An Improved Method for Checking HTS/uHTS Liquid-Handling Systems

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An efficient method is presented to determine precision and accuracy of multichannel liquid-handling systems under conditions near to application. The method consists of gravimetrical determination of accuracy and optical determination of precision based on the dilution of absorbing and fluorescent dye solutions in microplates. Mean delivery volume per well can be determined with precision better than a 0.04% coefficient of variation (CV). Optical signal precision, CV(S), is improved by multiwavelength measurements. Precision of absorbance measurement yields a better resolution than precision of fluorescence measurement (0.3% and 1.5%, respectively), indicating that absorbance measurements should be preferred. From CV(S), an upper bound of the precision of the volumes delivered is derived. Method performance is demonstrated with the dispenser CyBiTM-Drop and the pipetter CyBiTM-Well using different ejection principles; with commonly used fluids; with 96-, 384-, and 1536-well microplates; and with photometric and fluorometric indicators. Precision of the volumes delivered, as obtained with optimized methods, all plate formats, and both devices, is better than 2% CV with 2 μL set volume and about 1% CV with higher set volumes. (Journal of Biomolecular Screening 2004:726-733)

Key words: liquid-handling evaluation, microplates, HTS devices, multiwavelength

INTRODUCTION

INDUSTRIALIZED HIGH-THROUGHPUT AND ULTRA-HIGH-THROUGHPUT SCREENING strategies now enable pharmaceutical companies and scientific laboratories to rapidly screen hundreds of thousands of substances against biological targets. The highly parallel and strongly miniaturized assays used for such screening are based primarily on microplate technology. These systems require suitable liquid-handling techniques for microliter and submicroliter volumes of samples and reagents. The quality of the analytic results depends critically on the precision and accuracy of such devices.1,4

To assess the performance of pipettes, both gravimetric and photometric procedures are known.5,7 Currently, gravimetric methods are the gold standard for assessing pipette performance from 5 mL down to 1 μL and are used in GLP/GMP laboratories (ISO/FDIS 8655 part 5 and 6:2002(E)).8 They are commonly used for the calibration and characterization of single-channel pipettes and devices containing a few channels, down to a minimum volume of 50 μL.9 Photometric procedures were introduced, for example, by Fetzer9 and the patent specifications US 4,354,37610 and US 5,492,673.6

Because characterization of the above-mentioned highly parallel liquid-handling techniques must proceed under conditions near to high-throughput screening (HTS) application, it has to include pipetting microliters of sample and reagent liquids with conventional properties into microplates. However, gravimetric characterization of each single channel will lead to high errors and consume too much time and will therefore be impracticable. Photometric methods using microplate technology do not permit sufficiently reliable determination of the accuracy of the volumes delivered due to the restrictions of vertical photometry within microplates. Nevertheless, photometric detection using single indicators has been introduced as standard operating procedure for assessing multichannel liquid-handling techniques.11 There are a few approaches to overcome the influence of path-length variability on optical measurement precision found with microplates. Near-infrared absorbance of water can be used for path-length normalization.12,13 However, it is characterized by low signal levels that are not in the optimum measurement range with microplates commonly used, and it is prone to errors due to evaporation. Path-length equalization by lids14 introduces errors due to differences of the multitude of lid positions. The US patent 5,492,673 includes

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proposals for correcting light path errors of the cuvettes used and for procedures to prevent nonlinear dependence of absorbance on concentration as caused by the agglomeration of dye molecules or preservatives. The method proposed is restricted to calibration of automated liquid handlers with 1 to 96 channels. It requires use of special equipment furnished by the supplier, including a reader, special nonreusable microplates, 2 dyes different in optical properties, and a special sample solution (www.artel-usa.com/Documents/News/NRMVS.htm). By a current version of this system, light path lengths within all wells are determined from absorbances at one wavelength, and additional absorbance measurement at a second one permits calculation of the volumes dispensed. This system is also based on the use of special test liquids, special readers, and special microplates supplied from the inventor.

