RAPID VERIFICATION SYSTEM FOR CRITICAL VOLUME TRANSFERS WITHIN MICROTITER PLATE ASSAYS

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ABSTRACT

Technological advancements over the past decade have led to broad implementation of automated liquid handlers. With these systems, there has been a significant movement toward high-throughput and miniaturization of microtiter plate-based assays. Because high-throughput assays performed within a microtiter plate are volume dependent, the concentrations of biological and chemical components in these assays, as well as the associated dilution protocols, are volume dependent. Therefore, the accuracy and precision of individual volume aspirations and dispenses directly impact assay results. As a result, there is a tremendous need for analytical methodology that can be employed to measure the exact volume dispensed in each well of these plates. Presently, there are very few methodologies that can quickly and accurately interrogate dispensed volume in individual wells because validation of sub-microliter liquid volumes is an extremely challenging task. The ratiometric photometry approach implemented by the ARTEL Multichannel Verification System (MVS®) provides a standardized platform for simultaneously measuring both the accuracy and precision of volume delivery into 96-well or 384-well plates for volumes between 0.01 – 200 µL. Such measurements allow for assay integrity and proper interpretation of experimental results. This presentation will discuss the use of the MVS to help understand microtiter plate assay performance as well as many application-driven tasks employing critical volume transfers, including non-aqueous reagent use, dilution protocols, inter-device comparability (device 1 vs. device 2), and tip-to-tip reproducibility within one or more devices.

INTRODUCTION

The need to ensure quality in a laboratory process has become increasingly important, especially as it relates to the measurement of critical volume transfers within microtiter plate-based assays. Knowing the exact volume transferred will inherently lead to accurate and precise analysis of the experiment, *i.e.*, the results can be trusted. Too often, however, the importance of liquid delivery is overlooked. In these situations, imprecise or inaccurate liquid delivery may not be diagnosed and may lead to a false sense of performance (for either the assay or the liquid handler). Most assays depend on proper volume delivery, but in many situations, the liquid handler is never calibrated nor verified for the critical transfer volumes, which include aspirating, dispensing, diluting, mixing, and washing steps.

As throughput increases and assay volumes decrease, there are more demands for accuracy and precision of each volume transfer tasks. A volume verification system, such as the MVS, can be used to rapidly quantify most, if not all, of the critical volume transfers so that the dispensing performance of the liquid handler can be understood before the assay protocol begins. Furthermore, when the MVS is employed as a diagnostic tool, liquid handler performance and the need for maintenance become clear, reducing downstream troubleshooting, resource requirements, and economic loss.

The MVS is a complete system consisting of a plate reader, computer with software, barcode reader, an orbital plate shaker, sample and diluent solutions, a calibrator plate and a mobile cart. As discussed in this poster, the solutions are dispensed into the microtiter plate by the liquid handler, and the target volume is quantified well-by-well and tip-by-tip using dual-dye ratiometric photometry with a foundation based on the Beer-Lambert Law.

TARGET VOLUME CALCULATION

The volume verification approach is based on Beer's Law:

$$A_\lambda = (\varepsilon_\lambda \cdot C) \cdot l = a_\lambda \cdot l$$

where a_λ represents the absorbance per unit pathlength for the sample solution, l is the pathlength, ε_λ is the molar absorbtivity constant, C is the concentration, and A_λ is the measured absorbance.

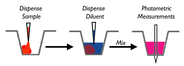
Two different dye-based solutions are employed:

- sample solution containing both red and blue dyes with absorbance per pathlength values a_r and a_b, respectively;
- diluent solution containing only blue dye at same concentration as in sample solution (a_b).

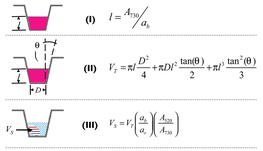
This dual-dye methodology requires absorbance readings for red dye at 520 nm (A_{520}) and blue dye at 730 nm (A_{730}).

Mixtures of any ratio of sample solution to diluent will result in the same concentration of blue dye, but a varying concentration of red dye. Thus, the blue dye is used as an internal standard.

The target volume of sample solution is added to the well before or after the diluent. The amount of target volume dispensed is quantitative and amount of diluent dispensed is approximate, or qualitative.



The absorbance values of both dyes at their respective wavelengths are collected independently for each well of the plate. For each well, the pathlength l is determined through equation (I). Equation (II) is then employed to determine the total volume (V_T) of the solution in each well by using the pathlength as well as the measured dimensions of the well, *i.e.*, diameter (D) and taper angle (θ). Equation (II) is specific, in this case, for the plates with truncated-cone shaped wells. The target volume of sample solution (Vs) is subsequently determined through ratiometric photometry.



For each plate type, the diluent is used to achieve the working volume, *i.e.*, 200 µL in a standard 96-well plate or 55 µL in a 384-well plate. Once both solutions are dispensed, they are thoroughly mixed before photometric measurements are acquired. In order to acquire absorbance measurements, the microplates must have optically-clear flat-bottom wells.

