...																						
"	F																								
4	G	n=1	...																						
"	H																								
5	I	n=1	...																						
"	J																								
6	K	n=1	...																						
"	L																								
7	M	n=1	...																						
"	N																								
8	O	n=1	...																						
"	P																								

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

Channel		1	2	3	4	5	6	7	8	9	10	11	12
1	A	101.80	98.74	99.77	102.56	100.39	100.77	101.84	99.58	99.89	101.29	98.51	100.18
2	B	100.87	100.09	101.66	101.49	100.48	102.71	102.29	100.76	101.50	100.93	99.52	101.43
3	C	95.25	94.88	97.48	97.79	95.91	96.40	97.85	96.17	96.84	97.76	96.78	96.09
4	D	99.65	99.01	99.25	99.27	97.87	98.65	98.67	99.47	99.38	100.02	98.67	99.72
5	E	97.23	95.58	97.31	94.82	95.22	96.15	95.87	96.99	95.25	97.16	95.97	96.32
6	F	100.28	98.30	99.68	98.71	100.17	99.32	100.22	99.55	100.88	99.71	99.99	99.57
7	G	101.41	98.92	97.73	100.47	100.89	100.34	100.91	98.98	99.60	100.29	100.65	100.10
8	H	102.86	101.91	101.86	103.22	103.57	101.54	101.31	102.44	103.22	103.17	101.35	101.77

Channel Statistics

Eqn 1	Eqn 2	Eqn 5
Mean	RSE	CV
100.444	0.44%	1.25%
101.144	1.14%	0.89%
96.600	-3.40%	1.03%
99.136	-0.86%	0.60%
96.154	-3.85%	0.90%
99.698	-0.30%	0.70%
100.025	0.03%	1.04%
102.351	2.35%	0.80%
max	2.35%	1.25%
min	-3.85%	

Run order statistics

	n=>	1	2	3	4	5	6	7	8	9	10	11	12
Eqn 7	Mean	99.92	98.43	99.34	99.79	99.31	99.49	99.87	99.24	99.57	100.04	98.93	99.40
Eqn 2	RSE	-0.08%	-1.57%	-0.66%	-0.21%	-0.69%	-0.51%	-0.13%	-0.76%	-0.43%	0.04%	-1.07%	-0.60%
Eqn 8	CV	2.53%	2.31%	1.79%	2.76%	2.80%	2.36%	2.22%	1.99%	2.55%	1.92%	1.87%	2.13%

Overall Statistics	
Grand Avg	Eqn 9
Mean	99.44

Eqn 11
CV - C2C
2.14%

Overall	Eqn 10
CV	2.20%

Figure A.4. Results and calculated statistics for example 1.

A.3.2 Statistics results

Channel statistics are shown the right of the data. The eight channel averages are calculated by applying formula 1 to each of the eight rows, and relative standard error is calculated by comparing each of these channel averages to the target volume using formula 2. Channel CV is calculated for each row using formula 5.

Run order statistics are shown below the data. The 12 run order averages are calculated using formula 7 and the relative standard error is calculated by using formula 2. The run order CV is calculated by applying formula 8 to the eight measurements in each column.

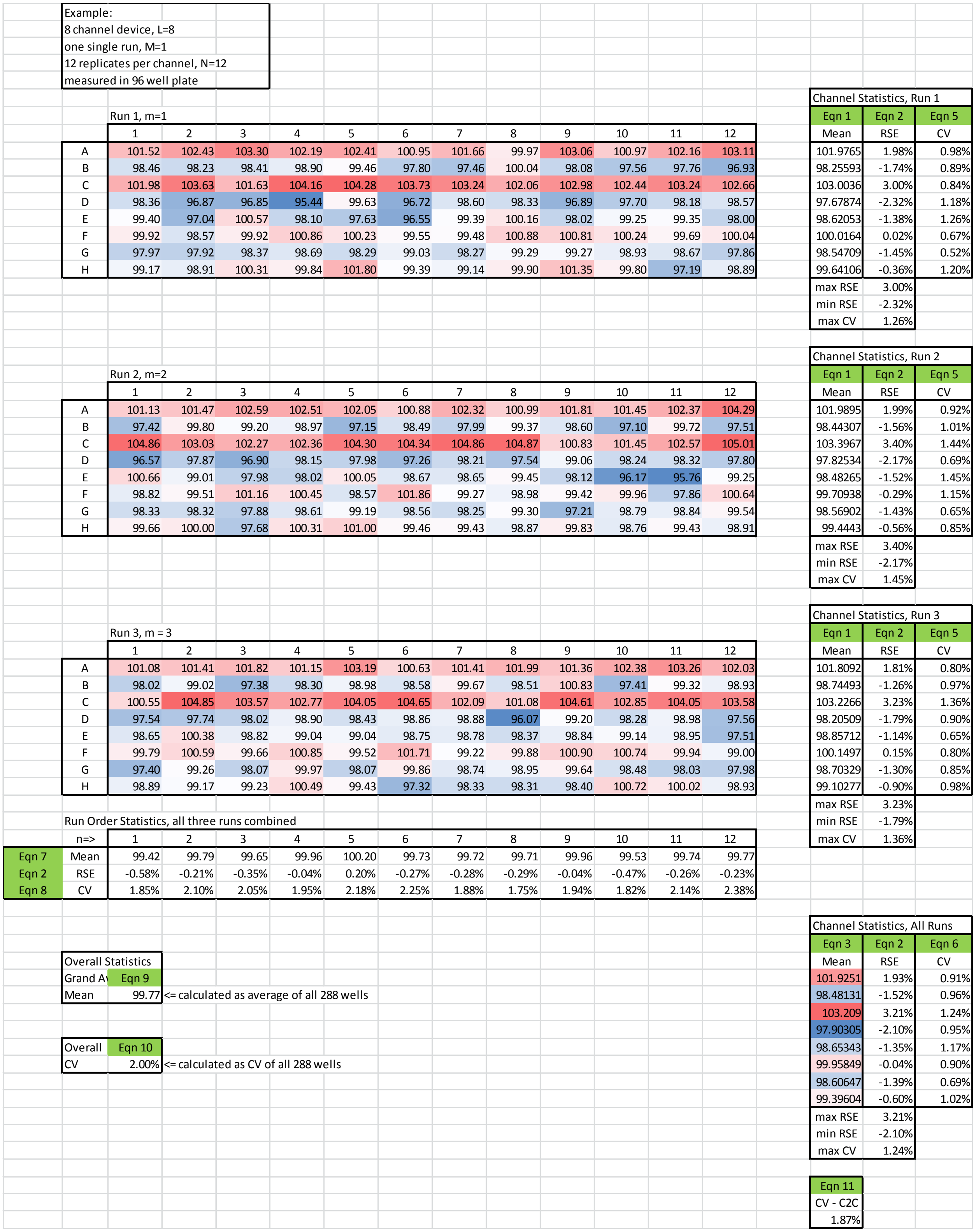
Overall statistics are shown at the bottom left part of Figure A.4. The grand average mean and overall CV are calculated using formulas 9 and 10, respectively.

