

*BioRobotics***IMPROVING ACCURACY BY USE OF TECHNIQUE CALIBRATION**

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Introduction

For any given assay, many laboratory and environmental conditions can contribute to the accuracy of dispensing with an automated liquid handler. Among the obvious are liquid type, technique, solution retention in the tips, laboratory humidity, and laboratory temperature. When determining the accuracy of a liquid handler, it may not be suitable to check accuracy gravimetrically. For volumes less than 10 μL , difficulties with gravimetric measurements are encountered due to volume loss through evaporation. As a result, it is desirable to use an indirect, spectrophotometric method to determine the accuracy of low-volume dispenses. This method can be performed for any volume and in any lab with access to a spectrophotometer. Assuming that the precision of a particular technique has been optimized to meet the requirements of an assay, then the accuracy of the dispense will be a matter of calibration. The Biomek[®] FX software offers a feature to manipulate the Scaling Factor and Offset of the volume displaced inside the mandrels for any particular technique, thus giving the user a method for adjusting the accuracy of the dispensed volume.

This Technical Bulletin will show the steps involved in improving the accuracy of the dispensed volume by using the calibration feature of the Biomek FX software. To accomplish this, an introduction to Techniques and the Technique Browser will be presented, followed by a more in-depth look at the Calibration tab within the Technique Editor. A detailed description of the process used to calibrate a pipetting technique will then be shown by example.

Briefly, the process of calibration involves:

1. The creation of a standard curve with a calibrated hand pipettor.
2. The Biomek FX is used to pipette the desired volume using the selected technique with the default Scaling Factor and Offset values of 1 and 0, respectively.
3. The actual volume dispensed is calculated by comparison of the $A_{510/650}$ from the plate dispensed with the Biomek FX to the standard curve.
4. A graph of the desired dispense volume vs. the actual volume dispensed is created and the slope and Y-intercept are determined.
5. These calculated values replace the default values for the Scaling Factor and Offset, respectively, in the technique.
6. Another dispense is performed with the Biomek FX using the same technique with the new Scaling Factor and Offset and determine if the accuracy requirement of the assay has been met.

Techniques and Technique Browser

Biomek FX makes use of Techniques within its software to control all the functions associated with pipetting. This use of techniques to control pipetting allows frequently used settings to be saved and reused in other methods rather than having to make the same modifications to each pipetting step within a method. The Biomek FX software, by default, selects the most appropriate technique to use for each pipetting step when a step is created. If necessary, changes can be made to a selected technique

in an individual step of a method by selecting the appropriate technique and then using the Customize button. This ability to use saved techniques, yet still modify parameters in a step or select a different technique altogether, provides the maximum flexibility and precise control of the pipetting process with the Biomek® FX. Some basic techniques and pipetting templates are provided with the Biomek FX software; however, users have the flexibility of using them as provided, modifying them, or creating new techniques and pipetting templates to meet the individual needs of any application or assay.

All criteria associated with the selection of the appropriate pipetting technique are set within the Technique Browser which can be accessed from the Tools menu of the Biomek FX software. When the Technique Browser is selected, a list of all available techniques, predefined and custom, that have been created are listed. Highlighting the name of a technique and selecting the Properties button will show which parameters have been designated as selection criteria for that technique. A technique may be created specifically for a single piece of labware or it may be used for any pipetting with the multichannel head. Double-clicking on a technique name or highlighting the technique and selecting the Edit button accesses the Technique Editor. In the Technique Editor, a drop-down list is used to select a pipetting template and six or seven tabs are displayed that are used to set values for the aspiration, dispense, mix, liquid level sensing, for Span-8 pod only, liquid type, and calibration. For this bulletin, we are concerned only with the Calibration tab. Additional information on the topic of creating and modifying techniques in the Technique Browser and Technique Editor can be found in the Biomek User's Manual or the Biomek FX Help which is found by accessing the Help menu of the Biomek FX software.

