Do You Really Know What Your Robot is Doing? The Importance of Paying Attention to Liquid Handling Details

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Introduction

The introduction of automation into biology and chemistry labs has arguably lead to significant advances in testing capabilities over the past 20+ years. Automation has certainly led to increased numbers of experiments, as compared to manual testing, particularly for pipetting operations. Because of this advantage, liquid handling robots have become commonplace even in small laboratories. However, in spite of all the advantages that something like a liquid handling robot brings to a laboratory, it also brings a different set of commonly overlooked challenges.

It may be argued that the largest challenge presented by using a liquid handling robot is the potentially incorrect assumption that the robot is doing what it is "supposed" to be doing. The robot may in fact be doing exactly what the user told it to do, but is that really what the user wanted? One may say that the real question is, do you *really* know how your robot is behaving, and particularly, do you *really* know how your robot is performing your assays?

This presentation is a follow-up to a poster we previously presented which discussed real case studies of how liquid handlers were performing, or rather misperforming, certain commonly employed test procedures.[1] Herein we will present even more examples of the importance of monitoring various commonly employed tasks, which are likely considered mundane and often assumed to have little bearing on overall robot performance. Specific examples that will be presented include: 1) proper pre-wetting of tips, 2) performance comparison between different robots running the same protocol, 3) protocol differences between high volume and low volume dispenses, and 4) effect of delivery mode. The examples presented herein will help users to think more about the specific tasks they are asking their robots to perform, and hopefully uncover certain steps that, if observed and controlled, will result in better performance.

How do you know?

Performance of a liquid handling robot is not determined through "osmosis", but requires specific and deliberate observation and testing.[2] While this may seem obvious, it is not always rigorously followed due to lack of time, resources, etc. However, without specific measurement and comparison, it is difficult to know the impact that various liquid handling steps may have on overall protocol performance.

For qualifying liquid handlers and/or assessing their performance, it is critical to measure both the accuracy *and* precision of each delivered volume. Said measurements are demonstrated herein using the Artel MVS® system, which determines both quantities in one experiment. Measures of accuracy and precision are often taken as the mean and standard deviation values from multiple data points, as defined in **Table 1**.

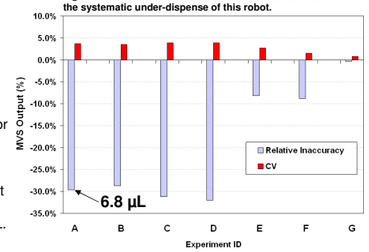
Table 1: Summary of Equations for Determining Accuracy and Precision.

Mean Volume	$\overline{V} = \frac{\sum_i^n V_i}{n}$
Standard Deviation	$STD = \sqrt{\frac{\sum_i^n (V_i - \overline{V})^2}{n-1}}$
Coefficient of variation	$CV = \frac{STD \times 100}{\overline{V}}$
Relative Inaccuracy[a]	$Inacc = \frac{(\overline{V} - V^P) \times 100}{V^P}$

[a] V^P is the target volume.

Accuracy can be defined as the degree of veracity (e.g., the measurement's *closeness* to a reference point or standard value), whereas precision can be defined as the agreement between multiple measurements of the same volume. While it may be argued that repeatability (i.e., precision) is the most important parameter to hit for a test protocol on a liquid handling robot, **Figure 1** clarifies how measurement of only precision may lead to a significant, systematic error in assay results. The data presented in **Figure 1** shows the measured precision and accuracy of multiple iterative changes made to a liquid delivery protocol for a given robot, with testing after each change. The key item of note is that had precision alone been the benchmark of robot performance, testing would have stopped after experiment A, and the volume delivered would have been 6.8 µL instead of the expected 10 µL.

Figure 1: Precision measurements alone would not uncover the systematic under-dispense of this robot.



Proper tip pre-wetting requires pressure equalization

Air displacement is a commonly used pipetting mechanism employed by many types of handheld and robotic pipettors. For this method of pipetting, two key physical parameters have significant impact on the volume delivered. These parameters are temperature and pressure of the air mass entrapped in the pipette tip.[3] This captive air mass is used to aspirate and dispense the target liquid volume. Whereas temperature is particularly troublesome for handheld pipettes due to heat transfer from the technician's hand, this parameter is typically more easily controlled in robotic systems. However, the pressure of the air mass inside the tip can have great impact on volumes delivered by any air displacement pipetting device. This internal pressure is strongly affected by evaporation of molecules from the sample solution, which occurs during the pressure drop required to achieve aspiration. The error associated with this evaporation is described in **Table 2**.

