Best Practices for the Use of Micropipettes

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Introduction

Mechanical action micropipettes are ubiquitous in laboratories and are used for many routine tasks, including the quantitative measurement and dispensing of analytical samples and reagents.
As concentrations of biological and chemical components in the prepared samples for analyses and assays are volume-dependent, incorrectly performed pipetting steps will directly impact the transferred volumes, and hence, the test results. The design and construction of piston-operated air-displacement pipettes (in this poster simply referred to as pipettes) render their performance susceptible to the pipetting technique and skills used by the operator of such devices.

This study evaluates a number of techniques that influence the accuracy and precision of the pipetted volume. The pipette operator has the ability to control all of these parameters by using the appropriate pipetting technique, as well as by choosing the appropriate pipette size and type of pipette tips.

Influence of the Air Cushion on Pipette Performance

Piston-operated air-displacement pipettes use an air cushion to couple the piston of the pipette to the aspirated liquid inside of the pipette tip. This air cushion, often referred to as captive air volume or dead air volume, is trapped within the pipette as soon as the tip is immersed in the sample solution. This captive air volume closely obeys the Ideal Gas Law (P_a is the pressure of the trapped gas, V_a its volume, n_a the number of moles and T_a the temperature of this gas):

$$P_a V_a = n_a R T_a$$

The ideal gas law allows to estimate the effects which temperature, evaporation, and the ratio of captive air volume to the pipette's set volume will have on the actually aspirated and delivered volume of a pipetting cycle.[1] The following techniques studied here directly influence the captive air volume: Pre-wetting of Pipette Tips, Temperature Dis-equilibrium, Hand Warming, and Immersion Depth of Pipette Tip. Since the total volume of the air cushion can vary widely depending on the type of pipette, the tip type and size, and the amount of the aspirated liquid aliquot, this study evaluated two different scenarios: one set of experiments was conducted with a 20 µL pipette set at 20 µL, the other set with a 100 µL pipette set at 20 µL.

Pre-Wetting of Pipette Tips

Sample solution in the pipette tip is susceptible to evaporation into the air cushion during and after aspiration. The evaporative loss of sample solution is dependent on the humidity of the captive air space, as well as the temperature of the sample solution. Repeated aspiration/dispense cycles will increase the humidity of the air in the pipette tip and shaft. Figure 1 shows the dispensed volumes of a 20 µL pipette set to 20 µL. Each dispense was performed with a new tip. The pipette dispensed on average 1.3% less volume when the tips were not pre-wetted, as compared to dispenses when the tip was "pre-wetted" three times prior to the dispense.
When using pipettes in particularly dry or warm environments, the error by not pre-wetting the tips can be significantly larger.[2]

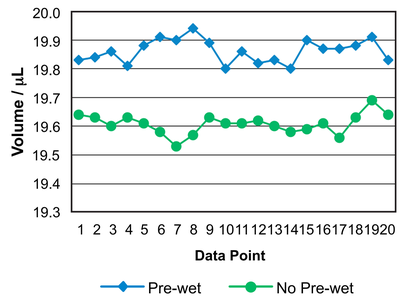


Figure 1. Volume deliveries of a 20 µL pipette. Each delivery used a new tip and was either pre-wetted three times or not pre-wetted prior to sample delivery.

Temperature Dis-equilibrium

For most accurate pipetting results, it is recommended that the pipette, the pipette tip, and the sample solution have been equilibrated for at least 2 hours and are within 0.5 °C of ambient temperature.[3] Many samples must be handled at specific high or low temperatures, however, and pipetting such samples can introduce significant errors in the delivered volume due to the expansion or contraction of the captive air volume and evaporation. Studies of this effect have been reported previously.[4] The present study evaluated the use of pipette tips, which had been cooled to 4 °C for 30 min prior to use. Pipetting with these cold tips led to significant under-delivery of sample with both pipettes, contributing up to -1.9% RI and 1.2% CV to the errors.

Heat Transfer / Hand Warming

Handling a pipette for prolonged periods of time will cause the barrel of the pipette to warm, leading to an expansion of the captive air volume. This heat transfer will ultimately impact the accuracy and precision, resulting in delivery of significantly smaller volumes than in the control experiment. The progressive warming of the pipette's barrel manifests itself by a trend toward smaller delivered volumes, and led to -1.1% RI and 0.8% CV in this study.

Immersion Depth of the Pipette Tip

Immersing the pipette tip to the proper depth during aspiration of the sample is important. Pipette calibration standards like ASTM E1154 recommend an immersion depth of 2-3 mm for pipetted volumes of 1-100 µL, 2-4 mm for 101-1000 µL, and 3-6 mm for volumes larger than 1 mL. In this study we evaluated immersion depths of 1 mm (too shallow) and 8 mm (too deep). Immersing the tip too little increases the risk of aspirating small amounts of air, while immersion it too deep increases the risk of carrying-over droplets on the outside of the tip, and/or forcing more sample in the tip due to increased hydrostatic pressure on the outside of the tip.
Either case leads to a significantly increased imprecision (up to 2.2% CV) of the delivered volumes.

