

# Why Is My Assay Failing? An Approach to Assay Equipment Optimization

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#### Abstract

Assays can produce unexpected or failing results for a multitude of reasons. Variability may be introduced at any point within the assay process. Some potential sources of error could include: 1) incorrect, contaminated or expired reagents, 2) technician error, 3) equipment error, etc. Even simple assays include multiple steps where errors could be introduced, which greatly increases the difficulty of finding the source of the error. Therefore, determining the cause of unexpected results commonly presents a time consuming and expensive task for laboratory researchers.

One approach to reducing assay failure is to ensure equipment errors are removed or at least reduced. This may be accomplished by optimizing the equipment employed prior to running the assay. Traditionally, tools used to optimize the performance of this equipment have been varied, not standardized and not traceable. In this poster presentation, the use of the Artel MVS® Multichannel Verification System and PCS® Pipette Calibration System will be discussed for optimization of the laboratory equipment used at each step of any given assay including plate washers, plate mixers, large volume liquid dispensers, manual pipettes and automated liquid handlers.

#### **Evacuation Efficiency Test**

For this test,  $200~\mu L$  of Range C was pre-filled in two different 96-well plate types – an MVS Verification Plate and a tissue culture treated 96-well plate. A one-time solution removal step was conducted for each plate and the residual sample solution was measured after the addition of and mixing with Diluent solution.

Test Results for Evacuation Efficiency Test within the MVS Data Manager software (with MVS Verification Plate):

Plate Type	MVS Verification Plate
Target Solution	Range C
Number of data points per channel	3
Mean volume for all Channels (μL)	1.262
Standard Deviation for all Channels (µL)	0.469

Test Results for Evacuation Efficiency Test within the MVS Data Manager software (with generic tissue-culture treated microplate):

Plate Type	Costar TC Treated 3603
Target Solution	Range C
Number of data points per channel	3
Mean volume for all Channels (μL)	1.311
Standard Deviation for all Channels (µL)	0.444

This experiment not only cites particular channels that may require cleaning, as highlighted by red shaded cells representing volumes two standard deviations greater than the inter-plate mean, but serves as a reminder that not all plate types perform equally. Thus, one generic, 96-well wash program might not be sufficient to efficiently aspirate all material from the wells of any plate type.





#### **Plate Washers**

Washing steps are vital to ensure reaction quality and integrity particularly when conducted in a microtiter plate. Operation and qualification performance tests often described by the manufacturer include dispense precision and evacuation efficiency tests. These tests are often conducted with the help of a balance and a single colorimetric dye. The MVS method of performance measurement generates more inclusive and informative output reports that present data in a well-by-well manner for ease of visualization and discovery.

#### **Methods for Artel MVS Use**

#### | Precision Testing

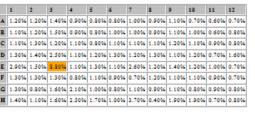
The MVS employs pre-mixed sample solutions at the appropriate concentration for the desired test. In this application, Range HV solution was used for the dispense test of 300  $\mu$ L. Once the sample solution was dispensed the plates were centrifuged to release any bubbles created from the manifold dispense, and then read in the MVS plate reader.

When observing the overall run statistics, the results passed the manufacturer's precision specifications of 4% for inter- plate CV. As illustrated in the results contained within the figure below, three plates were run in succession and data obtained highlights a failed channel with a %CV of 8.80% The well-by-well %CV report indicated that well E3 may require further attention to correct issues with the dispense channel.

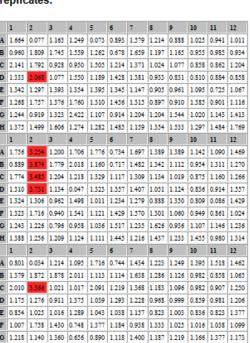
Test Results for Dispense Precision Test within the MVS Data Manager software (with MVS Verification Plate):

(with MVS Verification Plate):		+	
		_	
Target Volume (μL)	300		į
Target Solution	Range HV		I
Number of data points per channel	3		(
Mean volume for all Channels (μL)	303.1		I
Relative Inaccuracy for all Channels	1.03%		1
Standard Deviation for all Channels (µL)	5.5		(
Coefficient of Variation for all Channels	1.81%		I
Coefficient of Variation Pass/Fail Limit	4%		
Status based on channel results	Failed		ž
Status based on run statistics	Passed		1
		1	к

Channel-Specific Coefficient of Variation:



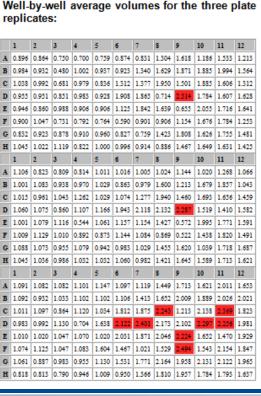
## Well-by-well average volumes for the three plate



I 1.059 1.398 1.404 3.178 1.226 1.156 1.450 1.078 1.275 1.170 1.182 1.333

Well-by-well average volumes for the three plate

1 2 3 4 5 6 7 8 9 10 11 12



#### **Plate Mixers**

When important assays are being conducted, the liquids dispensed into a microplate are often assumed to have mixed through simple diffusion or after only a cursory agitation of the plate. For assays results to be truly trustworthy every part of the methodology employed needs to be assessed for efficacy and repeatability including liquid mixing protocols.