Calibration Tab

The Calibration tab of the Technique Editor (Figure 1) shows the basic formula and the text boxes that allow one to change the Scaling Factor and Offset Volume of the calibration curve. The ability to manipulate these values allows a user to adjust the accuracy of the pipetting operations performed by the Biomek FX. If accurate, as well as precise, pipetting of a solution is critical to an assay, calibration may be necessary to compensate for properties, such as viscosity, of the solution. Additionally, the minimum pipetting height for the technique is set within this tab. As shown in Figure 1, the calibration equation is as follows:

$$V_{\text{displaced}} = V_{\text{desired}} * (\text{Scaling Factor}) + (\text{Offset Volume})$$

where

$V_{\text{displaced}}$ = Volume displaced in the mandrel;
equal to V_{desired} with Scaling Factor of 1
and Offset of 0

V_{desired} = Desired Volume
(the volume set in the method)

Scaling Factor = Slope, multiplier of V_{desired} used achieve an accurate dispense volume

Offset = Y-intercept, a fixed amount of overage which is pipetted

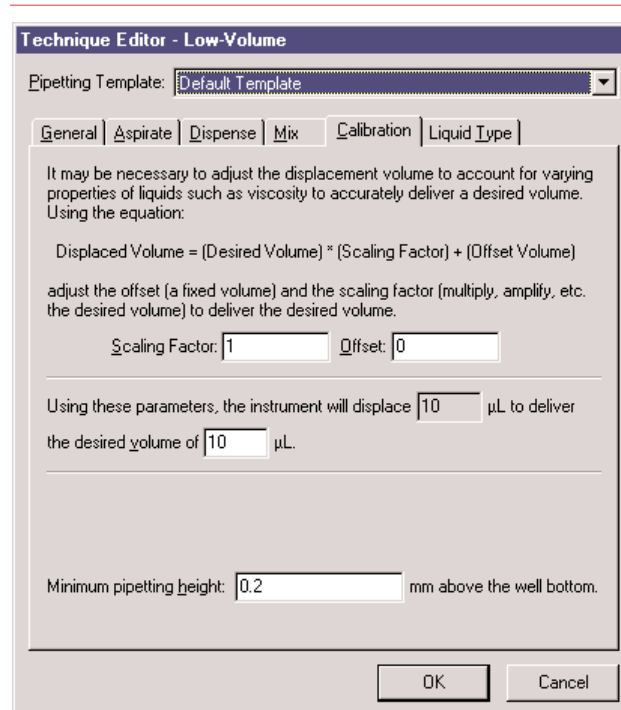


Figure 1. The Calibration tab within the Technique Editor of Biomek FX v2.1 software.

Calibration Process

In the following example, the technique was optimized with respect to precision for low-volume pipetting (0.25 to 1.0 µL) on a Biomek FX 96-Channel Disposable Tip Pipetting Head (20 µL) using Biomek AP96 P20 tips. Ideally, the Biomek FX should deliver the volume entered into the software accurately as well as precisely. Unfortunately, with low-volume dispensing, there are many factors such as viscosity of the solution, laboratory temperature, solution temperature, and laboratory humidity that make accurate low-volume pipetting difficult. Even though the precision was within specification, the dispensed volume was inaccurate by as much as 50% for a 1.0-µL dispense. The requirement for the assay was to achieve accuracy of ±10% at 0.25 µL and ±5% at 1.0 µL.

