The Use of a Dual Dye Photometric Calibration Method to Identify Possible Sample Dilution from an Automated Multichannel Liquid-handling System

Huijin Dong, Zheng Ouyang, Jane Liu, and Mohammed Jemal*
Bristol-Myers Squibb, New Brunswick, NJ

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A significant difference between the reported volumes delivered by a fluid-filled automated liquid-handling system with eight stainless steel fixed tips was observed when a gravimetric method and a dual dye photometric method were used to measure delivered volumes between 20 and 200 μL. A series of experiments, using the default pipetting parameters, was performed which demonstrated that the difference in the reported volume was due to a dilution effect by the system liquid of the liquid handler and did not indicate an error in the delivered volumes. This dilution effect led to a decrease in the reported volume by the dual dye method, which alerted the user to the problem of sample dilution. In contrast, the gravimetric method provided the expected volume, and therefore did not alert the user to the problem. Without optimization of pipetting parameters, the dilution issue can be significant, because the resultant change in the concentration of the compound(s) of interest in the sample could lead to an unidentified error in the sample assay. (JALA 2006;11:60–4)

*Correspondence: Huijin Dong, MS, Bristol-Myers Squibb, Bioanalytical and Discovery Analytical Sciences, 1 Squibb Drive, New Brunswick, NJ 08903, USA.

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Introduction

Automated liquid handling (ALH) systems are a vital component of the modern laboratory. These devices are used in a broad range of activities ranging from high-throughput screening in drug discovery/development studies to the monitoring of body fluids for diagnostic purposes. The accuracy and precision of an ALH system are critical parameters for the analyst, because the validity of all quantitative measurements taken by the laboratory is dependent on an accurate and precise delivery of the sample (reagent, buffer, etc.) to the microtiter plate.

A variety of methods are commonly used to determine the accuracy of pipettes and ALH systems including gravimetric,[1] photometric,[2,3] fluorometric,[2,3] and dual dye photometric methods.[4–6] Over the last few years, an innovative automated dual dye photometric method has been developed, which can rapidly test the performance of ALH systems using reagents that are traceable to national standards.[7,8] In our laboratories, we have recently completed an investigation comparing the gravimetric method with the dual dye method using a Tecan Genesis RSP 150 ALH system. The results indicated a consistent difference between the two methods, with the dual dye method reporting a lower delivered volume than the gravimetric method.

This paper describes a series of experiments that was performed to determine the method providing the correct volume for the dispensed liquid.

Observed data suggested that the difference between the results from the dual dye method and the gravimetric method was due to (1) a systemic error in one of these approaches and (2) the dilution of the sample by the Tecan ALH system liquid.

Experimental

ALH Systems

A Tecan Genesis RSP 150 ALH workstation with eight stainless steel fixed tips (Tecan Trading AG, Zurich, Switzerland), which operates on the principle of positive displacement of a system liquid, was the initial ALH tested. A default water liquid class, was used in the study, and a wash step was undertaken by the ALH prior to the delivery of the sample, unless otherwise noted. A Tomtec Quadra ALH system equipped with a 96-tip head (Tomtec, Inc., Hamden, CT), which operates on the principle of air displacement via a worm screw mechanism, was also used in this study. No wash step was incorporated for the disposable tips used by the Tomtec system. The volume of liquid withdrawn (aspirated) by either ALH was equal to the volume of liquid specified for dispensing.

Gravimetric Approach

With the Tecan system, the water or dye solution was deposited from each tip individually onto an appropriately configured balance pan and the weight of each sample determined. The density of all samples was assumed to be equal to that of water ($\rho = 1$ g/cm^3) and used to calculate the volume dispensed from each tip. Unless indicated otherwise, each tip dispensed a total of 12 data points, and the average volume calculated from all dispenses (eight tips, with 12 dispenses per tip) was used to represent the overall performance. The tips were washed before each dispense, unless otherwise noted. With the Tomtec system, the dye solution was dispensed into each well of a 96-well microtiter plate and weighed. The total weight of the plate less than the weight of the tare was the weight of whole plate.