The channel to channel CV is shown in the lower right and is calculated using the eight channel means in the column above the channel to channel CV cell. In this example the overall CV result is 2.20% which is only slightly larger than the channel to channel CV (2.14%). In contrast the maximum channel CV is only 1.25% (maximum of all the CVs calculated by formula 5). In this example, we see that channel to channel differences are the dominant contributor to overall CV, and the best way to reduce the overall CV would be to reduce the channel to channel 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.



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

Run 1, m=1

	1	2	3	4	5	6	7	8	9	10	11	12
A	101.52	102.43	103.30	102.19	102.41	100.95	101.66	99.97	103.06	100.97	102.16	103.11
B	98.46	98.23	98.41	98.90	99.46	97.80	97.46	100.04	98.08	97.56	97.76	96.93
C	101.98	103.63	101.63	104.16	104.28	103.73	103.24	102.06	102.98	102.44	103.24	102.66
D	98.36	96.87	96.85	95.44	99.63	96.72	98.60	98.33	96.89	97.70	98.18	98.57
E	99.40	97.04	100.57	98.10	97.63	96.55	99.39	100.16	98.02	99.25	99.35	98.00
F	99.92	98.57	99.92	100.86	100.23	99.55	99.48	100.88	100.81	100.24	99.69	100.04
G	97.97	97.92	98.37	98.69	98.29	99.03	98.27	99.29	99.27	98.93	98.67	97.86
H	99.17	98.91	100.31	99.84	101.80	99.39	99.14	99.90	101.35	99.80	97.19	98.89

Channel Statistics, Run 1

Eqn 1	Eqn 2	Eqn 5
Mean	RSE	CV
101.9765	1.98%	0.98%
98.25593	-1.74%	0.89%
103.0036	3.00%	0.84%
97.67874	-2.32%	1.18%
98.62053	-1.38%	1.26%
100.0164	0.02%	0.67%
98.54709	-1.45%	0.52%
99.64106	-0.36%	1.20%
max RSE	3.00%	
min RSE	-2.32%	
max CV	1.26%	

Run 2, m=2

	1	2	3	4	5	6	7	8	9	10	11	12
A	101.13	101.47	102.59	102.51	102.05	100.88	102.32	100.99	101.81	101.45	102.37	104.29
B	97.42	99.80	99.20	98.97	97.15	98.49	97.99	99.37	98.60	97.10	99.72	97.51
C	104.86	103.03	102.27	102.36	104.30	104.34	104.86	104.87	100.83	101.45	102.57	105.01
D	96.57	97.87	96.90	98.15	97.98	97.26	98.21	97.54	99.06	98.24	98.32	97.80
E	100.66	99.01	97.98	98.02	100.05	98.67	98.65	99.45	98.12	96.17	95.76	99.25
F	98.82	99.51	101.16	100.45	98.57	101.86	99.27	98.98	99.42	99.96	97.86	100.64
G	98.33	98.32	97.88	98.61	99.19	98.56	98.25	99.30	97.21	98.79	98.84	99.54
H	99.66	100.00	97.68	100.31	101.00	99.46	99.43	98.87	99.83	98.76	99.43	98.91

Channel Statistics, Run 2

Eqn 1	Eqn 2	Eqn 5
Mean	RSE	CV
101.9895	1.99%	0.92%
98.44307	-1.56%	1.01%
103.3967	3.40%	1.44%
97.82534	-2.17%	0.69%
98.48265	-1.52%	1.45%
99.70938	-0.29%	1.15%
98.56902	-1.43%	0.65%
99.4443	-0.56%	0.85%
max RSE	3.40%	
min RSE	-2.17%	
max CV	1.45%	

Run 3, m = 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	101.08	101.41	101.82	101.15	103.19	100.63	101.41	101.99	101.36	102.38	103.26	102.03
B	98.02	99.02	97.38	98.30	98.98	98.58	99.67	98.51	100.83	97.41	99.32	98.93
C	100.55	104.85	103.57	102.77	104.05	104.65	102.09	101.08	104.61	102.85	104.05	103.58
D	97.54	97.74	98.02	98.90	98.43	98.86	98.88	96.07	99.20	98.28	98.98	97.56
E	98.65	100.38	98.82	99.04	99.04	98.75	98.78	98.37	98.84	99.14	98.95	97.51
F	99.79	100.59	99.66	100.85	99.52	101.71	99.22	99.88	100.90	100.74	99.94	99.00
G	97.40	99.26	98.07	99.97	98.07	99.86	98.74	98.95	99.64	98.48	98.03	97.98
H	98.89	99.17	99.23	100.49	99.43	97.32	98.33	98.31	98.40	100.72	100.02	98.93