Table 2: Typical Values for Captive Air Ratios

Pipette Volume (µL)	Liquid Volume Setting (V_l, mL)	Captive Air Volume (V_a, µL)	Ratio (V_a / V_l)	Error [b]
1000	1000	1500	1.5	$e_p = \left(\frac{V_a}{V_l}\right)\left(\frac{\Delta P}{P_l}\right)$
1000	100	1500	15	
20	20	100	5	
20	2	100	50	$e_n = \left(\frac{V_a}{V_l}\right)\left(\frac{\Delta n}{n_a}\right)$
2	2	40	20	
2	0.2	40	200	

[a] See Reference 3.
[b] e_p, e_n are liquid delivery errors due to changes in pressure and evaporation, respectively.

The captive air ratios in **Table 2** corroborate the general recommendation of appropriately sizing the tip and head for the desired volume. Also, while it may not be apparent, **Table 2** can be used to understand the need for pre-wetting pipette tips, at least in terms of releasing the captive air pressure resulting from evaporated liquid vapor. This phenomenon is demonstrated in the data above and to the right. A 96-tip air displacement pipetting head, using 30 µL tips was programmed to dispense 0.5 µL. The first attempt at delivering this volume (shown as **Plate 1 Statistics**) included no pre-wet step. Instruction was provided regarding the need to include a three cycle pre-wet step, which was added to the protocol, and a second plate was dispensed (see **Plate 2 Statistics** and **Plate 2 Volumes**). However, it was observed during the three cycle pre-wet step that the dispense cycle was being conducted while the tips were still submerged in the sample solution. The resulting "bad" data shows no benefit of the pre-wet steps because the captive air volume never had opportunity to release the increased internal pressure caused by liquid evaporation. This increased pressure caused the captive air volume to increase, and in turn the liquid volume to decrease. The protocol was changed again, this time including a pre-wet dispense cycle occurring with the tips in air, thereby allowing the internal pressure to be released. These test results can be seen in the **Plate 3 Statistics** and **Plate 3 Volumes** above, and fall within the performance specifications for the robot being tested (accuracy = 0.35-0.65 µL, precision = +/-0.100 µL or +/-20%). Comparison of the "heat map" of volumes dispensed in plate 2 versus plate 3 demonstrates a significant improvement in uniformity of volumes delivered across the entire plate. The take home message: for air displacement pipetting devices, the best accuracy and precision will be achieved using a pre-wet protocol wherein solution is fully aspirated into the tip, then fully dispensed with the tip removed from the solution.

Plate 1 Statistics: Full Plate Summary

Target Volume (µL)	0.5
Mean Volume (µL)	0.173
Relative Inaccuracy	-65.40%
Standard Deviation (µL)	0.087
Coefficient of Variation	50.29%

Plate 2 Statistics: Full Plate Summary

Target Volume (µL)	0.5
Mean Volume (µL)	0.219
Relative Inaccuracy	-56.20%
Standard Deviation (µL)	0.105
Coefficient of Variation	47.95%

Plate 3 Statistics: Full Plate Summary

Target Volume (µL)	0.5
Mean Volume (µL)	0.561
Relative Inaccuracy	12.20%
Standard Deviation (µL)	0.071
Coefficient of Variation	12.66%

Volume Legend:

Plate 2 Volumes (µL):

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.221	0.296	0.282	0.282	0.264	0.855	0.315	0.11	0.983	0.82	0.583	0.249
B	0.352	0.284	0.289	0.284	0.261	0.282	0.305	0.345	0.299	0.331	0.855	0
C	0.336	0.28	0.325	0.178	0.247	0.284	0.341	0	0.193	0.041	0.266	0.354
D	0.291	0.869	0.311	0.243	0.255	0.325	0.295	0.301	0.262	0.246	0.262	0.311
E	0.306	0.867	0.282	0.239	0.295	0.306	0.276	0.266	0.266	0.311	0.26	0
F	0.301	0.318	0.337	0.057	0.227	0.168	0.281	0.239	0.001	0.057	0.34	0
G	0.145	0.326	0.304	0.157	0.201	0.26	0.124	0.001	0.251	0.323	0.241	0.085
H	0.255	0.271	0	0.198	0.283	0.297	0.198	0.213	0.246	0.271	0.162	0