Dual Dye Approach

The Artel MVS Multichannel Verification System (Artel, Inc., Westbrook, ME) was used to measure the volume dispensed by both ALH systems. The MVS system is composed of various components including dye solutions, characterized microtiter plates, and a microtiter plate reader. The operating principle of the MVS involves dispensing the target volume of a sample solution containing both red and blue dyes into the wells of a characterized microtiter plate. A diluent containing the same concentration of blue dye as in the sample solution is used to fill the wells to a total working volume. The dispensed solutions are then mixed, and the ratiometric absorbance of the two dyes present in the solutions is measured. Because the blue dye concentration is common between both solutions, and because this concentration is known, the pathlength of light through the total solution volume can be determined from the measured absorbance of the blue dye. Using this pathlength, in addition to the known dimensions of the wells in the characterized microtiter plates, the total volume of both sample and diluent solutions is calculated. Finally, the sample volume is determined by the MVS system using the known concentrations of blue and red dyes present in the sample and diluent solutions, the total volume, and the measured absorbance of both red and blue dyes. Thus, based on the measured ratiometric absorbance of the two dyes present in the mixed solutions and characterized dimensions of the microtiter wells, the MVS calculates the amount of sample solution that was dispensed into every well within the microtiter plate. A more detailed and complete discussion of the dual dye MVS method for the evaluation of ALH devices is presented in references 7 and 8.

For the Tecan ALH, each tip aspirated the target volume of MVS sample solution containing the two dyes (red and blue). This target volume was dispensed into one full column in a microtiter plate. This process was repeated until sample solution had been dispensed into every well in the plate (i.e., each tip dispensed 12 times across the plate, resulting in 96 filled wells). The diluent solution containing blue dye was then used to back-fill all wells to a total volume of 200 μL. The absorbance of the two dyes present in each sample was measured at 520 and 730 nm, and the volume of sample solution was automatically calculated by the MVS system. A similar process was followed using the Tomtec device, the difference being that the 96-tip head dispensed the MVS sample solution into every well with a single dispense, which was followed by back-filling with diluent.

Results and discussion

Our initial experiment was designed to compare the results from the MVS approach with those from the gravimetric approach using the Tecan ALH system. For this, the dye solution was used for the photometric approach and water for the gravimetric approach. The data, presented in Table 1, clearly demonstrate that the results from the MVS measurements are significantly lower than those from the gravimetric approach. It is interesting to note that the specification requirement of $\pm 5\%$ inaccuracy would be easily met by the gravimetric approach for both volumes of 20 and 200 μL, whereas the MVS approach would fail at 20 μL and barely pass at 200 μL.

To verify that there is no difference between the gravimetric results obtained from the delivery of water and the gravimetric results obtained from the delivery of the dye solution, an experiment was conducted in which 200 μL of water or the dye solution was delivered by the Tecan ALH. The results, summarized in Table 2 for the eight tips, each with 12 deliveries, show that there is no difference between the gravimetric results obtained using water or the dye solution.

It was believed that the difference reported by the two volume-verification (calibration) methods lies in the modality of sample delivery by the ALH, rather than an inherent error in

Table 1. Comparison between the MVS method and the gravimetry method—Tecan ALH using water liquid class

	MVS (μL)		Gravimetry (μL)	
Target volume	20	200	20	200
Mean volume (μL)	18.74	190.08	20.15	199.53
Inaccuracy (%)	−6.30	−4.96	0.75	−0.23
StDev	0.22	1.74	0.6	0.55
CV ($n = 96$) (%)	1.17	0.92	1.94	0.59

These solutions were delivered by the Tecan ALH using an aspirate/dispense protocol based on a water liquid class. Inaccuracy corresponds to 100 × (Observed volume − Target volume)/Target volume. Coefficient of variation (CV) corresponds to 100 × StDev/mean, where StDev is the standard deviation.