#### Method for MVS Use

In this study, the MVS was used to measure the absorbance of dye solutions in microplate wells at multiple time points. A pre-determined number of wells within a microplate were filled with a pre-mixed (i.e., constant absorbance) control solution comprised of Sample and Diluent Solutions. For the diffusion studies, the control solution was dispensed into 48 wells of the 384-well microplates and 24 wells of the 96-well microplates studied. The remaining wells were filled with 55  $\mu L$  of diluent, followed by 0.2  $\mu L$  of Range E sample solution. Absorbance measurements were then taken every 5 minutes using the MVS and the coefficient of variation (%CV) calculated.

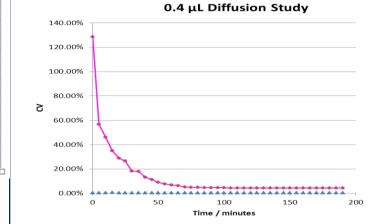
In the 2  $\mu$ L test cases (both for non-optimized and optimized studies), 48 wells in the microplate were filled with the pre-mixed control solution . The remaining wells were filled with 53  $\mu$ L of diluent and 2  $\mu$ L of Range C sample solution. The absorbance of the plate was measured immediately to determine the initial, unmixed absorbance in each well of the plate. The procedure for the optimized study was repeated for a sample volume of 0.2  $\mu$ L using Range E sample solution, dispensed into 55  $\mu$ L of diluent solution using a 5  $\mu$ L electronic syringe.

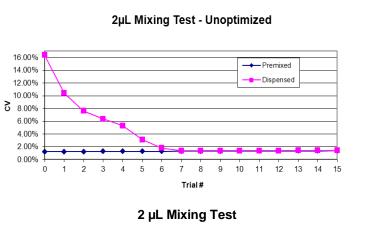
Non-optimized studies = Big Bear plate shaker set at 2200 RPM for 1 min.

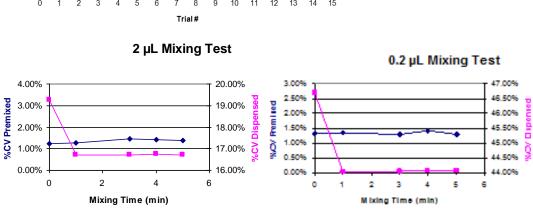
Non-optimized studies = Big Bear plate shaker set at 2200 RPM for 1 min.

Optimized studies = Q-Instruments shaker set at 2600 RPM for 1 min. Upon completion of the mixing cycle, the plate was immediately measured again to obtain the second set of absorbance values for each well. These steps were repeated until the %CV values remained unchanged.

The relative differences from measurement to measurement were used to determine the efficacy of the mixing cycles. When the %CV values were unchanging over multiple mix-read cycles, mixing was deemed complete, regardless of the magnitude of the %CV value. High %CV values were attributed to dispense inconsistencies, not mixing inefficiency as a result of using the dispensers at their lowest settings.







### **Large Volume Dispensers**

The use of bulk dispensers in the laboratory has become quite commonplace, especially for routine dispensing of buffers or other reagents into microplates. More commonly, however, the performance of bulk dispensers goes widely unchecked. Additionally, as assays become more miniaturized, there has been more emphasis on employing low-volume dispensers — especially for ease of use and simplicity that these systems offer. As with their bulk dispenser counterparts, many low-volume dispensers do not get checked for volume delivery performance, either due to lack of time, skill, or methodologies.

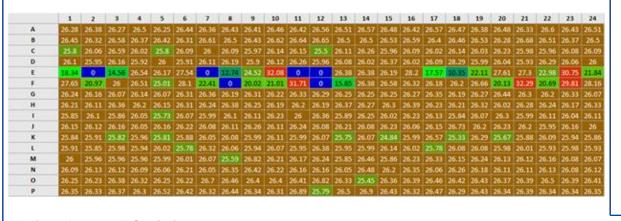
#### Method for MVS Use

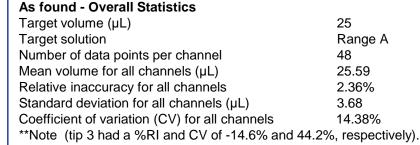
Described here are examples of checking the performance for high and low-volume dispensers using the MVS. Because not all channels perform the same way, channel-by-channel performance metrics become quite important when one is trying to understand exactly how a dispenser is behaving.