Here we introduce a method that combines gravimetric determination of the accuracy of the device by determining the sum of the volumes delivered by all channels and calculating their mean, as well as photometric determination of an upper bound of the precision of these volumes (i.e., of their coefficient of variation). The method may use photometry and fluorometry and can be applied to all solvents and microplates in use, precise microplate readers, and liquid handlers available. Normalized absorbance or fluorescence signals from mixtures of 2 dyes differing in optical properties are used to reduce vertical photometry restrictions in microplates. Although blank signals are weak (<10% and <0.2% of total signal for absorbance and fluorescence intensity, respectively), their possible presence is taken into account by the evaluation method applied. Examples conducted under application near conditions with different liquid-handling systems are presented.

MATERIALS AND METHODS

Materials

Diethanolamine and sodium phosphate (Pa) were obtained from Fluka (Buchs, Switzerland) and Roth (Karlsruhe, Germany), respectively. p-Nitrophenol was from Laborchennie Apolda (Apolda, Germany), phenolphthalein was from Schering (Berlin, Germany), and methylumbelliferone, DMSO, and fluorescein-Na were from Fluka. Transparent microplates with 96 and 384 wells (nos. 655101 and 781101, respectively), black 1536-well microplates with a transparent bottom (no. 783096, LoBase, µClear) for photometry, and black microplates with 96 wells (no. 655076), 384 wells (no. 781076), and 1536 wells (no. 782076, HiBase) for fluorometry were obtained from Greiner bio-one (Frickenhauen, Germany).

Devices

The microplate reader SpectraFluor Plus was from Tecan (Maennedorf, Switzerland). It was used for measuring absorbance at 405 nm ($A_1$, mainly p-nitrophenol), 540 nm ($A_2$, with phenolphthalein), 485 nm ($A_3$, with fluorescein), and 620 nm ($A_4$, blank absorbance) and for measuring fluorescence at 460 nm (excitation 365 nm; $F_1$, methylumbelliferone) and 535 nm (excitation 485 nm; $F_2$, fluorescein). Filters with pass band ±10 nm around the selected wavelength were used. The absorbance reader Spectrmax Plus™ and the fluorescence reader Spectrmax Gemini were from Molecular Devices (Sunnyvale, CA) and were used for comparative measurements. With these readers, absorbance measurements at 400 nm ($A_1$, mainly p-nitrophenol), 550 nm ($A_2$ with phenolphthalein), 492 nm ($A_3$ with fluorescein), and 620 nm ($A_4$, blank absorbance), as well as fluorescence measurements at 444 nm (cutoff 435 nm, 365 nm excitation, $F_1$, methylumbelliferone) and 520 nm (cutoff 515 nm, 488 nm excitation, $F_2$, fluorescein), were performed. The light-beam direction for fluorescence measurement was from top to bottom. The Centrifuge 5403 was supplied by Eppendorf AG (Hamburg, Germany). The microplate shaker Titramax 100 was from Heidolph Instruments (Schwabach, Germany). A balance Analytic AC 210 S from Sartorius GmbH (Göttingen, Germany) was used after calibrating it according to the manual supplied by the manufacturer. Microplates were weighed covered with tight lids. To eliminate electrostatic effects and to prevent the microplate border from touching the weighing chamber bottom, a 0.5-mm aluminum plate was placed between the microplate and weighing scale. The liquid-handling systems dispenser CyBiTM-Drop (10 µL) and the pipettors CyBiTM-Well with 96 channels (25 and 250 µL) and with 384 channels (25 µL) were from CyBio AG (Jena, Germany). The numbers in parentheses indicate the maximum volumes delivered per channel.

Standard conditions

The ejected sample mixtures contained dyes 1 and 2 together with solvents of interest (e.g., buffer or DMSO) or mixtures thereof. If not indicated otherwise, the same buffer composition was used for both sample and dilution medium. Dye 2 was used at the concentrations given in Table 1. The sample concentrations of dye 1 were such that mixing with the dilution fluid in the wells produced the concentrations indicated in Table 1. The dilution fluid, which was loaded into the wells in addition to the samples ejected by the liquid-handling system, contained the buffer and dye 2 at the concentrations given in Table 1. Final dye concentrations were selected to produce absorbance values $A_1$ = $A_2$ = 0.5 under experimental conditions. Final fluorescent dye concentrations were selected to produce nearly equal fluorescence intensities, $F_1$ = $F_2$, which were linear with concentration and near the middle of the dynamic range of the reader employed. Final volumes per well in 96-, 384-, and 1536-well microplates were 150, 50, and 8 µL, respectively.