one of the calibration methods. We theorized that the volume that was dispensed by the ALH was the correct amount, but the MVS approach reported a lower volume because the delivered solution was diluted by a small amount of the Tecan system liquid. This is because the MVS approach is based on the measurement of the absorbance, which is dependent on the concentration of the dye. Thus, the volume measured by the MVS approach would be lower due to the lowered concentration of the dye in the diluted solution. On the other hand, the overall volume delivered by the ALH does not change by the dilution caused by the system liquid. Therefore, the volume measured by the gravimetric approach will not be affected by the dilution.

To test the hypothesis, we performed three experiments described below.

Use of a Handheld Pipette to Deliver the Liquid in Place of the ALH

An Eppendorf handheld pipette was used to dispense eight volumes of solution (200 μL dye solution) onto a balance pan for a gravimetric measurement and into eight-well microtiter plate for the dual dye MVS measurement. The pipette was used as a ‘‘pseudo’’ transfer-standard to determine if the difference between the two methods was due to the Tecan ALH system or an inherent difference between the two verification methods. The mean value obtained via the MVS approach was 197.3 μL and that obtained via the gravimetric approach was 197.9 μL (Table 3). Because the difference between the two approaches was 0.3%, it is probable that there is no bias inherent in either approach. These results are consistent with data collected from a glass microsyringe, reported by Bradshaw et al.,[8] which also demonstrate the equivalence of the gravimetric and the MVS approaches.

Removal of the tip wash step in the operation of the ALH

Because dilution with Tecan system liquid was the suspected culprit of the observed bias between MVS and gravimetry, the effect of eliminating the wash step was investigated. This testing was carried out by dispensing a 200 μL test volume in the same manner as the initial experiment, but with no wash step used between each aspiration step. More specifically, the Tecan ALH was programmed to directly aspirate 200 μL from the reagent reservoir, and then dispense that 200 μL test volume into one column of a microtiter plate. The ALH then repeated this process, without washing the tips in between each transfer, until the plate was full (i.e., 12 dispense cycles).

As shown in Table 4, the mean volume reported by the MVS system increases across the cycles. Improvement of the MVS data when no wash step was used provides another piece of evidence pointing to unwanted dilution as the cause of the originally observed bias between MVS and gravimetry.

Table 2. Gravimetric comparison between water and dye solution delivered by Tecan ALH using water liquid class (target volume 200 μL)

ALH tip	Water (μL)	Dye solution (μL)
1	199.78	200.46
2	199.53	200.14
3	199.68	200.53
4	199.48	200.06
5	199.93	200.55
6	200.03	200.63
7	199.81	200.42
8	199.24	199.90
Mean volume	199.69	200.34
Inaccuracy (%)	−0.16	0.17
StDev	0.26	0.27
CV ($n = 8$) (%)	0.13	0.13

Each tip dispensed a total of 12 data points, and the average volume calculated from 12 dispenses was used to represent the overall performance. Inaccuracy corresponds to 100 × (Observed volume − Target volume)/Target volume. CV corresponds to 100 × StDev/mean, where StDev is the standard deviation.

Table 3. Comparison between the MVS method and the gravimetry method by manual delivery of dye solution (target volume 200 μL)

Dispense #	MVS (μL)	Gravimetry (μL)
1	196.2	197.8
2	199.3	198.7
3	195.5	198.0
4	194.6	198.8
5	196.3	198.7
6	198.3	198.7
7	200.4	196.2
8	197.8	196.2
Mean volume	197.3	197.9
Inaccuracy (%)	−1.35	−1.05
StDev	1.99	1.10
CV ($n = 8$) (%)	1.01	0.56

Eppendorf single channel hand pipette was used to deliver manually 200 μL of dye solution. Inaccuracy corresponds to 100 × (Observed volume − Target volume)/Target volume. CV corresponds to 100 × StDev/mean, where StDev is standard deviation.