Channel Statistics, Run 3

Eqn 1	Eqn 2	Eqn 5
Mean	RSE	CV
101.8092	1.81%	0.80%
98.74493	-1.26%	0.97%
103.2266	3.23%	1.36%
98.20509	-1.79%	0.90%
98.85712	-1.14%	0.65%
100.1497	0.15%	0.80%
98.70329	-1.30%	0.85%
99.10277	-0.90%	0.98%
max RSE	3.23%	
min RSE	-1.79%	
max CV	1.36%	

Run Order Statistics, all three runs combined

	n=>	1	2	3	4	5	6	7	8	9	10	11	12
Eqn 7	Mean	99.42	99.79	99.65	99.96	100.20	99.73	99.72	99.71	99.96	99.53	99.74	99.77
Eqn 2	RSE	-0.58%	-0.21%	-0.35%	-0.04%	0.20%	-0.27%	-0.28%	-0.29%	-0.04%	-0.47%	-0.26%	-0.23%
Eqn 8	CV	1.85%	2.10%	2.05%	1.95%	2.18%	2.25%	1.88%	1.75%	1.94%	1.82%	2.14%	2.38%

Channel Statistics, All Runs

Eqn 3	Eqn 2	Eqn 6
Mean	RSE	CV
101.9251	1.93%	0.91%
98.48131	-1.52%	0.96%
103.209	3.21%	1.24%
97.90305	-2.10%	0.95%
98.65343	-1.35%	1.17%
99.95849	-0.04%	0.90%
98.60647	-1.39%	0.69%
99.39604	-0.60%	1.02%
max RSE	3.21%	
min RSE	-2.10%	
max CV	1.24%	

Overall Statistics

Grand A\	Eqn 9
Mean	99.77

<= calculated as average of all 288 wells

Overall	Eqn 10
CV	2.00%

<= calculated as CV of all 288 wells

Eqn 11
CV - C2C
1.87%

Figure A.5. Results and calculated statistics for example 2.

A.4.2 Statistics results for example 2

Channel statistics are shown 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 standard error is calculated by comparing each of these channel averages to the target volume using formula 2. Channel CV is calculated for each row using formula 5.

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 averages are calculated using formula 7 which includes a summation over the M=3 runs; 24 data points are averaged for each of these calculated means. The relative standard error is calculated by using formula 2. The run order CV is calculated by applying formula 8 to the eight measurements in each column for each plate (24 volume measurement s points for each run order CV result).

Overall statistics are shown at the bottom left part of Figure A.5. The grand average mean and overall CV are calculated using formulas 9 and 10, 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 formulas 3 and 6.

Lastly, the the channel to channel CV is shown in the extreme lower right and is calculated using the eight channel means above channel to channel CV cell. When multiple runs are included in single experiment, formula 11 shows that the combined channel statistics should be used, as is illustrated in this example.

Like example 1, 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.

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.