In contrast to the sample fluid, the dilution fluid was added to 96- and 384-well microplates using a CyBiTM-Well with 96 channels (maximum volume 250 µL). For the 1536-well microplates, however, the CyBiTM-Well with 384 channels had to be used for
Table 1. Final Concentrations of Indicators in Microplates Ready for Measurement, Buffer Used with Dilution Fluid, and Readers Used

<table>
<thead>
<tr>
<th>Method</th>
<th>Microplate Format</th>
<th>Dye 1 Concentration (µM)</th>
<th>Dye 2 Concentration (µM)</th>
<th>Buffer Used with Dilution Fluid</th>
<th>Reader Used</th>
<th>Wavelength Used (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photometry</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>384</td>
<td>p-Nitrophenol</td>
<td>Phenolphthalein</td>
<td>DEA</td>
<td>SpectraFlor Plus</td>
<td>405</td>
</tr>
<tr>
<td>B</td>
<td>384</td>
<td>60</td>
<td>40</td>
<td>Pa</td>
<td>SpectraFlor Plus</td>
<td>405</td>
</tr>
<tr>
<td>C</td>
<td>96, 384</td>
<td>60</td>
<td>26</td>
<td>Pa</td>
<td>SpectraMax Plus</td>
<td>540</td>
</tr>
<tr>
<td>D</td>
<td>1536</td>
<td>90</td>
<td>50</td>
<td>Pa</td>
<td>SpectraFlor Plus</td>
<td>405</td>
</tr>
<tr>
<td>Fluorometry</td>
<td></td>
<td></td>
<td></td>
<td>DEA</td>
<td>SpectraFlor Plus</td>
<td>405</td>
</tr>
<tr>
<td>E</td>
<td>384, 1536</td>
<td>60</td>
<td>2</td>
<td>Pa</td>
<td>SpectraMax Gemini</td>
<td>488 ex/520 em</td>
</tr>
<tr>
<td>F</td>
<td>96</td>
<td>5.6</td>
<td>2.66</td>
<td>Pa</td>
<td>SpectraFlor Plus</td>
<td>485 ex/535 em</td>
</tr>
<tr>
<td>G</td>
<td>384, 1536</td>
<td>60</td>
<td>1.33</td>
<td>Pa</td>
<td>SpectraMax Gemini</td>
<td>488 ex/520 em</td>
</tr>
<tr>
<td>H</td>
<td>1536</td>
<td>188</td>
<td>1.33</td>
<td>Pa</td>
<td>SpectraFlor Plus</td>
<td>485 ex/535 em</td>
</tr>
</tbody>
</table>

DEA, diethanolamine/HCl, pH 10.0; Pa, 0.1 M Na-phosphate, pH 11.0.

both sample and dilution solution. Because errors of the volumes pipetted could remain undetected by the signal A1/A2 or F1/F2 if sample and dilution fluid were pipetted by the same channel, 1536-well microplates were rotated 180 degrees between pipetting both fluids. Mixing of sample and dilution medium was achieved by shaking the plates after sealing using the microplate shaker TitranaX 100 at its maximum rate for 15 min and 60 min in 96- and 384-well microplates, respectively. Mixing in 1536-well microplates was achieved solely by diffusion for 15 to 24 h. Pipetting the sample fluid into prefilled 1536-well microplates was done after centrifugating the plates for 5 min (2500 rpm). All fluids except DMSO were filtered before use (0.45 µm).

