Photometric and Gravimetric Liquid Handling Check Procedure to Determine the Random Error (Precision) and the Systematic Error (Trueness) of Automated Liquid Handling Systems (ALHS)

Introduction

The method describes a reliable liquid handling check procedure for Automated Liquid Handling Systems (ALHS) based on Annex B7 of the ISO IWA 15 proposal “Specification and method for the determination of volumetric performance of automated liquid handling systems” (1). In a first step the random error (precision) is determined by an absorbance measurement in microplates using p-Nitrophenol (synonym = 4-Nitrophenol, abbr. = p-NP). This dye is stable at room temperature and soluble in water (11.6 mg/mL at 20 °C) as well as in organic solvents like chloroform, methanol, DMSO, and ethanol. It has an absorbance peak at 405 nm at 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 signal variation of individual microplate wells.

QC standards

- Detailed standard method how to determine the liquid handling performance of Automated Liquid Handling Systems (ALHS)
- Based on ISO IWA 15 proposal
- Covers the evaluation of the multi-channel head formats 96, 384 and 1536
- Suitable for in-house liquid handling specification tests as well as Operational Qualification tests at customer laboratories

Figure 1: Relationship between trueness, precision and accuracy of an ALHS (1)
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Smaller test volumes are transferred in wells pre-filled with 0.1 N NaOH, where they should be dispersed homogeneously before measurement. The dye concentrations of the different test solutions are specifically adapted to the test volumes to give always a constant final dye concentration of 120 µM in all wells of the measurement plate, which is within the optimal dynamic range of the absorbance measurement. The random error (precision) is always determined at first in the evaluation of an ALHS followed by a gravimetric determination of the systematic error (trueness). In the common literature the term accuracy often also is used as synonym for trueness. The correct relationship between trueness, precision, and accuracy of an ALHS is described in (1) and shown in Figure 1. The microplate absorbance reader and the analytical balance should be calibrated at regular intervals and the test conditions must be considered strictly.

Method description

Test equipment

- Microplate photometer with 405 nm option
  For 96- and 384-well microplates, e. g. Tecan GENios Plus
  (resolution 0.0001 OD, linearity 0 – 3 OD ± 1.5% and 0.005 OD,
  precision 0 – 3.0 OD < 1.0%, and 0.005 OD, accuracy 0 – 2.0 OD ± 1.0% and 0.01 OD).
  For 96-, 384- and 1536-well microplates, e. g. BMG LABTECH SPECTROstar® Nano
  (OD range 0 – 4, accuracy < 1.0% at 2 OD, precision < 0.5% at 1 OD and < 0.8% at 2 OD).
  Measured absorbance values generally should be < 1.5, scheduled calibration is needed.

- Calibrated analytical balance, e. g. Mettler Toledo AG245
  (Capacity 41 g – 210 g, readability 0.1 – 0.01 mg, repeatability 0.1 – 0.2 mg, linearity ± 0.2 mg),
  scheduled calibration is needed.

- Calibrated manual pipettes to prepare the test solutions, scheduled calibration is needed.

- Original standard disposable tips corresponding with the head type under evaluation

- High quality transparent flat bottom polystyrene microplates
  e. g. Greiner Bio one # 655 010 (96 wells), # 781 101 (384 wells)
  or # 782 101 (1536 wells), respectively, vacuum packed microplates should be unpacked
  1 day before the measurement.

- High quality microplate sealing tape, e. g. Nunc # 236 366 provided by Thermo Scientific

- Microplate shaker, e. g. BioShake iQ

- Microplate centrifuge, e. g. SIGMA 6K 15
Test reagents

- p-Nitrophenol, MW 139.11 g/mol, spectrophotometric grade, e.g. Sigma-Aldrich # 1048-25G
- Sodium hydroxide (NaOH) pellets, MW 40.00 g/mol, p.a., e.g. Sigma-Aldrich # 30620-1kg-R, for preparation of 0.1 N NaOH as solvent and diluent, density = 1.004 g/cm³ at 20 °C
- DI water (purity type II, conductivity < 1 µS/cm), density = 0.998 g/cm³ at 20 °C
- Optional DMSO dried, purity ≥ 99.9%, density = 1.10 g/cm³ at 20 °C

All solutions are stable for at least 3 months and should be filtrated before use to be free of any solid particles. 0.1 N NaOH is used as standard solvent to prepare the test solutions, other solvents are possible (e.g. DMSO for simulation of compound transfers).

The final p-NP concentration in the wells of the measurement plate should be 120 µM to result in an absorbance value of about 1, which is within the optimal dynamic range.

