Single Channel Pipette Calibration and Operator Competency Assessment Using a Dual-Dye Ratiometric Photometry System

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Introduction

Handheld pipettes are the workhorse of most life sciences laboratories, and they are among the most ubiquitous laboratory instrumentation. They are the single most frequently-utilized of all calibrated laboratory instruments, yet they are often overlooked as potential sources of inaccuracy and imprecision in method development and validation. Proper pipette performance is essential to the generation of data that are both reliable and reproducible – two hallmarks of a laboratory’s reputation and success.

The success of analytical method development and validation, as well as routine assay execution is reliant upon two key requirements: first, that the instrumentation designated for the method is functioning properly1 and second, that the operators performing the development/validation are properly trained2,3. While instrument and operator qualification are regularly addressed in regulated laboratory environments, simple instruments such as handheld pipettes have a propensity to be overlooked, and their potentially compromising effects on data quality are often not considered. Many laboratories work under the assumption that operators will exercise good pipetting techniques without subjecting them to a specialized training and subsequent competency assessment program2,3. Similar to automated liquid handling platforms, pipettes can fail “silently” and unpredictably meaning their failure can potentially go undetected between calibration or preventive maintenance cycles. In fact, 90% of pipette failures are unexpected or unpredicted4. Providing pipetting technique training with regular retraining and competency assessments for operators, and regular performance verification/calibration of handheld pipettes are both considered to be best practices for minimizing error and improving data quality in method validation1. Training and routine assessment is essential for analysts performing routine assays. In this situation, samples are typically limited and time is of essence. Furthermore, operator training and ongoing pipetting practice ensures standardization among operators while regular calibrations and interim verifications identify malfunctioning pipettes in a timely manner and minimize the potential for compromised data.
Establishing the ideal pipette calibration frequency for a laboratory requires consideration of several factors. First and foremost, regulatory organizations such as the U.S. Food and Drug Administration (FDA) provide foundational guidance as to minimum requirements for instrument calibration. In addition to that, laboratories are encouraged to assess the Mean Time Between Failure (MTBF) and the target reliability level for liquid delivery for their particular laboratory and pipettes. MTBF is the average rate at which pipette failures occur, and can be determined by tracking a group of pipettes and noting how long it takes for each one to fail. Target reliability is expressed as a percentage; for example: 95% reliability indicates that 95% of the pipettes in a laboratory population are working correctly at any given time. By considering MTBF and target reliability for a pipette population, the example graph below (Figure 1) can be used to determine the suggested calibration frequency for a particular lab.

![Figure 1](image)

**Figure 1. Determination of calibration frequency of pipettes, based on MTBF and targeted reliability.**

For example, a set of pipettes with a MTBF of 4 years and a target reliability level of 95%, calibrating every 2 months is recommended in order to maintain the 95% reliability level in the laboratory, as indicated by the black arrow in Figure 1. This is a considerably shorter time frame than the typical calibration frequency of 6-12 months practiced by many laboratories. In this case, the analyst would perform interim verifications (e.g., weekly, daily, or before use) to ensure no silent failure has happened since the last calibration. Incorporating an interim verification substantially increases the reliability level. Interim verification is especially important when considering most labs either send pipettes away to a metrology lab or a third party service provider to satisfy calibrations and preventive maintenance requirements. Interim verifications can be performed in the same lab where the pipettes are used.
Performance Verification Methods for Handheld Pipettes

Performance verification of pipettes typically involves dispensing several replicates of liquid aliquots and measuring the volume of each dispensed aliquot. There are three widely accepted methods for pipette calibration and performance verification: gravimetric, photometric, and titrimetric methods. In gravimetric performance evaluation of pipettes, volumes of dispensed liquid are weighed on a balance in accordance with either ASTM or ISO standards. The weight of the liquid reported by the balance is then converted to mass, which can be used to calculate volume using known conversion factors for the specific liquid. Typically, deionized water is used in this method, and balances are readily available in a typical life sciences laboratory, making the gravimetric method a popular choice among performance verification methods.