Plate 3 Volumes (µL):

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.523	0.583	0.572	0.536	0.562	0.551	0.577	0.581	0.575	0.622	0.583	0.548
B	0.597	0.625	0.618	0.569	0.543	0.587	0.595	0.64	0.573	0.631	0.895	0.607
C	0.618	0.597	0.618	0.529	0.634	0.585	0.629	0.581	0.531	0.572	0.557	0.598
D	0.595	0.62	0.623	0.597	0.569	0.615	0.588	0.681	0.573	0.587	0.599	0.579
E	0.596	0.559	0.529	0.528	0.545	0.605	0.615	0.563	0.557	0.598	0.608	0.576
F	0.599	0.598	0.617	0.527	0.55	0.551	0.596	0.622	0.536	0.596	0.662	0.588
G	0.492	0.585	0.616	0.52	0.583	0.526	0.49	0.56	0.548	0.641	0.549	0.49
H	0.505	0.566	0.494	0.552	0.597	0.613	0.599	0.52	0.545	0.603	0.548	0.508

How well should different robots running the same assay protocol agree?

A common practice in automated laboratories is to run a given assay protocol on a defined robotic platform. The logic supporting this practice is that different platforms (i.e., different makes/models of liquid handlers) may require different programming steps to achieve the same result. Hence, selecting one type of robotic platform, or one type of pipetting head, to run all assays of a given type makes logical sense. But, in practice, how well do different robots from the same manufacturer perform a similar test?

Table 3 provides data collected from four identical 96-tip air displacement pipetting heads run by four separate robots. Robot 2 was a different model than the other robots, but the pipetting head was the same. Each robot performed the assay defined on the left, wherein the Protocol Name includes the test volume. Details on assay steps for each protocol type is not given herein. Regardless, it is interesting to note the degree of variation between the four different robots presented. A particular note of interest is the fine detail showing the slope and offset values used to achieve the tabulated results. A determination of the sensitivity of liquid delivery on final assay results would be recommended to ensure this pool of robots could indeed be used interchangeably to perform some of these assays, particularly at lower volumes.

Table 3: Comparison of four identical 96-tip pipetting heads used to perform various protocols.

Protocol Name	Robot 1	Robot 2	Robot 3	Robot 4
5 uL, Protocol A				
Relative Inaccuracy	-4.96%	-5.46%	-1.92%	2.80%
CV	1.96%	1.59%	1.47%	1.44%
New slope & offset values	*0.977 & 0.612*	*1.171 & -0.890*	*0.948 & 0.714*	*0.898 & 1.143*
10 uL, Protocol A				
Relative Inaccuracy	7.00%	5.40%	-1.40%	3.30%
CV	0.56%	0.66%	0.91%	0.58%
New slope & offset values	*0.977 & 0.612*	*1.171 & -0.890*	*0.948 & 0.714*	*0.898 & 1.143*
30 uL, Universal Protocol				
Relative Inaccuracy	-2.30%	-0.07%	1.07%	2.77%
CV	0.44%	0.57%	0.49%	0.65%
New slope & offset values	*1.08 & -1.36*	*1.05 & 0.19*	*1.053 & 0.677*	*not changed*
50 uL, Universal Protocol				
Relative Inaccuracy	0.00%	2.22%	-0.64%	1.94%
CV	0.60%	0.86%	1.13%	0.71%
New slope & offset values	*1.08 & -1.36*	*1.05 & 0.19*	*1.053 & 0.677*	*not changed*
100 uL, Universal Protocol				
Relative Inaccuracy	1.20%	0.70%	1.60%	0.99%
CV	0.59%	1.09%	0.49%	0.59%
New slope & offset values	*1.08 & -1.36*	*1.05 & 0.19*	*1.053 & 0.677*	*not changed*
100 uL, Protocol B				
Relative Inaccuracy	0.50%	3.70%	-3.10%	0.20%
CV	0.80%	0.87%	0.72%	0.60%
New slope & offset values	*1.013 & 4.559*	*1.0 & 0.1*	*not changed*	*not changed*
200 uL, Protocol B				
Relative Inaccuracy	0.65%	5.20%	-1.75%	2.95%
CV	0.55%	0.62%	0.56%	0.44%
New slope & offset values	*1.013 & 4.559*	*1.0 & 0.1*	*not changed*	*not changed*

Best accuracy at each target volume is highlighted.

How far will my protocol take me?