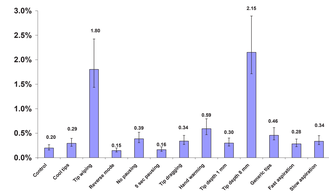


Figure 2. Imprecision (CV) of a 20 µL pipette set to 20 µL.

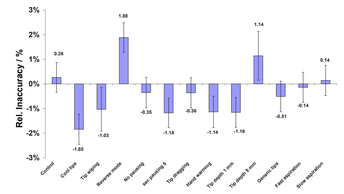


Figure 3. Inaccuracy of a 20 µL pipette set to 20 µL

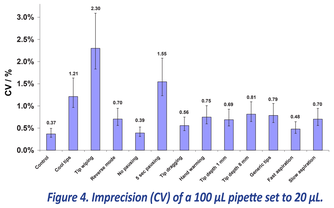


Figure 4. Imprecision (CV) of a 100 µL pipette set to 20 µL.

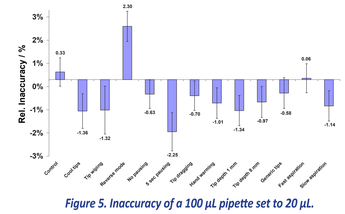


Figure 5. Inaccuracy of a 100 µL pipette set to 20 µL.

Experimental Conditions

- All tests reported in Figures 2 to 5 were conducted in a controlled calibration laboratory at 20.0 ± 1.0 °C and 45-65% relative humidity.
- Volume measurements were performed with an Artel PCS® Pipette Calibration System, using the photometric method according to ISO 8655-7.
- Only one parameter of the pipetting technique was varied in each experiment and compared to the control method described below; compounding of technique errors was not investigated in this study
- Two pipettes were evaluated in this study:
  (1) 20 µL pipette set at 20 µL
  (2) 100 µL pipette set at 20 µL
- The manufacturer's recommended tips were used.
- Experiments were carried out by trained operators.
- The control method used the following pipetting technique:
  - each pipette tip was pre-wet 3 times;
  - the tip was immersed 2 mm below the meniscus in the sample solution;
  - pipette was held in a vertical position during aspiration, and at a 45° angle during the dispense against the glass wall of the measurement cuvette;
  - forward mode of pipetting was used, with blow-out during dispensing.
- Each experiment was conducted with 30 replicates.
- A new pipette tip was used for each data point.
- Accuracy is reported as Relative Inaccuracy (RI) as percent difference to the set volume of the pipette.
- Precision is reported as the Coefficient of Variance (CV).

Forward and Reverse Mode

Using the appropriate pipetting mode has one of the biggest influences on the accuracy of the volume delivery. Forward mode describes the pipetting technique in which the plunger of the pipette is depressed to the first stop, the pipette tip then immersed in the sample solution, and the sample then aspirated by releasing the plunger. During delivery, the plunger is depressed beyond the first stop, to the so-called blow-out mode (second stop), forcing all the liquid out of the tip. Standard procedure for pipette calibration prescribes using this forward mode and aqueous sample solutions.

In reverse mode, the plunger is depressed beyond the first stop (to the second stop) before immersing the tip in the sample. This leads to aspirating additional sample volume into the tip. The desired volume is delivered by depressing the plunger to the first stop, retaining the additional sample in the tip.
This pipetting mode is recommended for use with viscous or volatile solutions, however using reverse mode with aqueous solutions leads to significant over-delivery of up to 2.3% RI and contributes up to 0.7% CV, as is obvious from Figures 3 and 5.

The authors recommend specifying in the SOP for each test, which pipette mode is to be used for the particular task(s) at hand. This will avoid errors induced by personal preference for one or the other pipetting mode.

Consistent Plunger Speed and Pressure

Depressing and releasing the plunger with consistent speed during aspiration and dispensing of the liquid aliquot is important for achieving precise and accurate results. The type of pipette, tip, and sample solution will determine the optimum pressure needed to move the plunger with a consistent and appropriate speed. Our studies indicate that a slow aspiration speed may result in under-delivery of up to -1.1% and contribute up to 0.7% to the imprecision.

Position of Tip during Aspirating and Dispensing

In order to ensure the optimum and undisturbed hydrodynamic flow of the sample solution during aspiration of the sample into the tip, the pipette should be held in a vertical position, and the tip should not touch the side or bottom of the vessel with the sample liquid. Further, it is important not to drag the tip along the wall of the source sample vessel, as this may lead up to -0.7% RI and 0.6% CV.