There are, however, several disadvantages to gravimetry, and these must be considered before the method is adopted for use in a laboratory environment. While gravimetric methods are generally reliable for larger volumes (>100 µL), accurate measurements of smaller volumes, such as micro- or nanoliter quantities, can be more problematic due to their propensity for rapid evaporation and vulnerability to a variety of environmental conditions. To ensure accurate measurement of microliter volumes, aliquots must be weighed on extremely sensitive balances, which can be affected by even the smallest of vibrations, drafts, electrostatic charges, or changes in the environmental conditions of the test room. These balances are typically housed on immobile marble weighing tables in a tightly controlled laboratory, meaning pipettes must then be removed from the lab or site where they are regularly used, transferred to the location of the balance, and equilibrated to the test room conditions for performance verification. This is in direct opposition to the recommended practice of calibrating pipettes in the lab where they are regularly used in order to maximize accuracy and precision and to minimize the impact of environmental changes. Furthermore, highly sensitive balances must be allowed sufficient time to settle after a sample dispense, before making the measurement, thereby decreasing the efficiency of this method and introducing a higher potential for error. Unfortunately, the balance settling time is user-defined and can be set to very short times, which has a direct effect on measurement uncertainty. While gravimetric analysis is certainly feasible, testing in the laboratory environment where the pipette will be used represents the true performance of the pipette.

Photometric calibration is another common method for evaluating pipette performance. In general, photometry relies upon the principle of absorbance of light and a known concentration of a chromophore (dye) in solution that absorbs light in the visible or UV spectrum. The pipette is used to deliver an aliquot of the dye to a receiving vessel; the exact delivered volume of which is considered to be an unknown. The absorbance change in the receiving vessel is read spectrophotometrically, and the volume of the aliquot can then be determined using mass balance calculations.

Instrumentation required for the photometric methods include a suitable UV-vis spectrophotometer, a measuring cell with known pathlength, a thermometer, and in case dye solutions are to be prepared in-house, an analytical balance and Class A volumetric glassware.
Approved methods of photometric calibration employ either one dye solution (single-dye photometry) or two (dual-dye ratiometric photometry). In single-dye photometry, a dye solution is dispensed into a receiving vessel (cuvette, microplate well, or measuring cell) containing a colorless diluent solution. Absorbance is analyzed, and the amount of light absorbed is proportional to the dye concentration allowing for the volume of dye added to be calculated using the principle of the Beer-Lambert Law. Single dye photometry is indeed precise and is less prone to environmental effects than gravimetry, however the use of two standardized dye solutions in ratiometric photometry allows for a higher degree of traceability, accuracy, and precision; even at extremely small liquid volumes\(^9\). For proper calibrations and verifications, photometric methods require well-characterized receiving vessels, calibrated and stable dye solutions, and a calibrated spectrophotometer and a calibrated analytical balance, some of which may not be readily available in every lab. Therefore, in-house photometric methods may be less cost effective than gravimetric methods for laboratories routinely measuring larger volumes, particularly greater than 100 µL. However, the precision and accuracy achieved when measuring small volumes, coupled with the cost effectiveness of quality data ultimately resulting from implementation of photometric calibration methods is unrivaled by gravimetry.

Artel’s Pipette Calibration System (PCS\(^a\)) employs the principle of dual-dye ratiometric photometry for immediate volume verification. The PCS Software allows for rapid and accurate volume measurement of aliquots dispensed by pipettes and is ideal for on-the-spot pipette verification (e.g., immediately before use, if the pipette is suspected of being damaged, has been soiled, then cleaned, etc.) as well as assessment of operator competency. Since the PCS can be employed in the analyst’s laboratory, pipette failures or analyst pipetting performance issues can be identified much quicker. When the proper dyes are used, as recommended in ISO 8655 Part 7, calibration by the dual-dye ratiometric photometry method offers a number of advantages over gravimetric calibration and is capable of delivering results with less than or equal to 0.6% inaccuracy and 0.3% CV, for volumes spanning 0.1 µL to 5000 µL.
Principles of the Beer-Lambert Law