Table 4. Tecan ALH performance as measured by the MVS approach with the elimination of the wash step

Target volume (200 μL)	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6	Cycle 7	Cycle 8	Cycle 9	Cycle 10	Cycle 11	Cycle 12
Tip 1	191.0	193.8	193.1	186.6	191.4	193.2	191.3	194.0	195.9	197.0	197.9	196.5
Tip 2	192.0	192.5	194.8	194.5	195.8	194.6	196.2	197.3	197.3	197.5	197.7	197.9
Tip 3	191.8	193.1	195.1	174.9	190.7	193.8	194.9	197.0	196.3	195.9	196.4	195.8
Tip 4	190.0	192.1	194.0	193.3	191.9	194.2	195.8	194.9	196.4	195.9	196.2	195.4
Tip 5	191.4	193.8	196.8	192.1	194.7	195.8	196.9	196.7	197.0	198.6	197.8	196.1
Tip 6	192.0	194.1	195.7	196.2	197.0	197.8	198.5	198.8	199.2	198.3	199.2	197.1
Tip 7	191.3	194.1	193.4	185.8	193.7	196.0	196.3	196.5	196.5	197.5	197.9	198.0
Tip 8	191.9	192.3	193.0	190.1	191.7	194.5	194.7	195.7	196.5	196.2	196.7	198.0
Mean volume	191.4	193.2	194.5	189.2	193.4	195.0	195.6	196.4	196.9	197.1	197.5	196.9
Inaccuracy (%)	−4.29	−3.39	−2.76	−5.41	−3.32	−2.51	−2.21	−1.82	−1.56	−1.44	−1.26	−1.58
StDev	0.68	0.83	1.35	6.82	2.30	1.47	2.10	1.49	1.03	1.05	0.99	1.05
CV (%)	0.36	0.43	0.70	3.61	1.19	0.76	1.07	0.76	0.52	0.53	0.50	0.53

Each tip dispensed a total of 12 data points. The wash steps were eliminated before each dispense. Inaccuracy corresponds to 100 × (Observed volume − Target volume)/Target volume. CV corresponds to 100 × StDev/mean, where StDev is the standard deviation.

The improved performance across the cycles might be explained by the fact that a portion of the system liquid is contaminated during the dispense step by the residual amount of the dye sample remaining in the delivery tube. This contaminated portion of the system liquid stays within the system because there is no postdispense wash step by which to expel it. Thus, by removing the post-dispense wash step, the next sample is aspirated with a system liquid, which has been contaminated with the dye sample from the previous dispense step. As the number of aspiration/dispense cycles increases, the concentration of dye in the system liquid also increases. Thus, even though the later dispense cycles are interacting with the system liquid in the same manner as the earlier cycles, they experience a smaller degree of dilution due to a higher concentration of dye contamination in the system liquid that comes into contact with the dye sample.

Use of a dierent ALH system

The final test compared the MVS and gravimetric approaches when used to measure the performance of an ALH system that operates on a completely different liquid delivery technology. The primary experiment was repeated with a 96-tip Tomtec ALH system, which uses an air displacement mechanism to deliver the desired volume. In this system, the dilution effect described above will not occur because there is no system liquid involved. In this experiment, the dye solution was dispensed into each well of a 96-well microtiter plate and weighed. The total weight of the plate less the weight of the tare was divided by 96 to get the weight per well. The same plate was then taken to the MVS system for volume measurement of each well by the photometric approach. As shown in Table 5, the gravimetric and MVS results agree very well. These results show that the MVS and the gravimetric approaches are equivalent when an ALH system with no system liquid is involved.