Whether a liquid delivery robot allows for "high level" programming only, or allows a user to program all aspects of robot movement, a key question that should be asked is, over what volume range will a given protocol be applicable? For some types of liquid delivery robots, users may be unfamiliar with control features such as "liquid classes", "slope and offset", or other adjustments available to hone protocol steps for optimal performance over specified volume ranges. However, it is important to recognize that whatever the robot may be, and whatever protocol may be running, there will be a volume limit beyond which a given set of parameters no longer apply.

Figure 2: Volume delivery achieved using different liquid delivery protocols.

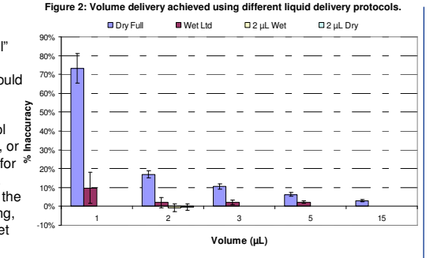


Table 4 demonstrates this principle quite well. For this single channel robot (which allows very little "low level" programming), a given test protocol performs very well from 200 µL to 20 µL. However, this same protocol "falls out of bed" when used for delivery at 2 µL. **Figure 2** demonstrates data for this same robot but using different liquid delivery protocols. The "Dry Full" protocol is the same as used for **Table 4** data. Three other protocols were tested, with the best results for the 2 µL volume coming from protocols specific to that volume (both dry and wet dispenses achieved acceptable results).

Table 4: Comparison of volume delivery performance using the same delivery protocol over a large volume range.

Target Volume (µL)	Tip Size (µL)	Number of data points per channel	Mean volume for all Channels (µL)	Relative Inaccuracy for all Channels	Standard Deviation for all Channels (µL)	Coefficient of Variation for all Channels
2	50	8	2.2352	11.76%	0.0528	2.36%
20	50	8	20.1546	0.77%	0.2486	1.23%
40	50	8	40.2845	0.71%	0.3638	0.90%
55	200	8	55.6068	1.10%	0.6258	1.13%
100	200	8	100.7002	0.70%	0.7351	0.73%
200	200	8	200.2775	0.14%	1.1606	0.58%

Delivery mode: Forward vs. Reverse?

Forward versus reverse mode pipetting has been a question discussed among the handheld pipetting community for some time now. It is recommended that the overall best pipetting of aqueous solutions for handheld pipettes is achieved using forward mode pipetting. However, is this the same for robotic pipettors?

The data presented in **Figure 3** and **Figure 4** illustrates the performance differences between these two commonly used liquid handling methods when implemented using a 96-tip air displacement device.[4] By comparing the precision and accuracy information directly, it can be concluded that liquid transfer accuracy is highly method dependent, especially at low volumes. In the case presented here, the reverse mode method was more accurate at the low volumes as compared to the direct, or forward mode method. The instrument's volume transfer precision, however, is much less dependent on method. It is only possible to make the comparison of the two methods when the accuracy and precision parameters are simultaneously measured as they are with the MVS. Thus, for this particular device using this particular protocol, the reverse mode is preferred.

Figure 3: Inaccuracy of Forward and Reverse mode pipetting over various volumes.

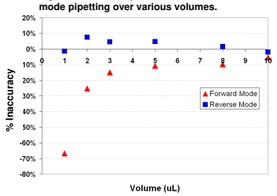
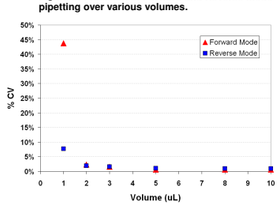


Figure 3: Precision of Forward and Reverse mode pipetting over various volumes.



Conclusions

Automated testing is a reality in today's laboratory environs. Modern laboratory tools such as automated liquid handling devices have greatly impacted the amount of testing that can be performed. However, such advanced equipment cannot be thought of as a "black box" that always produces the correct results. Getting the most out of automated liquid handling devices requires careful observation, and thoughtful control of key aspects. While such control points may be better suited to an engineering mind set, today's laboratory personnel cannot shirk the responsibility of ensuring that their test results have not been negatively impacted by the "black box".

The key message of this poster, and the poster from Reference 1 is that ultimate control over assay integrity *must* include an understanding of how your robotic pipettor is performing all steps throughout your assay. The examples provided herein show some important aspects of a delivery protocol that should be considered. The bottom line is that an automated liquid handling device need not be that "black box", but should be the well-controlled tool that does your bidding.

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