DIFFERENT PLATE TYPES MAY AFFECT THE MEASURED VOLUME

Three different, optically-clear, flat-bottom 96-well plate types, in replicates of three, were used to compare and correlate measured volume for 20 µL transfers.

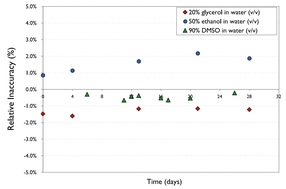
	Nunc (265300)	Greiner (655096)	BD-Falcon (353293)
plate A, columns 1-3	20.87	19.94	20.76
plate A, columns 4-6	20.84	20.09	20.78
plate A, columns 7-9	20.87	20.05	20.80
plate A, columns 9-12	20.87	20.13	20.68
plate B, columns 1-3	20.93	20.13	20.86
plate B, columns 4-6	20.93	20.18	20.82
plate B, columns 7-9	20.93	20.06	20.83
plate B, columns 9-12	20.83	20.07	20.79
plate C, columns 1-3	20.96	20.10	20.86
plate C, columns 4-6	20.96	20.16	20.86
plate C, columns 7-9	20.96	20.17	20.77
plate C, columns 9-12	20.93	20.19	20.74
Average (µL)	20.91	20.11	20.80
Stand Dev (µL)	0.05	0.07	0.05

* Each data point above represents an average of three replicates; i.e., there were 36 replicate dispenses (12 columns x 3 plates) for each plate type.

Careful selection of plate type and quality can have a significant impact on the measured target volume. Differences in measurement results might be observed if plate types, or lots, are interchanged during assay development. As above, there is roughly a 4% difference between the target volume dispensed into the Nunc and Greiner plates.

VOLUME VERIFICATION OF CUSTOM SOLUTIONS

By combining concentrated dye stocks with a solvent-of-interest, the MVS permits the user to verify target volume transfers of custom solutions. Using an 8-µL gravimetrically-calibrated syringe as the volume transfer standard, three very different custom solutions were prepared and wet-dispensed into 192 µL of diluent. The solutions were 20% glycerol in water (v/v), 90% DMSO in water (v/v), and 50% ethanol in water (v/v). Each solution was dispensed in replicates of 8, into a 96-well ARTEL Verification Plate multiple times within a 4-week period.



The graph shows the stability in the measurement — performance, as shown by relative inaccuracy, did not degrade during the testing period. Individual users can use the MVS to assess device performance for specific or proprietary solutions at assay-specific target volumes. Though there are some limitations, the ability to precisely and accurately determine the volume of complex and/or non-aqueous liquids dispensed from a liquid handling device is a definite advantage over many other volume verification techniques.

TARGET VOLUME TRANSFER FROM A CUSTOM PLATE TO OPTICALLY-CLEAR FLAT-BOTTOM PLATES

Using a gravimetrically-calibrated syringe, a target volume was dispensed, in replicates of 8, to two control plates and three custom plate types.

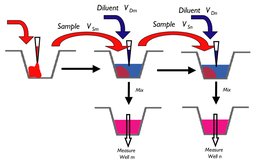
Experimental ID[1]	a	b	c	d	e
Target Volume of Gravimetrically-Calibrated Syringe (µL)	7.97	7.97	7.97	7.97	7.97
Number of replicate dispenses	8	8	8	8	8
Plate Type for target volume	96-well Artel VP	96-well Artel VP	96-well, round-bottom	384-well, "inverted" pyramid wells	384-well, conical tube like wells
Description of target volume dispense	wet dispense into 192 µL Diluent	dry dispense into empty well	dry dispense into empty well	dry dispense into empty well	dry dispense into empty well
Number of Diluent additions; Volume of diluent; number of pipette mixing cycles before transfer	NA	1 x 192 µL; no mix cycles	4 x 48 µL; 3 mix cycles	7 x 20 µL; 3 mix cycles	5 x 20 µL; 3 mix cycles
Plate Type used to receive transferred target volume	NA - no transfer	NA - no transfer	96-well Artel VP	96-well Artel VP	96-well Artel VP
Amount of Diluent added to Artel VP before shaking and volume verification (µL)	0 µL	0 µL	0 µL	50 µL	90 µL
Mean volume (µL)	**7.994**	**7.984**	**7.999**	**8.004**	**8.029**
Relative Inaccuracy	**0.30%**	**0.18%**	**0.36%**	**0.43%**	**0.74%**
Standard Deviation (µL)	**0.022**	**0.02**	**0.023**	**0.025**	**0.028**
Coefficient of Variation	**0.28%**	**0.25%**	**0.29%**	**0.31%**	**0.35%**

[1] (a) control, wet dispense into optically-clear, flat-bottom 96-well Artel Verification Plate (VP); (b) control, dry dispense into 96-well Artel VP; (c) 96-well plate, Corning 3790 round-bottom, optically opaque; (d) 384-well plate, Matrical very conical

By using successive diluent additions and subsequent mix cycles, the target volume was transferred to a plate type for photometric measurements to successfully quantify the original target volume dispense.