**Gravimetric determination of mean volume ejected**

The total weight, Wtot, of the sample fluid ejected simultaneously into all wells was determined under conditions that reduced evaporation prior to optical measurement. When prefilled microplates were used, weight loss of the prefilled dilution medium by evaporation, between filling and weighing, was determined with reference plates handled in parallel. The mean volume pipetted per channel, Vmean, is calculated as

\[ V_{\text{mean}} = W_{\text{t}}/(N \times p) \]

where N is the number of channels, and p is the fluid density. Sample fluid densities were determined by a pyknometer. Weighing exhibited a mean standard deviation of 0.066 mg independent of the weight with all microplate formats. Because the precision of the mean weight of fluid delivered per channel is the same as the precision of the total weight of fluid delivered into the microplate, it improves as N increases. With a sample volume of 0.5 µL ± 0.5 mg, the CV calculated is 0.036% and 0.008%, and with a volume of 1 µL, it is 0.018% and 0.004% for N = 384 and 1536, respectively.

**RESULTS**

**Principle of the method**

**Liquid handler check using absorbance measurements**

**Step 1: Pipetting dye solutions.** Each channel of the liquid-handling device was loaded with the so-called sample solution containing dye 1 and dye 2 at concentrations c10 and c20, respectively. The liquid-handling device under inspection was set to deliver the volume, V, into each well of the plate. In addition, each well was loaded with the volume, V0, of the dilution solution containing only dye 2 at concentration c20. Thus, the resulting concentration of dye 1 depends on V,

\[ c_1 = V \cdot c_{10} / (V_0 + V) \]

whereas the concentration of dye 2 remains unchanged, c2 = c20, for each well. Absorbance of dye 1 and dye 2 at each well was used to indicate the volume pipetted and to eliminate the unknown light-path length, respectively.

**Step 2: Gravimetric determination of the mean volume pipetted; mixing of sample and dilution fluid (see relevant sections above).**

**Step 3: Determination of normalized absorbance signals.** Dye 1 and dye 2, together with 2 wavelengths, λ1 and λ2, were chosen so that dye 1 absorbed light at λ1 and had minimal absorbance at λ2 and vice versa. Thus, according to Lambert-Beer’s law, the absorbances A1 and A2 measured at λ1 and λ2, respectively, are
\[ A_1 \equiv I \cdot c_1 \cdot e_1 + A_{bl,1} \]  

(3)

and

\[ A_2 \equiv I \cdot c_{20} \cdot e_2 + A_{bl,2} \]  

(4)

where \( e_1 \) and \( A_{bl,1} \) are the absorbance coefficients of the respective dyes at \( A \) and the blank absorbances produced by well bottom, solvent, and so forth, respectively, and \( I \) is the light-path length within the liquid. With negligible blank absorbances, one gets from equations (3) and (4) a normalized signal \( S_2 \), which is essentially not influenced by the unknown light-path length \( l \):

\[ S_2 = A_1/A_2 \equiv c_1 \cdot e_1/(c_{20} \cdot e_2) \]  

(5)

If blank absorbances are to be considered as well, a third wavelength, \( A_{bl} \), can be chosen so that dyes 1 and 2 do not contribute to the absorbance measured, \( A_1 \). If the blank value essentially does not depend on wavelength, the normalized signal

\[ S_3 = (A_1 - A_2)/(A_1 - A_0) \equiv c_1 \cdot e_1/(c_{20} \cdot e_2) \]  

(6)

will be free from the influences of both light-path length and blank absorbance.

For the present study, \( CV(S_3) \) was not used because it turned out to be not superior to \( CV(S_2) \) (see below).

**Step 4: Evaluation.**

**Accuracy evaluated from gravimetry.** The accuracy was evaluated from \( V_{n0} - V_{av} \), where \( V_{av} \) is the set-point volume (i.e., the volume to be dispensed as specified by the liquid handler’s supplier).

**Precision evaluated from photometry.** For evaluating measurements made under real conditions, additional factors had to be taken into account.