When dispensing the sample using forward mode, it is recommended to touch the pipette tip against the side of the receptacle, while the pipette may be held at a 45° angle. With the exception of pipetting very small volumes, it is not recommended to immerse the tip into already present solution in the receiving vessel. This technique may lead to over-delivery if droplets are clinging to the outside of the pipette tip, and significantly increases the risk of cross-contamination.

Pause after Aspirating

Once the aliquot of sample solution has been aspirated into the pipette tip, it is important to pause for about 1 second with the tip still immersed in the source liquid, allowing the sample to "settle" in the tip. Removing the pipette tip prior to allowing the vibrational motion of the liquid to settle will introduce errors in the precision and accuracy, up to -0.6% RI and 0.4% CV in our studies. Allowing the tip to remain in the liquid for too long, however, will result in significant under-delivery, up to -2.3% RI and 1.6% CV. The magnitude of these errors depends on the pipette tip, temperature, sample type (vapor pressure), speed of aspiration, and the sample volume.

Tip Wiping

The practice of wiping the pipette tip after aspiration with an absorbent laboratory cloth is a wide-spread habit. Due to the high propensity of introducing large errors through this technique, it should be very carefully evaluated whether this is really necessary. If it is determined that a particular sample is prone to forming droplets on the outside of the pipette tip that need to be wiped off, extreme care should be exercised in not touching the tip orifice, as it is very easy to wick out some of the sample solution through the tip orifice by moving the tissue in its close vicinity.

Despite exercising great care when wiping off the pipette tips in our study, it introduced over 2.3% of CV and consistently lead to under-delivery of up to -1.3% RI.

Pipette Tip Quality

For the most accurate and precise pipetting results, the pipette manufacturer's tips should be used. Achieving a proper seal between the pipette's nose cone and the tip is critical for good performance. Generic tips may seemingly fit on a pipette, but due to different taper angles of the nose cone and tip, a poor seal is established, resulting in errors. In our study, the generic tips fit on the pipettes but still introduced errors of up to -0.6% RI and 0.8% CV, which would be additive to all other pipetting errors.

Claimed pipette performance assumes the use of manufacturer's recommended tips.
Regardless of the type of tip used in the lab, it is imperative that a pipette is calibrated with this very same tip type and under the conditions of its use in the lab in order to avoid errors while using the pipette for analytical tests.

Pipette Size

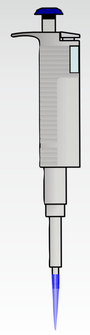
Adjustable volume pipettes can be used over a large range of volumes. Manually operated pipettes usually allow the user to select volumes as low as 10% of the pipette's nominal volume. Several electronically operated pipettes offer an even wider range of selectable volumes.
Best pipette performance, however, is achieved at or near the nominal volume of a pipette.
For best results, it is recommended to use variable-volume pipettes only to the nominal volume of the next available, smaller denomination of pipette.

Best Pipetting Practices

The results of this study demonstrate that even minor variances in the operating technique of handheld air-displacement pipettes can result in measurable errors in accuracy and precision. This study did not evaluate errors resulting from combining multiple of the discussed technique variations, although this is commonly observed in the field. Compounded errors can easily reach 12%, and are often even larger as data from field surveys suggests.

The following steps will ensure the most accurate and precise results:

- **Pre-wet tips at least three times**
- **Use proper pipetting mode**
- **Work at temperature equilibrium**
- **Immerse tips to proper depth**
- **Aspirate with pipette in vertical position**
- **Pause after aspirating**
- **Do not touch vessel wall during and after aspiration**
- **Use consistent plunger speed and pressure**
- **Minimize heat transfer from hands**
- **Avoid tip wiping**
- **Examine tip prior to dispensing**
- **Use high-quality pipette tips**
- **Use proper pipette size**



References

[1] Rodrigues, G., Curtis, R. *Instrument Performance Verification: Micropipettes.* Practical Approaches to Method Validation and Essential Instrument Qualification, Chan, C.C. (ed.), John Wiley & Sons, Inc., New York 2010, pp. 327-346.
[2] Carle, A.B. *Dry Heat and Humidity –Lab Environmental Conditions as Sources of Error*, Lab Business 2009, online at: labbusinessmag.com/articles/winter09dryheat/dryheat.html
[3] ISO 8655-2:2002 and ISO 8655-6:2002; Piston-Operated Volumetric Apparatus; Part 2: Piston Pipettes; Part 6: Gravimetric Methods for the Determination of Measurement Error. ISO, Geneva, Switzerland.
[4] Carle, A.B. *Minimizing Liquid Delivery Risk: Laboratory Environmental Conditions as Sources of Error; Part 1-Barometric Pressure and Thermal Disequilibrium*, Am. Lab. News 2008, 40 (3), 8-10.

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