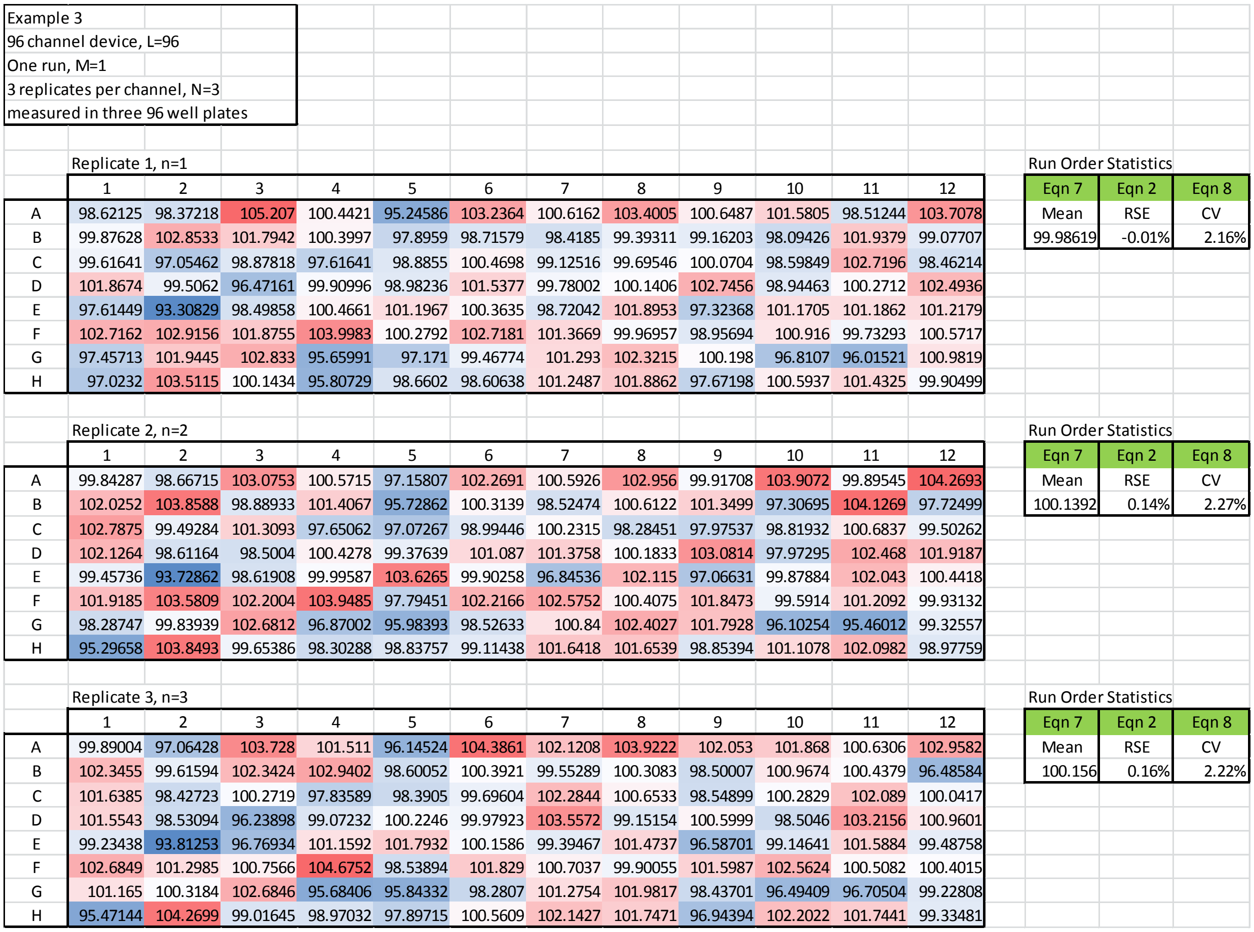
A.5.2 Statistics results for example 3

Run order statistics are shown 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 formula 5. 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 (0,21% up to 2,36%). 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 mean and overall CV use formulas 9 and 10, 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	98.62125	98.37218	105.207	100.4421	95.24586	103.2364	100.6162	103.4005	100.6487	101.5805	98.51244	103.7078
B	99.87628	102.8533	101.7942	100.3997	97.8959	98.71579	98.4185	99.39311	99.16203	98.09426	101.9379	99.07707
C	99.61641	97.05462	98.87818	97.61641	98.8855	100.4698	99.12516	99.69546	100.0704	98.59849	102.7196	98.46214
D	101.8674	99.5062	96.47161	99.90996	98.98236	101.5377	99.78002	100.1406	102.7456	98.94463	100.2712	102.4936
E	97.61449	93.30829	98.49858	100.4661	101.1967	100.3635	98.72042	101.8953	97.32368	101.1705	101.1862	101.2179
F	102.7162	102.9156	101.8755	103.9983	100.2792	102.7181	101.3669	99.96957	98.95694	100.916	99.73293	100.5717
G	97.45713	101.9445	102.833	95.65991	97.171	99.46774	101.293	102.3215	100.198	96.8107	96.01521	100.9819
H	97.0232	103.5115	100.1434	95.80729	98.6602	98.60638	101.2487	101.8862	97.67198	100.5937	101.4325	99.90499

Run Order Statistics

Eqn 7	Eqn 2	Eqn 8
Mean	RSE	CV
99.98619	-0.01%	2.16%

Replicate 2, n=2

	1	2	3	4	5	6	7	8	9	10	11	12
A	99.84287	98.66715	103.0753	100.5715	97.15807	102.2691	100.5926	102.956	99.91708	103.9072	99.89545	104.2693
B	102.0252	103.8588	98.88933	101.4067	95.72862	100.3139	98.52474	100.6122	101.3499	97.30695	104.1269	97.72499
C	102.7875	99.49284	101.3093	97.65062	97.07267	98.99446	100.2315	98.28451	97.97537	98.81932	100.6837	99.50262
D	102.1264	98.61164	98.5004	100.4278	99.37639	101.087	101.3758	100.1833	103.0814	97.97295	102.468	101.9187
E	99.45736	93.72862	98.61908	99.99587	103.6265	99.90258	96.84536	102.115	97.06631	99.87884	102.043	100.4418
F	101.9185	103.5809	102.2004	103.9485	97.79451	102.2166	102.5752	100.4075	101.8473	99.5914	101.2092	99.93132
G	98.28747	99.83939	102.6812	96.87002	95.98393	98.52633	100.84	102.4027	101.7928	96.10254	95.46012	99.32557
H	95.29658	103.8493	99.65386	98.30288	98.83757	99.11438	101.6418	101.6539	98.85394	101.1078	102.0982	98.97759