Every well of a 384-well plate was filled with 30  $\mu$ L of diluent using an 8-tip 1200- $\mu$ L Eppendorf repeater pipette. The plate was then centrifuged at1500 rpm for 15 s. The dispenser under test was primed with Range A for 30 s and then it was used to deliver 25  $\mu$ L to every well of the plate (48 replicate dispenses per tip). The dispenser delivered volume simultaneously to rows A (tip 1), C (tip 2), E (tip 3), etc. in sequential order from columns 1 to 24. When the dispenser reached column 24, volume delivery continued in order from column 24 back to column 1 by dispensing in rows B (tip 1), D (tip 2), F (tip 3), etc.

Using the MVS and its associated heat map feature, tip 3 appeared to be misperforming and may have been compromised in some fashion, which resulted in missed wells and excess dye being delivered to other wells. Some wells contained no volume and others contained up to 30  $\mu L$  per well (see below). To correct the performance of the tip, the cartridge was flushed using the "prime" button with ethanol-water mixture followed by a 1-minute flush of DI water. The system's "prime" and "empty" buttons were used in sequential fashion during the ethanol washing procedure to potentially disrupt any clogs from salts or other left over reagents in the tubes and tips. After the cartridge was cleaned and deemed acceptable for use, multiple volumes were checked using the 1-200  $\mu L$  small volume tubing cartridge, which according to the manufacturer, is suitable for volumes between 1-200  $\mu L$  in either 96-well or 384-well plates.

As shown in the two tables below, the accuracy and CV are about the same for the tested volumes ranges in both 96-well and 384-well plates (where the accuracy for each noted volume is between 3.6 and 6.4% high; CV <1% at all volumes greater than 1  $\mu$ L). In both testing scenarios, the CV for the 1-  $\mu$ L dispense is high (16%) and based on this testing, this particular dispenser should not be used at such low volumes in order to avoid questionable assay results in future testing.





Post wash procedure – Overall Statistics	
Target volume (µL)	25
Target solution	Range A
Number of data points per channel	48
Mean volume for all channels (µL)	26.26
Relative inaccuracy for all channels	5.04%
Standard deviation for all channels (µL)	0.2
Coefficient of variation (CV) for all channels	0.76%

#### **Handheld Pipettes**

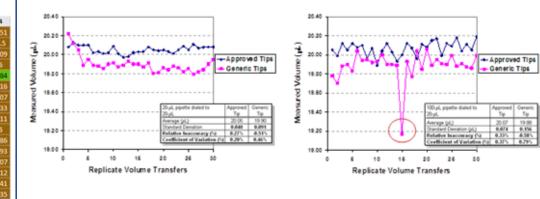
The type, characteristics, and quality of disposable pipette tips can directly affect the volume transferred to and from an assay, regardless of operator skill or type of pipette employed. All pipette tips are not created equal (see **Figure 1**), nor are all assays. Failure to use proper tips for a given type of pipette can lead to an inadequate seal between the pipette and tip, causing inaccurate volume transfers due to leakage and sample loss<sup>1</sup>. The need for the best performing tip is likely proportional to the importance of having *both* accuracy and precision in volume delivery for specific assay steps.

Artel's dual-dye photometric technology for simultaneously measuring accuracy and precision of volume transfers allows users to objectively correlate volume transfer performance to tip type, enabling users to not only select the optimal tip type based on their needs, but also allows for troubleshooting specific volume transfer steps when tip reliability is in question.

In this study, the volumes dispensed by two different pipettes each using two different pipette tip types were compared. A standard pipetting method (described below) was used with each pipette to dispense 20 mL of Artel dye solution and the transferred volume was subsequently measured with the Artel PCS. Pipette 1 was a 20-mL single-channel pipette dialed to 20 mL and Pipette 2 was a 100-mL single-channel pipette dialed to 20 mL. For each pipette, one tip type was "manufacturer approved" for the specific pipette and the second tip type was generic. Both pipettes were from different manufacturers.

If the user intends to evaluate tip types with a handheld, manual pipette, the user should follow proper pipetting technique for pre-wetting, tip depth pipette angle during aspiration, pause steps, etc. as discussed in reference 2. For all measurements, standard (forward) mode pipetting was employed.

The results indicate that in each example, the vendor approved pipette tips showed slightly better volume transfer performance compared to generic tips for both the 20-mL pipette dialed to 20 mL (Figure below left) and the 100-mL pipette dialed to 20 mL (Figure below right). In both cases, the generic tips demonstrated a higher degree of variability compared to the manufacturer approved tips (demonstrated by higher CVs, see inset tables) using the same pipetting technique and conditions. Additionally, for one generic tip in the 100-mL pipette experiment, the volume transfer performance was noticeably different than the rest (Figure below right).