The data from the above experiments confirm the hypothesis that the bias between the MVS and gravimetric approaches is due to a dilution by the system liquid. When the Tecan ALH system, which operates on the principle of positive displacement by the system liquid, is used, a dilution of the sample can occur. As the system liquid is pulled back to draw the air gap and sample, it is possible that a sheath of liquid remains on the inner cannula walls. The sample picks up this small volume of extraneous liquid and is thereby diluted. While the volume that is delivered does not change, the concentration of the analyte in the sample does. Thus, the volume reported by the MVS method, which is concentration dependent, is less than the actual delivered volume. If the

Table 5. Comparison between dual dye method and gravimetry method—Tomtec ALH

	MVS (mean of all channels μL)				Gravimetry (whole plate weight g)			
Target value	20	50	100	200	1.9200	4.8000	9.600	19.200
Measurement 1	17.50	46.57	92.96	196.88	1.6358	4.5852	8.9587	18.9222
Measurement 2	17.47	46.49	93.16	196.57	1.6401	4.4774	8.9312	18.8777
Mean	17.49	46.53	93.06	196.73	1.6380	4.5313	8.9450	18.9000
Inaccuracy (%)	−12.58	−6.94	−6.94	−1.64	−14.69	−5.60	−6.82	−1.56
StDev	0.021	0.057	0.141	0.219	0.003	0.076	0.019	0.031
CV ($n = 2$) (%)	0.12	0.12	0.15	0.11	0.19	1.68	0.22	0.17

Inaccuracy corresponds to 100 × (Observed volume − Target value)/Target value. CV corresponds to 100 × StDev/mean, where StDev is the standard deviation.

analyst uses a gravimetric approach to measure the dispensed volume, the reported volume would be accurate, but the change in the analyte concentration would not be noted. In contrast, if the MVS approach was used for calibration, the reported volume might not be correct. When both methods are used, the discrepancy would alert the analyst to a potential problem of dilution of the delivered sample, which could affect the validity of the subsequent assay results if the pipetting parameters are not subsequently adjusted to eliminate the observed dilution.

It should be noted the Tecan ALH system is free of sample dilution effects when configured with disposable tips, where the system liquid does not enter the tips.[9] It should also be pointed out that there are advantages of using a fluid-filled system.[10–12] However, proper configuration of such systems is critical. Tecan does recommend pipetting parameters that can be used to reduce or eliminate the dilution effect shown in this paper.[9] We are currently conducting additional experiments to further optimize these parameters so that we can take full advantage of fixed tips. These findings will be presented in a future publication.

An additional benefit of the MVS approach for the Tomtec system should be noted. Because the Tomtec system consists of 96 tips, a typical gravimetric analysis cannot provide information on the performance of each individual tip. Instead, the aggregate volume dispensed into the entire plate is gravimetrically measured. This aggregate weight is divided by the number of wells to give the average weight per well. By comparison, the MVS approach consists of measuring the absorbance of dye solutions in every well, thereby allowing for comparison of the performance of individual tips. Because a measurement has been made for all 96 tips, the average of all tips can be reported, along with a deviation of each tip about that mean. Because the MVS approach provides data for each tip, the operator can readily determine that there is a problem with a specific tip position. By contrast, the gravimetric data alone allow no determination on the spread of tip performance about the calculated mean. The other advantage of the MVS method is that the accuracy of the reported volume is only slightly affected by the evaporation of the sample dispensed by the ALH system, whereas the reported volume by gravimetric approach would be lowered by the evaporation of the delivered sample, which could happen during whole plate weighing unless appropriate precautions are taken.

Conclusions

Our data clearly indicate that the gravimetric and MVS approaches for verification of the volume delivered by an ALH system agree when the integrity of the dispensed sample is maintained. The difference observed between the reported volume from the MVS approach and the gravimetric approach is attributed to sample dilution with the system liquid in the Tecan ALH system with fixed tips. It is important to note that while the gravimetric method reports the correct volumes delivered by the ALH system, it does not alert the analyst to the potential dilution of the sample. This point presents a significant risk for the analyst, because if a dilution occurs the concentration of the analyte is changed during the delivery by the ALH.

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