Run Order Statistics

Eqn 7	Eqn 2	Eqn 8
Mean	RSE	CV
100.1392	0.14%	2.27%

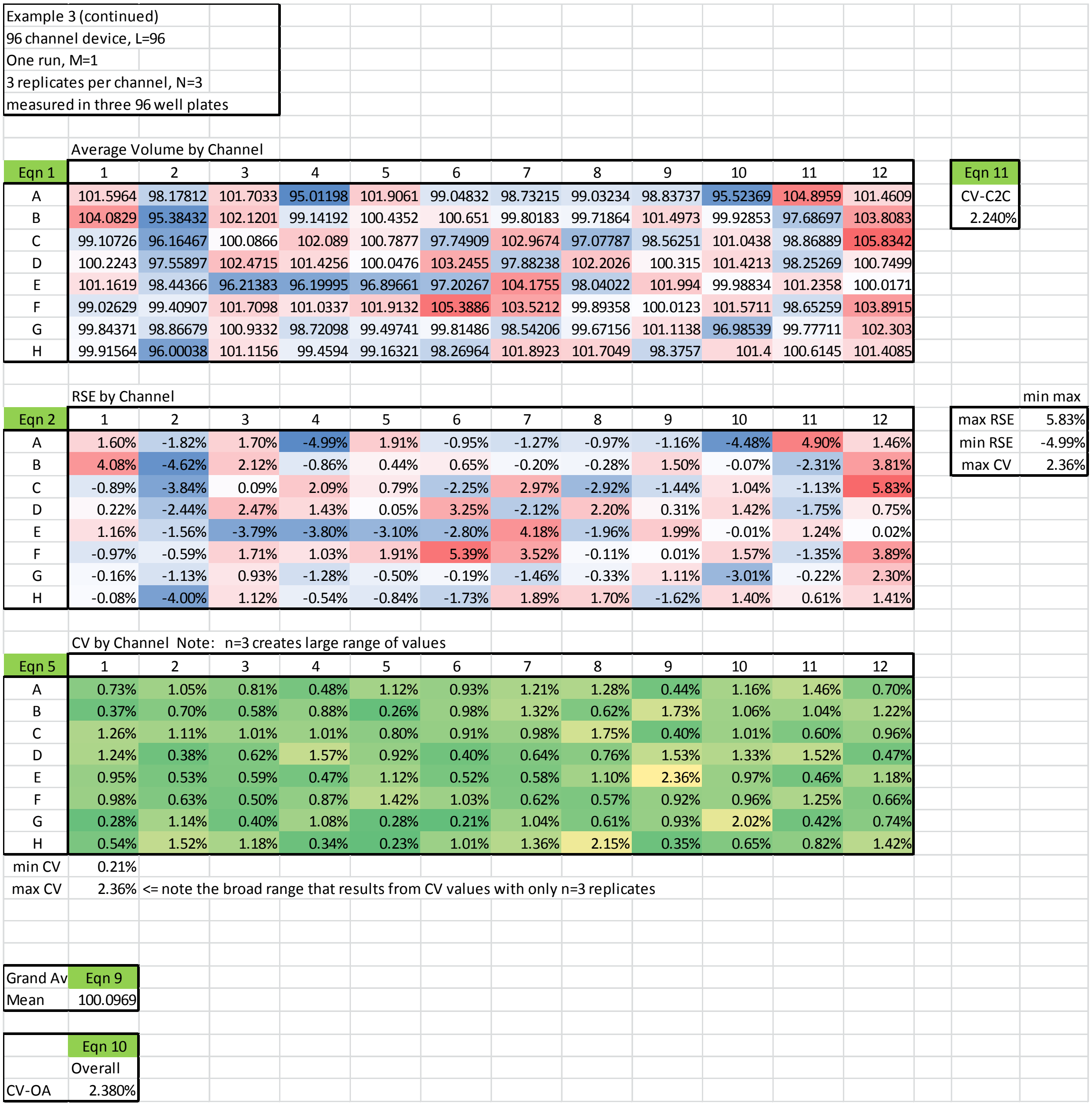
Replicate 3, n=3

	1	2	3	4	5	6	7	8	9	10	11	12
A	99.89004	97.06428	103.728	101.511	96.14524	104.3861	102.1208	103.9222	102.053	101.868	100.6306	102.9582
B	102.3455	99.61594	102.3424	102.9402	98.60052	100.3921	99.55289	100.3083	98.50007	100.9674	100.4379	96.48584
C	101.6385	98.42723	100.2719	97.83589	98.3905	99.69604	102.2844	100.6533	98.54899	100.2829	102.089	100.0417
D	101.5543	98.53094	96.23898	99.07232	100.2246	99.97923	103.5572	99.15154	100.5999	98.5046	103.2156	100.9601
E	99.23438	93.81253	96.76934	101.1592	101.7932	100.1586	99.39467	101.4737	96.58701	99.14641	101.5884	99.48758
F	102.6849	101.2985	100.7566	104.6752	98.53894	101.829	100.7037	99.90055	101.5987	102.5624	100.5082	100.4015
G	101.165	100.3184	102.6846	95.68406	95.84332	98.2807	101.2754	101.9817	98.43701	96.49409	96.70504	99.22808
H	95.47144	104.2699	99.01645	98.97032	97.89715	100.5609	102.1427	101.7471	96.94394	102.2022	101.7441	99.33481

Run Order Statistics

Eqn 7	Eqn 2	Eqn 8
Mean	RSE	CV
100.156	0.16%	2.22%

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

Eqn 1	1	2	3	4	5	6	7	8	9	10	11	12
A	101.5964	98.17812	101.7033	95.01198	101.9061	99.04832	98.73215	99.03234	98.83737	95.52369	104.8959	101.4609
B	104.0829	95.38432	102.1201	99.14192	100.4352	100.651	99.80183	99.71864	101.4973	99.92853	97.68697	103.8083
C	99.10726	96.16467	100.0866	102.089	100.7877	97.74909	102.9674	97.07787	98.56251	101.0438	98.86889	105.8342
D	100.2243	97.55897	102.4715	101.4256	100.0476	103.2455	97.88238	102.2026	100.315	101.4213	98.25269	100.7499
E	101.1619	98.44366	96.21383	96.19995	96.89661	97.20267	104.1755	98.04022	101.994	99.98834	101.2358	100.0171
F	99.02629	99.40907	101.7098	101.0337	101.9132	105.3886	103.5212	99.89358	100.0123	101.5711	98.65259	103.8915
G	99.84371	98.86679	100.9332	98.72098	99.49741	99.81486	98.54206	99.67156	101.1138	96.98539	99.77711	102.303
H	99.91564	96.00038	101.1156	99.4594	99.16321	98.26964	101.8923	101.7049	98.3757	101.4	100.6145	101.4085

Eqn 11
CV-C2C
2.240%

RSE by Channel

Eqn 2	1	2	3	4	5	6	7	8	9	10	11	12
A	1.60%	-1.82%	1.70%	-4.99%	1.91%	-0.95%	-1.27%	-0.97%	-1.16%	-4.48%	4.90%	1.46%
B	4.08%	-4.62%	2.12%	-0.86%	0.44%	0.65%	-0.20%	-0.28%	1.50%	-0.07%	-2.31%	3.81%
C	-0.89%	-3.84%	0.09%	2.09%	0.79%	-2.25%	2.97%	-2.92%	-1.44%	1.04%	-1.13%	5.83%
D	0.22%	-2.44%	2.47%	1.43%	0.05%	3.25%	-2.12%	2.20%	0.31%	1.42%	-1.75%	0.75%
E	1.16%	-1.56%	-3.79%	-3.80%	-3.10%	-2.80%	4.18%	-1.96%	1.99%	-0.01%	1.24%	0.02%
F	-0.97%	-0.59%	1.71%	1.03%	1.91%	5.39%	3.52%	-0.11%	0.01%	1.57%	-1.35%	3.89%
G	-0.16%	-1.13%	0.93%	-1.28%	-0.50%	-0.19%	-1.46%	-0.33%	1.11%	-3.01%	-0.22%	2.30%
H	-0.08%	-4.00%	1.12%	-0.54%	-0.84%	-1.73%	1.89%	1.70%	-1.62%	1.40%	0.61%	1.41%