200 150 100 50

12 12 12 12

Coefficient of variation (CV) for all channels | 0.62% | 0.51% | 0.53% | 0.67% | 16.22%

Coefficient of variation (CV) for all channels | 0.99% | 0.81% | 0.72% | 0.83% | 16.72% |

208.5 156.8 104.49 52.44 1.0638

4.25% | 4.53% | 4.49% | 4.88% | 6.38%

1.3 0.8 0.55 0.35 0.1725

 55
 40
 25
 10
 1

 48
 48
 48
 48
 48

57.76 42.02 26.27 10.655 1.0359

5.02% 5.05% 5.08% 6.55% 3.59%

0.57 0.34 0.19 0.088 0.1732

Testing 1-200 uL in a 96-well plate

Number of data points per channel

Mean volume for all channels (μL)

Relative inaccuracy for all channels

Standard deviation for all channels (µL)

Testing 1-55 uL in a 384-well plate

Number of data points per channel

Mean volume for all channels (μL)

Relative inaccuracy for all channels

Standard deviation for all channels (μL)

Target volume (μL)

Target volume (μL)

#### **Automated Liquid Handlers**

Described here, three different tip types were compared using a 384-channel liquid handler. After a 384-tip head had gone through a preventive maintenance service from the manufacturer of the liquid handler, it was tested for dry dispensing 1 mL of DMSO dye solution into flat-bottom microplates. In multiple liquid handler runs three different disposable tip types were employed:

(a) manufacturer-approved 10-mL tips;(b) 10-mL tips from an alternative vendor X; and(c) 30-mL tips from an alternative vendor Y.

Two replicates were collected per tip, *i.e.*, two full 384-well plates were used to assess volume transfer performance based on tip type. The two destination plates, the tip types under test, and DMSO sample reservoir were placed on the deck of the liquid handler. The liquid handler was programmed to load tips, aspirate the DMSO solution, and dispense it into the first test plate. Before repeating the volume transfer to the second plate, the tips were discarded and new tips were loaded (i.e., non-prewetted, new tips were employed for single dispensing target volume for each replicate).

For each testing experiment, *identical* liquid handler methods and parameters were employed. For instance, the aspirate rate(s), air gap volume(s), dispense rate(s), tip touch(es), etc. stayed exactly the same for every dispense and the only variable changed was the tip type. It is likely (but not known) that the head's Z direction (positioning up and down) could have been adjusted if the tips were of different lengths so that each tip type was almost pinned on the bottom of the well during the dispense.

After two plates were filled for the first tip type, a second tip type was loaded onto the deck and the method was repeated (same for the third tip type). In all, six 384-well plates were filled. After the target volume of DMSO sample solution was dispensed, 54 mL of MVS Diluent (non-quantitative addition) was dispensed into every well of the 384-well plates. The volumes were measured on a well-by-well basis using the Artel MVS.

Based on the performance results – specifically CV values across the plate –the 10-mL tips from alternative vendor X were chosen for the 1-mL DMSO transfer. In other collected data (not shown), the %CV values for vendor X's tips were not as good as CV values for manufacturer approved tips when dispensing < 1 mL (300 and 500 nL). In the end different tip types for different volumes were selected to maintain a cost effective approach while remaining compliant with established tolerances.

 Table 1. Evaluating Three Different Tip Types on a 384-tip robot in a high-throughput laboratory

	(a) 10-山 approved tips	(b) 10-此 tips from alternative vendor X	(c) 30-µL tips from alternative ∨endor Y
Target Volume (μL)	1	1	1
Average (µL)	0.973	0.969	0.979
Relative Inaccuracy (%)	-2.70%	-3.10%	-2.10%
Standard Deviation (µL)	0.022	0.025	0.077
Coefficient of Variation (%)	2.26%	2.58%	7.87%

#### Conclusions

Ensuring the performance of equipment used in every step of an assay is essential to the success of the test. The dual-dye ratiometric technology employed by Artel products provides the most universally applicable means by which to determine as-found performance of virtually all laboratory equipment. Additionally, the Artel technology allows the user to optimize instrument performance thereby minimizing variability introduced to assays by equipment.

#### References:

- 1. Khovanath, K., Knaide, T., Albert, K., Artel application note: "Performance Qualification of an Automated Plate Washer Using the Artel MVS", Artel document number: 12A6115.
- 2. Knaide, T., Wilkinson, M. Parshley, R., "Artel MVS as a Tool For Measuring Liquid Mixing Efficacy in Microplates", Artel document number: 12A6128.
- 3. Knaide, T., Albert, K., "Quantifying the Impact of Pipette Tip Type Using Dual Dye Ratiometric Technology", Artel document number: 12A5952.

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