QUANTIFYING SERIAL DILUTIONS

This section of the poster serves to briefly highlight the concept of measuring serial dilutions across portions of a microtiter plate. Though all the calculations are not shown, it is important to know that by using different sample solutions, the accuracy of serial dilutions can be measured when the solutions are diluted up to 1:2000.



A general expression for dilution steps is given by:

$$R_{mn} = \frac{A_{Rm}}{A_{Rn}} \cdot \frac{A_{Bn}}{A_{Bm}}$$

If a characterized highly concentrated dye is initially employed, which is beyond the measurable absorbance range of the reader, then the dilution ratio expression becomes:

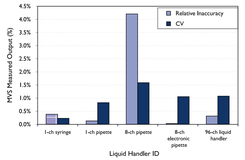
$$R_{1n} = \frac{a_R}{a_B} \cdot \frac{A_{Bn}}{A_{Rn}}$$

Total Dilution	0	1:2	1:8	1:32	1:128	1:512	1:2048
Volume Transferred	200	100	100	100	100	100	100
Volume Remaining	100	100	100	100	100	100	100
% Inacc from initial	0.78%	-1.07%	-2.38%	-3.33%	-2.55%	-0.16%	-0.91%
% Inacc of transfer		-1.83%	-1.45%	-1.35%	-0.04%	-0.24%	0.32%
%CV tip-to-tip	0.39%	0.54%	0.30%	0.35%	0.38%	0.41%	1.10%

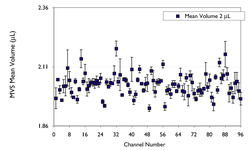
Conceptually, the results of this approach are independent of well size, shape and consistency as well as the interaction effects between the solution and the plate materials, *i.e.*, meniscus or air pockets, unless the light beam is clipped. The success of this analysis is highly dependent on the quality of mixing of the two dyes within the well at each step.

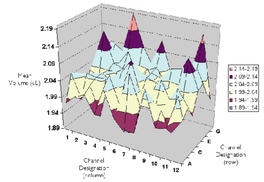
DEVICE-TO-DEVICE AND TIP-TO-TIP COMPARISONS

Five different pipettors, including both manual and automated devices, were directly compared for dispensing performance when transferring a target volume of 8 µL. Because the MVS' verification measurement results are traceable to international standards, performance can be directly compared regardless of liquid handler make, model, manufacturer, or location. The five pipettors all show slight differences in performance and these differences could be very important if the devices were used in parallel, *i.e.*, in assay scale-up, transfer, or for preparing reproducible samples.



The accuracy and precision performance information for each pipette tip should always be known for each critical target volume transfer. The MVS quantifies transferred volume on a tip-by-tip basis so the exact volume, and variation on volume, is measured for each well. The data below show the mean volume (± standard deviation) per tip for a 96-channel liquid handler dispensing 2 µL into 3 replicate 96-well ARTEL Verification Plates. On the top is a concatenated plot for tips 1 through 96 and the surface plot on the bottom offers visual insight to potential dispense patterns, such as edge effects or areas of high/low tip dispensing.





INTEGRATING A ROBUST VERIFICATION METHOD

There are many other places where a robust volume verification system, or method, should be implemented, such as:

- automation method development
- diagnostics and troubleshooting
- before, during, and after system maintenance or service
- evaluating new volume transfer strategies
- optimizing and validating automation transfer parameters
- when volume transfer accuracy is critical
- when volume transfer precision is critical
- periodic system calibration over the desired volume range
- understanding or learning a system's baseline performance
- plate reformatting or hit-picking
- frequent quick volume 'spot' checks (interim performance checks)
- monitoring dispense trending or drift patterns
- proving performance for internal/external sources
- comparing factory and site acceptance testing for new liquid handlers
- critically transferring aqueous, non-aqueous, or proprietary liquids
- removing volume transfer as a source of assay variability
- repetitive transfer tasks (preparing clone plates from source plates)
- assay scale-up (compare bench top assay to automated assay)
- evaluating new microplates or different lots of plates

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DISCUSSIONS AND CONCLUSIONS

This poster addresses some important factors and their associated applications in understanding volume verification in microtiter plates. More importantly, the specific applications and results discussed herein are intended to represent the types of critical information that can be learned with regard to any volume transfer. A robust and reliable volume verification method allows for measurement of transferred volumes at all levels in assay development, from a pure research level to a highly-regulated laboratory environment, and different information may be required for different types of volume transfer applications. The goal is to emphasize the importance of achieving liquid delivery quality assurance through accurate and precise transfer of critical target volumes within microtiter plates.

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