	min max
max RSE	5.83%
min RSE	-4.99%
max CV	2.36%

CV by Channel Note: n=3 creates large range of values

Eqn 5	1	2	3	4	5	6	7	8	9	10	11	12
A	0.73%	1.05%	0.81%	0.48%	1.12%	0.93%	1.21%	1.28%	0.44%	1.16%	1.46%	0.70%
B	0.37%	0.70%	0.58%	0.88%	0.26%	0.98%	1.32%	0.62%	1.73%	1.06%	1.04%	1.22%
C	1.26%	1.11%	1.01%	1.01%	0.80%	0.91%	0.98%	1.75%	0.40%	1.01%	0.60%	0.96%
D	1.24%	0.38%	0.62%	1.57%	0.92%	0.40%	0.64%	0.76%	1.53%	1.33%	1.52%	0.47%
E	0.95%	0.53%	0.59%	0.47%	1.12%	0.52%	0.58%	1.10%	2.36%	0.97%	0.46%	1.18%
F	0.98%	0.63%	0.50%	0.87%	1.42%	1.03%	0.62%	0.57%	0.92%	0.96%	1.25%	0.66%
G	0.28%	1.14%	0.40%	1.08%	0.28%	0.21%	1.04%	0.61%	0.93%	2.02%	0.42%	0.74%
H	0.54%	1.52%	1.18%	0.34%	0.23%	1.01%	1.36%	2.15%	0.35%	0.65%	0.82%	1.42%
min CV	0.21%											
max CV	2.36%	<= note the broad range that results from CV values with only n=3 replicates										

Grand Av	Eqn 9
Mean	100.0969

	Eqn 10
	Overall
CV-OA	2.380%

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

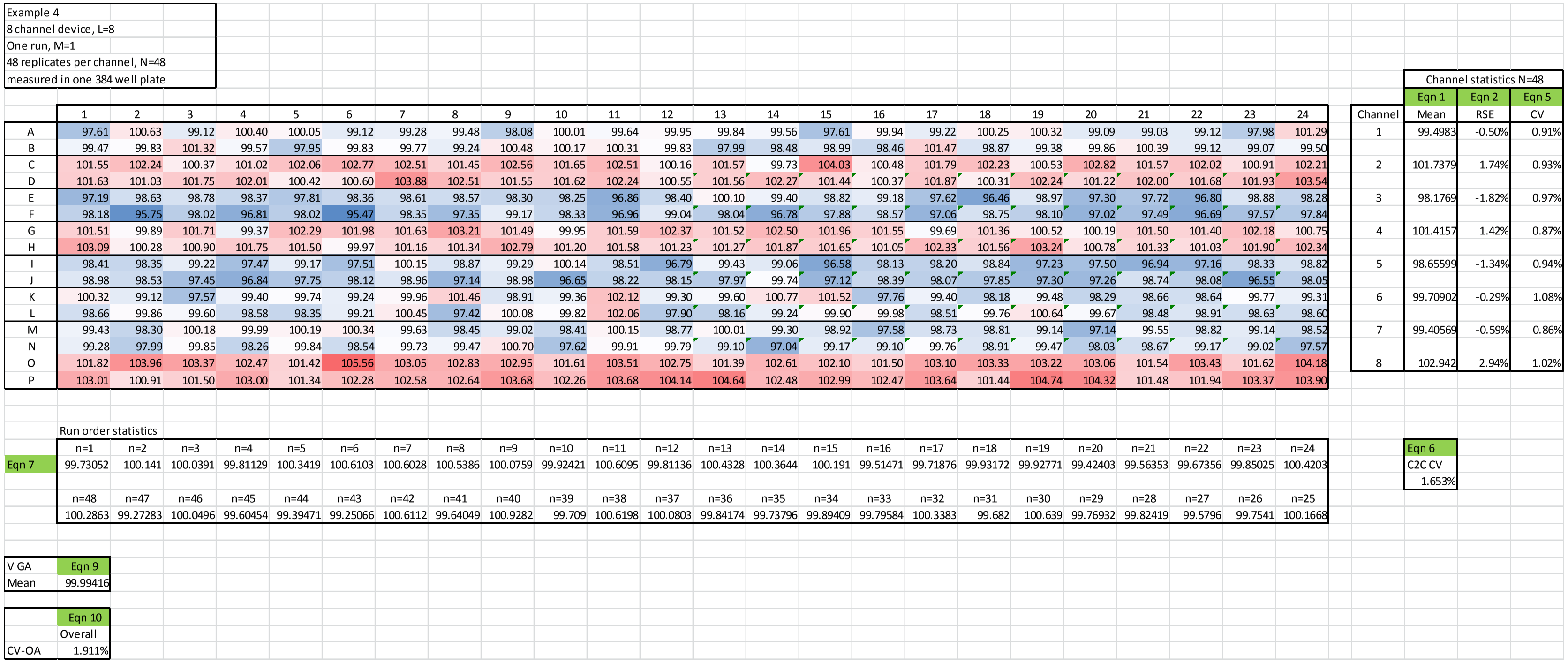
The experimental design for example 4 is an eight channel head delivering 48 replicates into one 384 well plate. The plate layout is as shown in Figure A.3 with the pattern shown as traveling left to right down the first row, then moving downward and traveling back in the reverse direction on the second 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 formulas. 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, which results in 48 run order statistics.