The corresponding p-NP concentrations for the different test volumes in the microplate formats 96, 384 and 1536 are summarized in Table 1.

<table>
<thead>
<tr>
<th>Test volume [µL]</th>
<th>96-well microplates with 200 µL final volume/well</th>
<th>384-well microplates with 50 µL final volume/well</th>
<th>1536-well microplates with 8 µL final volume/well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward volume 0.1 N NaOH [µL/well]</td>
<td>p-NP concentration [mM]</td>
<td>Forward volume 0.1 N NaOH [µL/well]</td>
</tr>
<tr>
<td>200.00</td>
<td>-</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>100.00</td>
<td>100.00</td>
<td>0.24</td>
<td></td>
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<tr>
<td>50.00</td>
<td>150.00</td>
<td>0.48</td>
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<tr>
<td>0.50</td>
<td>199.50</td>
<td>8.00</td>
<td>49.00</td>
</tr>
</tbody>
</table>

Table 1: Recommended p-Nitrophenol test solutions for measuring different test volumes in 96-well, 384-well or 1536-well microplates
Test conditions
The tests should be performed preferably in an air-conditioned room (e.g. at 20 °C and 60% humidity) or at room temperature under constant environmental conditions (temperature variation < ± 1 °C, humidity variation < ± 10%). All test equipment, disposables and reagents should be in equilibrium with these environmental conditions for at least 1 hour. Temperature and humidity at the beginning and the end of the tests should be documented in the test report. The analytical balance should be placed nearby the liquid handling device under evaluation and lids should be used to limit evaporation.

Test execution
To verify the specification of a pipetting head the test should be performed at least with the smallest specified volume for the pipetting head under evaluation. One or even more higher volumes can be included in the tests e.g., the application specific volume of interest. According to our experience the liquid handling performance of higher test volumes is comparable or better. The results should be calculated from three measurement plates per test volume.

Photometric determination of the random error (precision)
The precision (random error) of an ALHS describes the variation of the liquid transfer from well to well. At first the measurement plates should be prefilled with the recommended forward volume (see Table 1) using the reverse pipetting mode considering the test volume and the final volume in the respective microplate format. A calibrated manual or electronic multi-channel pipette should be used for this. The pipetting head under evaluation itself can be used exceptionally to prefill the measurement plates. The forward volumes generally are in the uncritical higher volume range and in every case quality issues would become obvious in the test result.

The pipetting methods should be set-up in the software of the liquid handling device with the following rules and parameter settings:
- One set of new standard tips per test volume
- Use single pipetting mode and reverse pipetting for all transfers
- Reduce piston speed to default speed/3
- Reduce vertical speed for moving tips out of the liquid to default speed/3
- Prime tips at least 5 times with the maximum tip volume
- Implement a break of 1 s after every aspiration and dispensing step
- Final immersion depth of the tips in the liquid should be 1 mm – 2 mm
- Reverse pipetting of the test volume with overstroke
- Pipetting the test volume in the measuremt plate without blow-out of the test volume in the measurement plate
- Ejecting the residual volume back with blow-out into the source reservoir or waste to empty the tips completely, move the tips out of the liquid and set pistons back to start position (zero)
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- Immediate sealing of the measurement plate
- Shaking of the plates for at least 10 min (careful acceleration and microplate format adapted final speed to avoid splashing)
- Centrifugation of the measurement plates to remove bubbles and to align meniscus (e.g. 2 min at 2000 rpm)
- Read-out not earlier than 30 min after finishing the pipetting procedure

If there are any obvious drifts in the microplate absorption values please cross-check the reader performance by turning the test plate through 180 degrees, independent of the scheduled service and manufacturer calibration. If the drift remains the same this indicates a reader calibration issue.

Gravimetric determination of the systematic error (trueness)

The trueness as described here defines the agreement between the mean transferred volume in the corresponding destination wells of a microplate and the volume setting per well in the pipetting method. The variation of the trueness from well to well is defined by the random error (precision) of the pipetting device, which should be checked at first and should be in the specified CV range before determination of the trueness.

Immediately after the prefilling of the measurement plates with the forward volume (see Table 1) the tare weight should be read after the stabilization of the balance. Then the test solution should be transferred as described in the previous chapter “Photometric determination of the random error (precision)” and the microplate weight should be read again immediately. The time interval from weighing before and after the test solution transfer should not be greater than 15 s to limit the evaporation to be less than 1‰ of the test volume, otherwise the evaporation should be measured at the specific environmental conditions and the calculation of the systematic error should be corrected respectively.