Standard Curve

In order to determine the actual volume dispensed by the Biomek® FX with the selected technique, a standard curve was created with a hand pipettor to use as a means of comparison. To create the standard curve, an operator using the Artel PCS® Pipette Calibration System calibrated a hand pipettor for volumes of 5, 10, 15, 20, and 30 μL . To perform the hand pipetting for the standard curve used in the calibration process, the pipette operator was required to have a coefficient of variation (CV) of 1.5% or less, with regard to precision, at each volume. Each laboratory can establish precision requirements depending on the assay requirements, the skill of the pipette operator, and the volumes pipetted. The actual volumes pipetted by the operator with the hand pipettor were found to be 4.97, 9.89, 14.67, 19.65, and 29.70 μL , respectively. If a pipette calibration system is not available, an assumption can be made that the pipette is delivering the volume specified if it has been calibrated and maintained according to good laboratory practice (GLP) standards and the operator is qualified by meeting established precision requirements. An aqueous solution of Eosin Y, disodium salt (Sigma, cat. no. E6003), was made to a concentration that delivered the maximum absorbance (A) at a dispense volume of 2 μL for the linear range of the plate reader, a SPECTRAmax® PLUS³⁸⁴. A surfactant, EDTA (Sigma, cat. no. E5134) at 5.0 g/L, was added to the solution. This 2 μL dye solution, used for pipetting with the Biomek FX, was diluted 1:20 using volumetric flasks, thus making a 40 μL dye that was used for creation of the standard curve. The standard curve can also be created by pipetting the desired dispense volumes, 0.25 to 1.0 μL , with a hand pipettor. However, it is difficult to pipette precisely with a 0.1 to 2.0 μL hand pipettor and many labs do not have access to this pipettor. The dilution method is presented as an alternative to pipetting small sample volumes. The dispensed samples were measured at 510 nm. In addition, a reading is taken at 650 nm to determine background. This background value is subtracted from the value obtained at 510 nm for all calculations. When the dispenses are normalized to the same final volume in the well, 100 μL , with water, the following physical property is true:

When $A_{510/650}(40 \mu\text{L}) = A_{510/650}(2 \mu\text{L})$

then $V_{40 \mu\text{L}} = 20 * V_{2 \mu\text{L}}$

Furthermore, the graph of $A_{510/650}(40 \mu\text{L})$ vs. $V_{40 \mu\text{L}}$ is linear (Figure 2), given in the form of

$$A_{510/650}(40 \mu\text{L}) = m * V_{40 \mu\text{L}} + b$$

where $m = \text{slope}$

$b = \text{Y-intercept}$

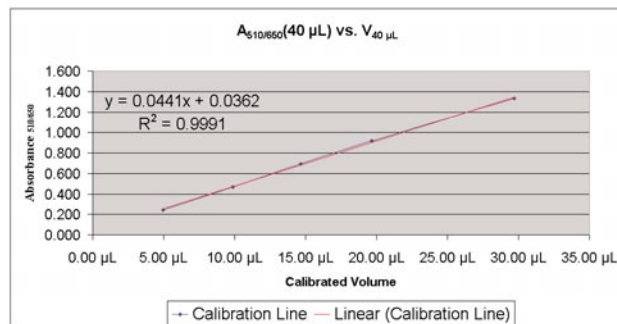


Figure 2. Standard curve made with a hand pipettor from 5.00 to 30.00 μL .

Initial Dispense Assay

Once the standard curve has been created, the next step of the process is to pipette with the automated liquid handler to create the curve of $V_{\text{displaced}}$ vs. V_{actual} for the optimized, with respect to precision, technique using the default calibration settings, a Scaling Factor of 1 and an Offset of 0 μL . Several volumes over the range covered in the calibration should be dispensed for the initial assay. The desired dispense range for this assay was 0.25 to 1.0 μL . Previous experimentation established that the assay conditions tested here require displaced volumes between 0.8 μL and 1.4 μL to yield actual volumes in the desired range of 0.25 μL to 1.0 μL .

The assay consisted of three plates pipetted at each desired volume. As a result of using the default calibration settings, the desired volume is equal to the displaced volume. From the $A_{510/650}$ values in the standard curve and the mathematical relationship described previously between the absorbance of the dye used for the assay and the dye used for the standard curve, one can use the $A_{510/650}(40 \mu\text{L})$ vs. $V_{40 \mu\text{L}}$ curve to calculate the actual volumes dispensed from the unit. The $A_{510/650}$ value used in the calculation of V_{actual} is the average $A_{510/650}$ across all wells of the three plates dispensed on the automated liquid handler.

The calculations are as follows:

$$A_{510/650}(40 \mu\text{L}) = 0.0441 * V_{40 \mu\text{L}} + 0.0362$$

And when

$$A_{510/650}(40 \mu\text{L}) = A_{510/650}(2 \mu\text{L})$$

then

$$V_{40 \mu\text{L}} = 20 * V_{2 \mu\text{L}}$$

Therefore

$$A_{510/650}(2 \mu\text{L}) = 0.0441 * (20 * V_{2 \mu\text{L}}) + 0.0362$$

So

$$V_{2 \mu\text{L}} = (A_{510/650}(2 \mu\text{L}) - 0.0362)/0.882$$

Although the standard curve shown only includes volumes from 5 to 30 μL using the 40- μL dye, or 0.25 to 1.5 μL for the 2- μL dye, experimental values for actual volumes falling outside that range can be calculated due to the linearity of the standard curve.