(i) A small absorbance coefficient, \( e_{20} \), of dye 2 (i.e., phenolphthalein or fluorescein) at the wavelength used for measuring dye 1 (i.e., p-nitrophenol) was taken into account by changing equation (3). Thus,

\[ A_1 = I \cdot c_1 \cdot e_1 + I \cdot c_{20} \cdot e_{20} + A_{bl,1} \]  

(7)

\[ A_2 = I \cdot c_{20} \cdot e_2 + A_{bl,2} \]  

(8)

(ii) Taking account of possible well-to-well variations of \( V_{in}, A_{in} \) well shape, and so on, an upper bound for \( CV(V) \) was derived from \( CV(S_2) \); applying the error propagation law to the linear expansion of \( S_2 \), one gets the variance of the signal \( S_2 \):

\[ Var(S_2) \equiv (\partial S_2/\partial V)^2 \cdot Var(V) + (\partial S_2/\partial V_{in})^2 \cdot Var(V_{in}) + \text{further terms.} \]  

(9)

Thus,

\[ Var(S_2) \geq (\partial S_2/\partial V)^2 \cdot Var(V) \]  

(10)

\[ Var(V) \leq Var(S_2)/(\partial S_2/\partial V)^2 \]  

(11)

and

\[ CV(V) \leq CV(S_2)/(\partial S_2/\partial V) \cdot V_{in}/S_{20} = \text{upper bound for } CV(V), \]  

(12)

where \( V_{in} \) and \( S_{20} \) are mean values over all wells of the plate. The derivative, \( \partial S_2/\partial V \), can be determined experimentally by measuring \( S_2 \) with solutions corresponding to prescribed constant \( V_{in} \) and different \( V \) values, adapting a smooth curve, and evaluating its derivative numerically. This derivative can also be determined mathematically by taking the derivative of \( S_2 = A_1/A_2 \) with respect to \( V \), with \( A_1 \) and \( A_2 \) according to equations (7) and (8), \( I = f(V + V_{in}) \) = light-path length within liquid according to the conical shape of the wells, and \( c_1 = c_{10} \cdot V/(V + V_{in}) \).

To compare 2- versus 3-wavelength measurements, we compared \( CV(V) \) bounds obtained from \( S_2 = A_1/A_2 \) with those obtained from \( A_1 \) alone. \( \partial A_1/\partial V \) was determined using the above-described procedure, except that \( S_2 \) was replaced by \( A_1 \). Mathematically determined derivatives were in accordance with those determined experimentally for 96- and 384-well plates. Differences found with 1536-well plates are most likely due to small differences between plates (see Discussion).

**Liquid handler check using fluorescence measurements**

Similarly to measurements using absorbance, dye 1 and dye 2 were chosen so that dye 1 contributes to the fluorescence excited at \( \lambda_{ex1} \) and emitted at \( \lambda_{em1} \) but not to the fluorescence excited at \( \lambda_{em2} \) and emitted at \( \lambda_{em2} \) and vice versa.

**Step 1 (pipetting) and step 2 (gravimetry and mixing).** These were done as described above.

**Step 3: Determination of normalized fluorescence signals.** Assuming that the fluorescence intensities emitted by dyes 1 and 2 can be described by

\[ F_1 = J_1 \cdot G \cdot c_1 + F_{bl,1} \]  

(13)

and

\[ F_2 = J_2 \cdot G \cdot c_{20} + F_{bl,2} \]  

(14)

where

\[ J_1 = \text{factor characteristic of dye number } i \text{ comprising the ratio of emitted/absorbed photons and so forth;} \]

\[ G = \text{factor characteristic of a channel comprising incoming light intensity, effects of geometry of fluorescent fluid, and so forth;} \] and

\[ F_{bl,1} \text{ and } F_{bl,2} = \text{blank fluorescence intensities; normalized fluorescence signals can be evaluated analogously to absorbances, except that blanks are extremely difficult to be measured and depend strongly on wavelength so that no useful third wavelength exists.} \]

The normalized fluorescence signal was therefore calculated as

\[ S_2 = F_1/F_2 \equiv c_1 \cdot J_1/(c_{20} \cdot J_2) \]  

(15)

**Step 4: Evaluation.** As with absorbance measurement, the accuracy was evaluated from \( V_{n0} - V_{av} \), where \( V_{av} \) is the set-point volume, and upper bounds for \( CV(V) \) were evaluated from \( CV(S_2) \)
Table 2. Resolution of the Method: Examples of the Precision of Different Optical Signals in Microplates