The Beer-Lambert Law states that when light is passed through a solution containing a chromophore, there is a linear relationship that exists between the concentration of the chromophore in solution and the amount of light energy the solution can absorb. Mathematically, the law is expressed as:

$$A_\lambda = \varepsilon_\lambda lC$$

In this equation, $A_\lambda$ represents the absorbance of the chromophore at a specific wavelength, or $\lambda$. The molar extinction coefficient of the chromophore at the designated wavelength is represented by $\varepsilon_\lambda$. $l$ is the pathlength of the light traveling through the solution, and $C$ is the concentration of the chromophore in solution. Therefore, the absorbance of the chromophore at a certain wavelength is directly proportional to the product of the extinction coefficient, the pathlength, and the concentration of the chromophore.

A common application for this principle is the determination of an unknown concentration. When both the pathlength and the extinction coefficient are known, the equation can be manipulated to solve for the concentration of the chromophore in solution by reading its absorbance at a certain wavelength.$^\dagger$

Dual-Dye Ratiometric Photometry

Artel’s PCS employs the principle of dual-dye ratiometric photometry with proprietary dye chemistry to offer rapid, user-friendly, traceable, and robust volume measurement. This method of pipette calibration, as described in ISO 8655-7, is recognized for its precision, accuracy, and robustness of measurement.$^\dagger$ The absorbance of the two dyes (red and blue, with absorbance maxima at 520 nm and 730 nm, respectively) are examined under defined conditions and applied to the Beer-Lambert Law to generate data that is ultimately indicative of both the accuracy and precision of a handheld pipette. In dual-dye ratiometric photometry, the closely-controlled concentrations of both the red and blue dyes are known, and the molar extinction coefficients for the dyes are known and constant. The absorbance per unit pathlength can be obtained for each dye, allowing the volume of red dye dispensed by the pipette (and thus its accuracy and precision) to be calculated.$^\dagger,12-15$
PCS Components

The PCS from Artel is designed to be compact and portable. Its small footprint facilitates not only its use in any laboratory, but also its transport between laboratories allowing for pipettes to be calibrated in the exact location where they are used. Results provided by the PCS are traceable to the SI through NIST and NPL standards, and pipette calibrations performed with the PCS conform to a variety of regulatory requirements such as CLIA and CAP. The PCS is frequently used in laboratories complying with 21 CFR Part 11, as well as laboratories accredited to various clinical, calibration, testing, and quality management standards developed by AABB, CAP, CLSI, and ISO. The components of the PCS system are listed below:

1. The Artel PCS instrument reliably reads and reports absorbance at 520 nm and 730 nm. The instrument has an onboard barcode scanner, automated open/close lid, and vial-mixing capabilities.

2. Artel PCS Software drives the PCS and allows for scheduling of pipette calibrations, interim performance verifications, pipette inventory management, pipette operator training and competency assessments, and documentation management. The software facilitates compliance with 21 CFR Part 11 through the implementation of electronic signatures, audit trail, unique user accounts with passwords, and secure databases. It also allows e-mail notifications, customized user level controls, and a fully digital review, approval and sign-off process.

3. A laptop computer compatible with PCS Software allows the PCS instrument to be easily transported from lab to lab. The software can also be deployed on a network, which facilitates remote access, reporting, and review by quality management.

4. A label printer is included for generation of barcode and pipette calibration labels.

5. PCS sample solutions accommodating volume ranges from 0.1 µL to 5,000 µL (Table 1). The results from using the PCS sample solutions are traceable to the International System of Units (SI) through NIST and NPL standards.

6. The PCS Instrument Calibrator Kit allows for quick and easy instrument calibration by the user. The Calibrator Kit contains 4 vials of different calibration solutions that remain sealed and are reusable so that the user can calibrate the PCS as frequently as needed. Since the PCS can be easily calibrated on site there is no need for a technical specialist to perform the calibration.