The data from the initial dispense assay with the Biomek[®] FX using the default settings for the Scaling Factor and Offset, 1 and 0, respectively, is shown in Table 1.

Slope and Offset Calculation

Since the objective is to obtain $V_{\text{actual}} = V_{\text{desired}}$, a new graph is created for $V_{\text{displaced}}$ vs. V_{actual} using the data from Table 1. The values from the graph shown in Figure 3 for slope and Y-intercept are used as the new Scaling Factor and Offset, respectively, in the Technique Editor.

$$\text{Scaling Factor} = 0.808 \quad \text{Offset} = 0.470$$

Once the default values for Scaling Factor and Offset are replaced by the experimentally determined values in the Technique Editor, the software will calculate the new values for $V_{\text{displaced}}$ automatically. The assay is repeated and V_{actual} is calculated in the same manner as previously described using the standard curve.

Table 1. Actual Volume Dispensed by the Biomek FX as Determined by Comparison of OD Values to the Standard Curve

Desired Volume	Displaced Volume	Average $A_{510/650}$ (2 μL Dye)	Actual ($V_2 \mu\text{L}$) Volume	Accuracy
0.80 μL	0.80 μL	0.387	0.398 μL	-50.3%
1.00 μL	1.00 μL	0.625	0.667 μL	-33.3%
1.20 μL	1.20 μL	0.843	0.915 μL	-23.8%
1.40 μL	1.40 μL	1.041	1.139 μL	-18.6%

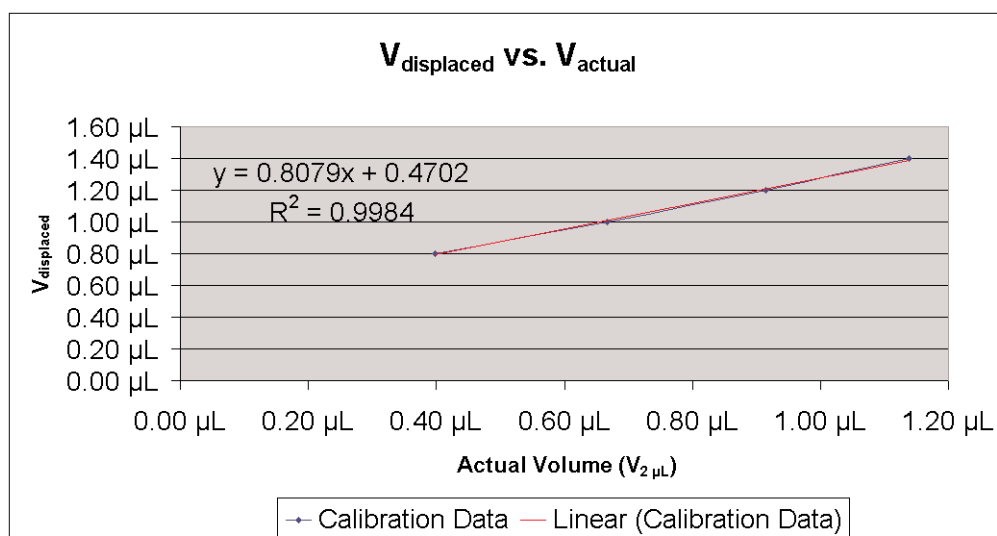


Figure 3. Graph of displaced volume vs. actual volume as calculated in Table 1.

Since the accuracy requirement has been achieved, further iterations of the calibration process will not be performed. If the accuracy requirement had not been met, $V_{\text{displaced}}$ vs. V_{actual} would be graphed again using the data from Table 2. A new Scaling Factor and Offset would be calculated from the graph, Figure 4, and these values, 0.762 and 0.480, would be substituted for the current Scaling Factor and Offset, respectively, in the Technique Editor.