<table>
<thead>
<tr>
<th>Microplate Format</th>
<th>Methods (cf. Table 1)</th>
<th>Photometry, CV(S), %</th>
<th>Fluorometry, CV(S), %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S = A₁</td>
<td>S = A₂</td>
<td>S = S₁</td>
</tr>
<tr>
<td>96</td>
<td>C, F</td>
<td>0.60</td>
<td>0.63</td>
</tr>
<tr>
<td>384</td>
<td>A, E</td>
<td>1.06</td>
<td>0.96</td>
</tr>
<tr>
<td>1536</td>
<td>D, E</td>
<td>1.28</td>
<td>1.19</td>
</tr>
<tr>
<td>384</td>
<td>B, G</td>
<td>0.88</td>
<td>0.89</td>
</tr>
<tr>
<td>1536</td>
<td>H</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1536</td>
<td>D, G</td>
<td>1.29</td>
<td>1.30</td>
</tr>
</tbody>
</table>

Homogeneous mixtures of dye 1 and dye 2 were applied. For mixture compositions and volumes, see Materials and Methods, Standard Conditions section. A₁, A₂, F₁, and F₂: absorbances and fluorescence intensities of all wells of the microplate. S₁ and S₂: normalized signals (cf. Principle of the Method section). — = not determined.

a. The mixture also contained 25% (v/v) DMSO.
b. The mixture also contained 4% (v/v) DMSO.

Table 3. Precision and Accuracy of the Dispenser CyBi™-Drop with 384-Well Microplates

<table>
<thead>
<tr>
<th>Mean Volume (µL)</th>
<th>V̄mean − V̄ref (‰)</th>
<th>Photometry</th>
<th>Fluorometry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CV(V) %</td>
<td>S = A₁</td>
<td>S = S₁</td>
</tr>
<tr>
<td>10.04</td>
<td>-0.4</td>
<td>1.35</td>
<td>0.95</td>
</tr>
<tr>
<td>4.97</td>
<td>-0.6</td>
<td>1.19</td>
<td>0.67</td>
</tr>
<tr>
<td>0.95</td>
<td>-5.0</td>
<td>1.40</td>
<td>1.13</td>
</tr>
</tbody>
</table>

Methods A and E were used (cf. Materials and Methods section). Precision values: upper bounds of CV(V) were determined from CV(S) using ∂S/∂V; obtained mathematically and experimentally for photometry and fluorometry, respectively (cf. Evaluation section). Where the upper bound of CV(V) seems to be identical to CV(S), ∂S/∂V + V̄ref/100 was found very close to 1.

and CV(F₁), where ∂S₁/∂V and ∂F₁/∂V were determined experimentally as indicated above.

Resolution of the method

To check the resolution (i.e., the precision of the measurement system), we loaded the microplate wells in 1 step with final volumes of premixed dye mixtures, thus circumventing delivery of small volumes, V, together with the concomitant variations of the resulting dye concentrations. Table 2 shows examples of the optimum precision of optical signals obtained under this condition. From the results, one sees that (1) the between-well variability of optical signals was reduced significantly by 2 wavelength measurements and quotient formation, (2) absorbance measurement at 3 wavelengths and difference-quotient formation did not yield further improvement under the conditions used, and (3) liquid-handling devices yielding photometric precision better than 0.3% CV cannot be characterized correctly with any plate format. Liquid-handling devices yielding precision better than 0.5% CV with 96-well microplates and 1% to 1.6% CV with 384-well or 1536-well microplates, respectively, cannot be characterized using fluorometry (Table 2).