<table>
<thead>
<tr>
<th>Sample Solution</th>
<th>Pipette Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200 µL - 5,000 µL</td>
</tr>
<tr>
<td>2</td>
<td>50 µL - 199 µL</td>
</tr>
<tr>
<td>3</td>
<td>10 µL - 49.9 µL</td>
</tr>
<tr>
<td>4</td>
<td>2.0 µL - 9.9 µL</td>
</tr>
<tr>
<td>5</td>
<td>0.50 µL - 1.99 µL</td>
</tr>
<tr>
<td>6</td>
<td>0.10 µL - 0.499 µL</td>
</tr>
</tbody>
</table>
PCS Workflow

The operation of the PCS follows a facile workflow that can be completed within minutes. Guided by easy-to-follow instructions, the entire process can be seamlessly introduced into any laboratory environment. The basic workflow includes five elements (e.g., select pipette, select task, scan reagents, dispense sample, and review results), where the basic process is shown in Figure 2.

- Identify the pipette in the software
- Scan barcode on pipette

- Select the task: calibrate, train/assess, or measure volume
- Specify the test volume

- Scan Blank (blue) solution
- Scan Sample (red) solution

- Dispense Sample (red) solution
- Obtain real-time results

- Digitally review, approve, and sign off on calibration data and reports

Figure 2. PCS workflow summary.
**PCS Applications**

The PCS has several useful applications that can be performed in any laboratory, depending on needs.

**Calibration** is traditionally performed by an in-house metrology lab or by a third party service provider. This function is performed at routine intervals by a qualified specialist during, and sometimes as part of preventive maintenance. The calibration procedure typically consists of a performance check (as-found calibration), followed by device maintenance and adjustment, if needed, then the final as-left calibration. This is often an iterative approach.

**Training and competency assessment** is a necessary mechanism to ensure standardized pipetting technique across various pipette operators and labs within an organization. Training is especially important for new employees regardless of experience. Additionally, the PCS is used to verify operator competency, which usually occurs at regular, pre-scheduled intervals. It can also be administered as a result of increasing assay variability, or as part of a CAPA (corrective and preventive action), if within a cGMP regulated laboratory. By taking a proactive stance and establishing a training and competency assessment program, regardless of whether an issue occurred, the cost of failure is minimized. Furthermore, a training and competency assessment program makes users constantly aware of the importance of pipetting technique, thus improving the likelihood of assay success. An example of the importance of training is shown in Figure 3. This example highlights the results of two short course cohorts, where the participants pipetted as they normally would without guidance (Pre-Training). After evaluating results and discussing proper technique, the same two cohorts pipetted again, with instructor guidance (Post-Training). As can be seen from these results, there is a drastic improvement in pipetting performance regardless of experience.

**Interim performance verifications** can be used to supplement full calibration programs. By performing verifications between calibration cycles, users can spot pipette malfunctions as early as possible, thus shortening the time a corrective action needs to take place for non-conforming performance. Interim performance verifications are especially important in regulated labs, where the cost of failure is higher. Interim performance verifications can also be used to determine calibration frequency, and results of interim verifications often become part of a pipette’s official performance record.

**Figure 3. Effect of training on pipetting performance for two different cohorts of industry short course attendees (●Cohort 1 and ●Cohort 2).**
Quick check is an application that is often used in many laboratories, whereby a user checks the performance because something is suspect. Examples include dropping a pipette on the lab bench or floor, trying new pipette tips, routine assay results show an out of specification trend, etc.

Summary

This whitepaper presents the dual-dye ratiometric photometry technology behind the industry-leading Artel PCS. The PCS platform provides a fully integrated and streamlined approach to pipette calibrations, interim verifications, operator training and competency assessments, as well as results that are traceable to the SI through NIST and NPL standards. The workflow includes an easy-to-follow software interface and allows an all-digital review and approval system and allows for seamless integration into any regulated environment which uses handheld pipettes. The PCS facilitates a laboratory’s compliance with all regulatory requirements for pipette calibration, interim verification, operator training and competency assessments, as well as electronic record keeping according to 21 CFR part 11. The PCS assists the laboratory manager in maintaining an audit-ready state of pipette calibration and operator training documentation, and by increasing confidence in the laboratory’s data integrity.
References


Artel designs, develops, manufactures, distributes and supports analytical systems including instruments, consumables and software for quality assurance applications in laboratory liquid handling. The Company also provides related training and on-site services.

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