Another assay would be performed and the accuracy would be determined in the same manner as previously described. If, after multiple iterations, the accuracy requirement cannot be met for all volumes in the dispense range, multiple techniques, with specific volume ranges, can be created in the Technique Browser. One could create several techniques with contiguous volume ranges such as 0.25 to 0.5 μL , 0.5 to 1 μL , and 1 to 1.5 μL . With each technique, the same pipetting template and parameters could be used with individualized calibration numbers, Scaling Factor, and Offset, for each range. These features offer maximum flexibility for improving the accuracy of pipetting on the Biomek® FX.

Table 2. Actual Volume Dispensed after Using Experimentally Determined Scaling Factor and Offset

<i>Desired Volume</i>	<i>Displaced Volume</i>	<i>Average $A_{510/650}$ (2 μL Dye)</i>	<i>Actual (V_2 μL) Volume</i>	<i>Accuracy</i>
0.25 μL	0.67 μL	0.250	0.242 μL	-3.1%
0.50 μL	0.87 μL	0.503	0.529 μL	5.8%
0.75 μL	1.08 μL	0.734	0.791 μL	5.5%
1.00 μL	1.28 μL	0.952	1.037 μL	3.7%

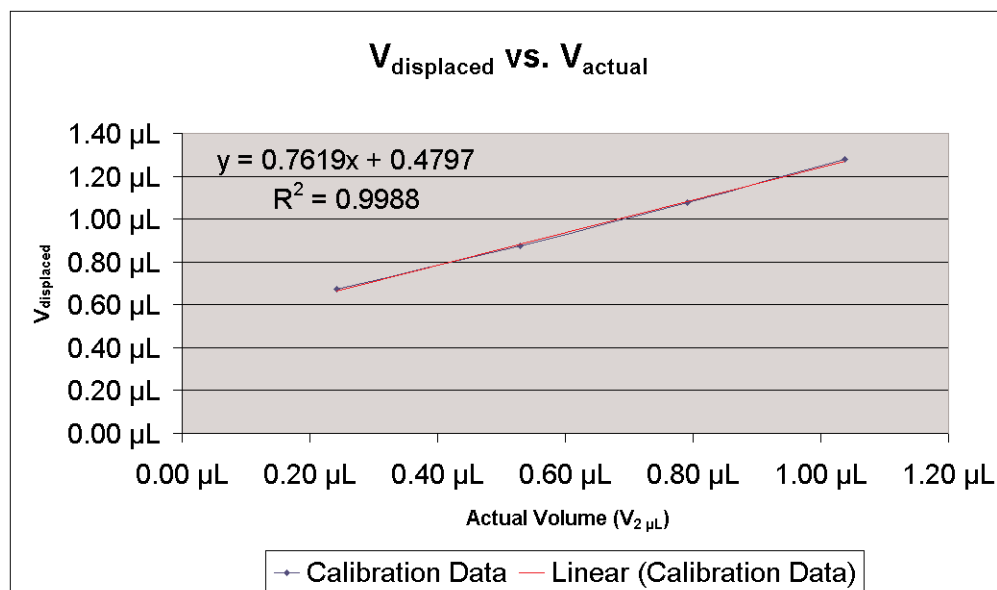


Figure 4. Graph of displaced volume vs. actual volume as calculated in Table 2.

Conclusion

This bulletin demonstrates, by example, the calibration feature of the Biomek® FX software by presenting the results of an actual dispense assay before and after modifying the calibration settings to improve the accuracy of the volume dispensed. Any laboratory with access to a spectrophotometer can use this same method to calibrate a predefined or custom pipetting technique that is used in any assay automated with the Biomek FX. This calibration procedure, as demonstrated, is flexible enough to be used with any volume, any solution, any dye, and any type of labware that is compatible with the spectrophotometer. The ability to adjust the accuracy of pipetting with the Biomek FX is just one of the many features which the Biomek FX software offers to give the user precise, yet flexible, control of all aspects the pipetting process. Other parameters that control and can be modified to improve pipetting for a particular assay can be found within the Technique Browser, Technique Editor, Liquid Type Editor, and Pipetting Template Editor.

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