In addition, the CV of absorbance due to optical measurement errors was determined by repeated measurements at 1 channel with light-absorbing foils instead of dye solutions and was found to be around 0.22% at 405 nm (A₁) and 0.14% at 540 nm (A₂). If only such errors were present with microplates, the squared CV values of the absorbances should add to approximately the squared CV of their quotient, CV(S₁). The difference between the purely photometric value thus expected for CV(S₁), 0.26%, and the values given in Table 2 is within the experimental error and indicates that further errors affecting A₁ and A₂ are either correlated (e.g., between-well light-path differences) or otherwise negligible. Although fluorescence intensities (F₁) show very different precision with 384- and 1536-well microplates, quotient formation improved the precision to similar levels. Further improvement to better than 1% could be achieved neither with alternate 384-well microplates (nos. 784076, 783092, and 783076) nor by inverting the measurement direction to bottom → top or using other readers (results not shown).
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Table 4. Precision and Accuracy of the Pipettor CyBi™-Well 96 with 96-Well Microplates

<table>
<thead>
<tr>
<th>Accuracy</th>
<th>Photometry</th>
<th>Fluorometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Volume (µL)</td>
<td>V_{mean} - V_{set} (%)</td>
<td>S = A_{i}</td>
</tr>
<tr>
<td>9.89</td>
<td>-1.1</td>
<td>CV(V)≤S</td>
</tr>
<tr>
<td>4.97</td>
<td>-0.6</td>
<td>CV(V)≤S</td>
</tr>
<tr>
<td>2.93</td>
<td>-2.2</td>
<td>CV(V)≤S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV(V)≤S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV(V)≤S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV(V)≤S</td>
</tr>
</tbody>
</table>

Methods C and F were used (cf. Materials and Methods section). The sample medium was pipetted into prefilled wells. For precision values, see legend to Table 3. Where the upper bound of CV(V) seems to be identical to CV(S), δS/V • V_{set}/S_{set} was found very close to 1.

Table 5. Precision and Accuracy of the Pipettor CyBi™-Well 384 with 384-Well Microplates

<table>
<thead>
<tr>
<th>Accuracy</th>
<th>Photometry</th>
<th>Fluorometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Volume (µL)</td>
<td>V_{mean} - V_{set} (%)</td>
<td>S = A_{i}</td>
</tr>
<tr>
<td>6.97</td>
<td>-0.4</td>
<td>CV(V)≤S</td>
</tr>
<tr>
<td>5.00</td>
<td>0</td>
<td>CV(V)≤S</td>
</tr>
<tr>
<td>1.95</td>
<td>-2.5</td>
<td>CV(V)≤S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV(V)≤S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV(V)≤S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV(S)</td>
</tr>
</tbody>
</table>

Methods C and H were used (cf. Materials and Methods section). The sample medium was pipetted into prefilled wells. For precision values, see legend to Table 3. Where the upper bound of CV(V) seems to be identical to CV(S), δS/V • V_{set}/S_{set} was found very close to 1.

Table 6. Precision of the Pipettor CyBi™-Well 384 with Photometric Measurement of 1536-Well Microplates

<table>
<thead>
<tr>
<th>Pipetting Mode</th>
<th>Solvent Used for Sample Fluid</th>
<th>Mean Volume (µL)</th>
<th>Photometry</th>
<th>Fluorometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Into prefilled wells</td>
<td>Pa</td>
<td>1.81</td>
<td>CV(V)≤S</td>
<td>3.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CV(V)≤S</td>
<td>2.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CV(V)≤S</td>
<td>4.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CV(V)≤S</td>
<td>4.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CV(V)≤S</td>
<td>3.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CV(V)≤S</td>
<td>2.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CV(V)≤S</td>
<td>4.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CV(S)</td>
<td>4.15</td>
</tr>
</tbody>
</table>

Method D was used. Pa: sample buffer was the same as the dilution buffer. DMSO: sample fluid was practically 100% (v/v) DMSO. Precision values: upper bounds of CV(V) were determined from CV(S) using δS/V obtained experimentally (cf. Evaluation section).

Application of the method

Dispenser CyBi™-Drop. Because this dispenser shoots the sample volumes into the wells, the volumes delivered do not depend on pipetting into dry or prefilled microplates. Results are summarized in Table 3.

Pipettors CyBi™-Well with 96 (25 µL max) and 384 channels (25 µL max). Precision and accuracy with watery fluids depend on the set volume, as summarized in Tables 4 and 5. In addition, these parameters depend on solvent composition, plate type (not shown), and mode of ejection (e.g., pipetting into dry or prefilled microplates) (Table 6).