Calculation of grand average mean and overall CV use formulas 9 and 10 , 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	13	14	15	16	17	18	19	20	21	22	23	24
A	97.61	100.63	99.12	100.40	100.05	99.12	99.28	99.48	98.08	100.01	99.64	99.95	99.84	99.56	97.61	99.94	99.22	100.25	100.32	99.09	99.03	99.12	97.98	101.29
B	99.47	99.83	101.32	99.57	97.95	99.83	99.77	99.24	100.48	100.17	100.31	99.83	97.99	98.48	98.99	98.46	101.47	98.87	99.38	99.86	100.39	99.12	99.07	99.50
C	101.55	102.24	100.37	101.02	102.06	102.77	102.51	101.45	102.56	101.65	102.51	100.16	101.57	99.73	104.03	100.48	101.79	102.23	100.53	102.82	101.57	102.02	100.91	102.21
D	101.63	101.03	101.75	102.01	100.42	100.60	103.88	102.51	101.55	101.62	102.24	100.55	101.56	102.27	101.44	100.37	101.87	100.31	102.24	101.22	102.00	101.68	101.93	103.54
E	97.19	98.63	98.78	98.37	97.81	98.36	98.61	98.57	98.30	98.25	96.86	98.40	100.10	99.40	98.82	99.18	97.62	96.46	98.97	97.30	97.72	96.80	98.88	98.28
F	98.18	95.75	98.02	96.81	98.02	95.47	98.35	97.35	99.17	98.33	96.96	99.04	98.04	96.78	97.88	98.57	97.06	98.75	98.10	97.02	97.49	96.69	97.57	97.84
G	101.51	99.89	101.71	99.37	102.29	101.98	101.63	103.21	101.49	99.95	101.59	102.37	101.52	102.50	101.96	101.55	99.69	101.36	100.52	100.19	101.50	101.40	102.18	100.75
H	103.09	100.28	100.90	101.75	101.50	99.97	101.16	101.34	102.79	101.20	101.58	101.23	101.27	101.87	101.65	101.05	102.33	101.56	103.24	100.78	101.33	101.03	101.90	102.34
I	98.41	98.35	99.22	97.47	99.17	97.51	100.15	98.87	99.29	100.14	98.51	96.79	99.43	99.06	96.58	98.13	98.20	98.84	97.23	97.50	96.94	97.16	98.33	98.82
J	98.98	98.53	97.45	96.84	97.75	98.12	98.96	97.14	98.98	96.65	98.22	98.15	97.97	99.74	97.12	98.39	98.07	97.85	97.30	97.26	98.74	98.08	96.55	98.05
K	100.32	99.12	97.57	99.40	99.74	99.24	99.96	101.46	98.91	99.36	102.12	99.30	99.60	100.77	101.52	97.76	99.40	98.18	99.48	98.29	98.66	98.64	99.77	99.31
L	98.66	99.86	99.60	98.58	98.35	99.21	100.45	97.42	100.08	99.82	102.06	97.90	98.16	99.24	99.90	99.98	98.51	99.76	100.64	99.67	98.48	98.91	98.63	98.60
M	99.43	98.30	100.18	99.99	100.19	100.34	99.63	98.45	99.02	98.41	100.15	98.77	100.01	99.30	98.92	97.58	98.73	98.81	99.14	97.14	99.55	98.82	99.14	98.52
N	99.28	97.99	99.85	98.26	99.84	98.54	99.73	99.47	100.70	97.62	99.91	99.79	99.10	97.04	99.17	99.10	99.76	98.91	99.47	98.03	98.67	99.17	99.02	97.57
O	101.82	103.96	103.37	102.47	101.42	105.56	103.05	102.83	102.95	101.61	103.51	102.75	101.39	102.61	102.10	101.50	103.10	103.33	103.22	103.06	101.54	103.43	101.62	104.18
P	103.01	100.91	101.50	103.00	101.34	102.28	102.58	102.64	103.68	102.26	103.68	104.14	104.64	102.48	102.99	102.47	103.64	101.44	104.74	104.32	101.48	101.94	103.37	103.90

	Channel statistics N=48		
	Eqn 1	Eqn 2	Eqn 5
Channel	Mean	RSE	CV
1	99.4983	-0.50%	0.91%
2	101.7379	1.74%	0.93%
3	98.1769	-1.82%	0.97%
4	101.4157	1.42%	0.87%
5	98.65599	-1.34%	0.94%
6	99.70902	-0.29%	1.08%
7	99.40569	-0.59%	0.86%
8	102.942	2.94%	1.02%

Run order statistics

	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=13	n=14	n=15	n=16	n=17	n=18	n=19	n=20	n=21	n=22	n=23	n=24
Eqn 7	99.73052	100.141	100.0391	99.81129	100.3419	100.6103	100.6028	100.5386	100.0759	99.92421	100.6095	99.81136	100.4328	100.3644	100.191	99.51471	99.71876	99.93172	99.92771	99.42403	99.56353	99.67356	99.85025	100.4203
	n=48	n=47	n=46	n=45	n=44	n=43	n=42	n=41	n=40	n=39	n=38	n=37	n=36	n=35	n=34	n=33	n=32	n=31	n=30	n=29	n=28	n=27	n=26	n=25
	100.2863	99.27283	100.0496	99.60454	99.39471	99.25066	100.6112	99.64049	100.9282	99.709	100.6198	100.0803	99.84174	99.73796	99.89409	99.79584	100.3383	99.682	100.639	99.76932	99.82419	99.5796	99.7541	100.1668

Eqn 6
C2C CV
1.653%

V GA	Eqn 9
Mean	99.99416

	Eqn 10
	Overall
CV-OA	1.911%

Figure A.8. Results and calculated statistics for example 4.

NOTE TO REVIEWERS

Annex B is distributed as a separate document for this review. All page numbers following this note are not correctly reflected in the Table of Contents, which indexes the entire document. No comments to the apparent mis-numbering are needed during this review.

Thank you!

Annex C
(normative)

Calculation of liquid volumes from balance readings

Balance readings for the weight of a liquid are converted to volume by Formula C.1.

$$V_L = Z \cdot W_L \qquad \text{(C.1)}$$

Where:

V_L is the volume of liquid in units of µl

Z is the appropriate correction factor for converting weight to volume of liquid in units of µl/mg

W_L is the weight of the liquid as measured on the balance in units of mg

The conversion factor can be calculated generally using Formula C.2.

$$Z = \frac{[1 - \rho_a / \rho_s]}{[\rho_l - \rho_a]} \qquad \text{(C.2)}$$

Where:

ρ_a is the density of air at the conditions of weighing, in units of kg/m^3

ρ_s is the density of the mass standards used to calibrate the balance, in units of kg/m^3

ρ_l is the density of the test liquid air at the conditions of weighing, in units of kg/m^3

The density of air should be calculated using the current version of the CIPM air density formula where barometric pressure, air temperature, and relative humidity are required inputs for using this formula **[Ref.1 Picard, et al]**. The density of the mass standards should be obtained from the balance calibration report, or mass calibration report for the standards.