It is possible to combine the determination of the systematic error with the determination of the random error via weighing the measurement plates before and after the transfer of the test solution. Here, the specific density of the test solution should be considered in the calculation of the trueness (see point “Test reagents” and “Test evaluation”) and the results are acceptable only if the random error (precision) is in the specified range.
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Test evaluation

The random error (precision) is calculated as relative coefficient of variation (CV in %) according to

\[ CV = \frac{\sigma}{S_m} \times 100\% \]

where \( CV \) = coefficient of variation, \( \sigma \) = standard deviation

\[ \sigma = \frac{\sum_{i=1}^{n}(S_i - S_m)^2}{n-1} \]

\( S_m \) = mean OD signal, \( S_n \) = OD signal of a single well, \( n \) = number of wells

The systematic error (trueness) is calculated as follows:

\[ Accuracy = \frac{V_m - V_{adj}}{V_{adj}} \times 100\% \]

\( V_{adj} \) = volume per well which is adjusted in the method

\( V_m \) = volume per well determined by weighing the microplate and calculated according to

\[ V_m = \frac{m_{plate}^{disp} - m_{plate}^0}{N \rho_{liquid}(T)} \]

\( m_{plate}^0 \) = weight of the microplate before the test solution transfer

\( m_{plate}^{disp} \) = weight of the microplate after the test solution transfer

\( \rho_{liquid}(T) \) = density at 20 °C, \( N \) = number of wells

Test report

The test report should include at least the following details:

- Title
- Name and serial number of tested ALHS under evaluation (base unit and head type)
- Software and firmware version of the tested ALHS under evaluation (base unit and head type)
- Tip type, order number
- Test environment (laboratory, temperature and humidity before and after the test)
- Reader / Balance name, serial number and last/next calibration date
- Test volume and test solution (p-NP concentration solved in 0,1 N NaOH or another solvent)
- Test script with all liquid handling parameters (e.g. pre-wetting of tips, reverse pipetting, piston speed, breaks, vertical speed settings ...)
- Test results, test criteria and evaluation of the results
- Test date, name of test operator
- Name, function and signature of authorized person
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Possible error sources

- Bubbles or uneven liquid surfaces in the wells
- Splashing due to heavy shaking or inattentive removal of the sealing tape
- Inhomogeneous distribution of the test solution in the buffer, e.g. because of too short shaking or waiting time
- Incorrect liquid handling parameter settings
- Non filtrated test reagents
- Incorrect tip type
- Evaporation
- Incorrect or instable environmental conditions

Method traceability

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

Method uncertainty

The uncertainty of the photometric and gravimetric method was determined according to EN ISO 8655-6:2002.

To determine the uncertainty of the photometric method a 96-well microplate was filled homogeneously with 200 µL of 120 µM p-NP solution in 0.1 N NaOH per well. The microplate was centrifuged to align the meniscus and then the absorbance at 405 nm was read 10 times immediately under defined environmental conditions (see point 2.3). To evaluate the uncertainty of the method the standard deviation of the 10 OD values per well was calculated well-specific and resulted in an average variation of ± 0.0014 OD, which is within the manufacturer’s specification of the used absorbance reader (see point 2.1).

The same test was repeated with a 384-well microplate filled homogeneously with 50 µL of 120 µM p-NP solution in 0.1 N NaOH per well. The average variation here was ± 0.002 OD. With a 1536-well microplate filled homogeneously with 8 µL of 120 µM p-NP solution in 0.1 N NaOH per well the average variation was ± 0.005 OD, thus passing the manufacturer’s specification (see point Test equipment).

The uncertainty of the photometric method is independent from the test volume because all volumes are tested with a microplate format specific constant final measurement volume and a constant p-NP final concentration of 120 µM.
The uncertainty of the gravimetric method was determined by 10 times weighing of a sealed test microplate and calculation the standard variation of the different results. The repeatability was within the manufacturer’s specification (see point 2.1) resulting in an uncertainty of the gravimetric method of < 1% for the volume range 0.1 µL – 2 µL and < 0.1% for the volume range > 2 µL – 200 µL.

References

(1) ISO IWA 15 proposal “Specification and method for the determination of volumetric performance of automated liquid handling systems”, published in October 2015, 119 pages, can be obtained from the Standards catalogue in the ISO Store: www.iso.org/standard/65552.html, brochure see www.IWA15.org

(2) Photometric and Gravimetric Liquid Handling Check Procedure to Determine the Random Error (Precision) and the Systematic Error (Accuracy) of Automated Liquid Handling Systems. Analytik Jena AG 2014. Annex B7 in (1), published as informative section at http://iwa15.liquidhandling.org (last accessed on 29 April 2015)