Although the velocity of pistons and vertical microplate movement were adapted to the pipetted medium, the piston stroke was only adjusted to the set volume without taking into account effects of surface tension, adhesive forces, viscosity, and so on. This method works well with set volumes of 2 µL and more, but the weighing results show that for pipetting smaller volumes into
1536-well microplates, the pipettor has to be recalibrated, corresponding to the sample fluids and pipetting modes used (results not shown).

DISCUSSION

Evaluation of HTS liquid-handling devices requires measurement under HTS conditions, including use of microplates. Unfortunately, such measurements with single indicators are burdened by restrictions such as undefined path length, caused not only by the variability of the pipetted volumes themselves but also by evaporation, meniscus formation, optical meniscus effects, bubbles, scratches, and so forth. Here we describe a simple multilayer method by which errors due to the variability of path length can be significantly reduced. For the quotient of absorbances measured at different wavelengths, $A_1/A_2$, it could be shown that its CV over the microplate approached values very close to the CV of the reader itself (Table 2). Measurement of blank absorbances at a third wavelength and use of the difference quotient did not yield significantly better results (Table 2), suggesting that the influence of blanks caused by bubbles, scratches, and so on was negligible under the conditions applied. This improved measurement method was used to characterize the precision of HTS liquid-handling devices. The quotient of absorbances, $A_1/A_2$, turned out to characterize the precision of the volumes delivered more precisely than $A_1$ alone. The same holds for the quotient of fluorescence intensities, $F_1/F_2$, as compared to $F_1$ alone.

Besides variation of the volume delivered, $V$, variation of other parameters (e.g., the dilution volume) can contribute to the variation of the absorbance or fluorescence signal, $S$. Thus, from $CV(S)$ only an upper bound for $CV(V)$ can be obtained, making use of the partial derivative of $S$ with respect to $V$. For absorbance measurement and $S = A_1/A_2$ or $S = A_1$, theoretical values of that derivative were calculated in accordance with experimental ones for 96- and 384-well microplates. For 1536-well plates, differences between theoretical and experimental results were found possible due to between-plate differences of reader adjustment relative to the pattern of wells and/or differing microplate measures, well shapes, and so forth. The accuracy of the liquid-handling device under inspection (i.e., the relative deviation of the mean volume delivered from the set point) was determined with high accuracy by weighing. With sample volume 0.5 μL ± 0.5 mg, the CV due to weighing is 0.036% and 0.008% for 384- and 1536-well microplates, respectively.

From a practical point of view, selection of the actual buffer indicator system must be performed carefully with respect to temperature dependence of the pH buffer, pH stability in small sample volumes, pH dependence of optical indicators, long-term stability of the dyes used under the conditions selected, and influence of solvents on the optical indicators. For example, if phenolphthalein instead of fluorescein were used as a second indicator for the measurements with 1536-well microplates reported in Table 6, decreased precision would be obtained because of a slight instability of phenolphthalein absorbance with respect to time and pH that becomes apparent under the conditions used with these microplates (i.e., mixing by diffusion for 15-24 h in contrast to mixing by shaking for 1 h with 384-well microplates). Furthermore, phenolphthalein bleaches out in mixtures with more than 4% (v/v) DMSO, and the absorbance maximum of fluorescein shifts slightly with increasing DMSO concentration. Therefore, use of phenolphthalein absorbance and fluorescein fluorescence in the presence of high DMSO content was and should be avoided. As outlined by Taylor et al., the position of the light path of the microplate reader with respect to the pattern of wells is crucial, especially with 1536-well plates, and has to be adjusted exactly for the actual plate batch used.

CONCLUSIONS

The suitability of the method proposed is demonstrated with 2 multichannel liquid-handling devices using different ejection principles, commonly used sample fluids, different plate formats, and photometric and fluorometric indicators. Accuracy and precision of the liquid-handling devices are shown to depend on the medium handled, the plate type, and the delivery method (i.e., dispensing into prefilled or dry plates). For testing liquid-handling devices exhibiting a usual range of precision, we have shown that absorbance signals should be preferred to fluorescence signals because of their higher precision.

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REFERENCES


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