If the test liquid is anything other than pure water, the density should be measured using a 5 or 6 decimal place density meter which has been calibrated using certified standards of known uncertainty which span the range of the measurement. The density measurement must be applicable to the liquid temperature at the time of liquid dispensing.

In the special case where the test liquid is pure water, the density of water may be calculated using the accepted CIPM formula **[Ref. 2 Tanaka, et al]**. If the water is exposed to air, the Bignell correction should be applied to correct for the effect of air saturation **[Ref. 3 Jones and Harris]**. If the barometric pressure deviates from one atmosphere, the Kell formula should also be applied **[Ref. 3 Jones and Harris]**. This formula for density only applies if no other chemical components have been added to the water. Tables of Z correction factors may be used if the accuracy of the table is sufficient for the intended use.

Selected values for Z correction factors are given in Table C.1.

NOTE: The values in Table C.1 are calculated using the formulas and references of this annex. The following simplifying assumptions are used to calculate the values in Table C.1:

- The liquid being measured is pure water
- The pure water has been exposed to air and become air-saturated
- Isotope ratios in water are same as surface mean ocean water (SMOW)
- Relative humidity in air is 50%
- Carbon dioxide concentration in air is 400 µmol / mol
- Air temperature is identical to water temperature
- Balance is calibrated using a weight with density of 8000 kg / m^3

Table C.1. *Z* correction factors for pure air-saturated water in units of µl per mg.

Temperature °C	**Air pressure** kPa					
	80	**85**	**90**	**95**	**100**	**105**
15,0	1,001 76	1,001 81	1,001 86	1,001 91	1,001 96	1,002 01
16,0	1,001 91	1,001 96	1,002 01	1,002 06	1,002 11	1,002 16
17,0	1,002 08	1,002 13	1,002 18	1,002 23	1,002 28	1,002 33
18,0	1,002 25	1,002 30	1,002 35	1,002 40	1,002 46	1,002 51
19,0	1,002 44	1,002 49	1,002 54	1,002 59	1,002 64	1,002 69
20,0	1,002 64	1,002 69	1,002 74	1,002 79	1,002 84	1,002 89
21,0	1,002 85	1,002 90	1,002 95	1,003 00	1,003 05	1,003 10
22,0	1,003 07	1,003 12	1,003 17	1,003 22	1,003 27	1,003 32
23,0	1,003 30	1,003 35	1,003 40	1,003 45	1,003 50	1,003 55
24,0	1,003 54	1,003 59	1,003 64	1,003 69	1,003 74	1,003 79
25,0	1,003 79	1,003 84	1,003 89	1,003 94	1,003 99	1,004 04
26,0	1,004 05	1,004 10	1,004 15	1,004 20	1,004 25	1,004 30
27,0	1,004 32	1,004 37	1,004 42	1,004 47	1,004 52	1,004 57
28,0	1,004 60	1,004 65	1,004 70	1,004 75	1,004 80	1,004 85
29,0	1,004 89	1,004 94	1,004 99	1,005 04	1,005 08	1,005 13
30,0	1,005 19	1,005 23	1,005 28	1,005 33	1,005 38	1,005 43

Annex D
(informative)

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[7] Liang, D., Steinert, C., Bammesberger, S., Tanguy, L., Ernst, A., Zengerle, R., & Koltay, P. (2013). Novel gravimetric measurement technique for quantitative volume calibration in the sub-microliter range. *Measurement Science and Technology*, *24*(2), 025301.

[8] JCGM 100:2008 Evaluation of measurement data - Guide to the expression of uncertainty in measurement.

The following references pertain to Method B.6 (Gravimetry and Photometry with Tartrazine)

[1] ISO 8655-6:2002 Piston-operated volumetric apparatus - Part 6: Gravimetric methods for the determination of measurement error.

The following references pertain to Method B.7 (Photometry and Gravimetry with p-nitrophenol)

-none reported- {editorial info only}

The following references pertain to Method B.8 (Gravimetry and Photometry with Ponceau S)

-none reported- {editorial info only}

The following references pertain to Method B.9 (Pressure sensing)

[1] The Stratec Tholos® device is covered by patents listed at www.google.com/patents/US8561459

The following references pertain to Method B.10 (Calorimetric measurement)

[1] Patent application US20140208824.

[2] Patent application EP2762843.

The following references pertain to Method B.11 (Optical image analysis)

Kerstin Thurow, Thomas Krüger, and Norbert Stoll, *An Optical Approach for the Determination of Droplet Volumes in Nanodispensing*, Journal of Automated Methods and Management in Chemistry, vol. 2009, Article ID 198732, 10 pages, 2009. doi:10.1155/2009/198732.

The following references pertain to Method B.12 (Gravimetry and photometry)

Toolpoint Photometric Volume Check Procedure, 2008. Toolpoint, Switzerland.

Toolpoint Gravimetric Volume Check Procedure, 2008. Toolpoint, Switzerland

The following references pertain to Method B.13 (Photometry with Tartrazine)

[1] Sofia MJ. Leveraging Compound Management Capabilities in Support of Drug Discovery: From Sample Archive to Sample Distribution — Driving Efficiency and Improving Productivity. Laboratory Robotics Interest Group Meeting Oral Presentation, Jan. 2005.

[2] Holman JW, Mifflin TE, Felder RA, Demers LM. Evaluation of an automated preanalytical robotic workstation at two academic health centers. Clin Chem. 2002 Mar; 48(3):540-8.

The following references pertain to Annex C

[1] Picard, A., R S Davis, M. Gläser, and K. Fujii. "Revised Formula for the Density of Moist Air (CIPM-2007)" *Metrologia*, 2008, 149-55.

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