C2 Biotechnologies, LLC

Using biotechnology to improve the quality of life

SOP-111

Pipetting Small Volumes

Procedures used to pipette small volumes of liquid.

Section 1

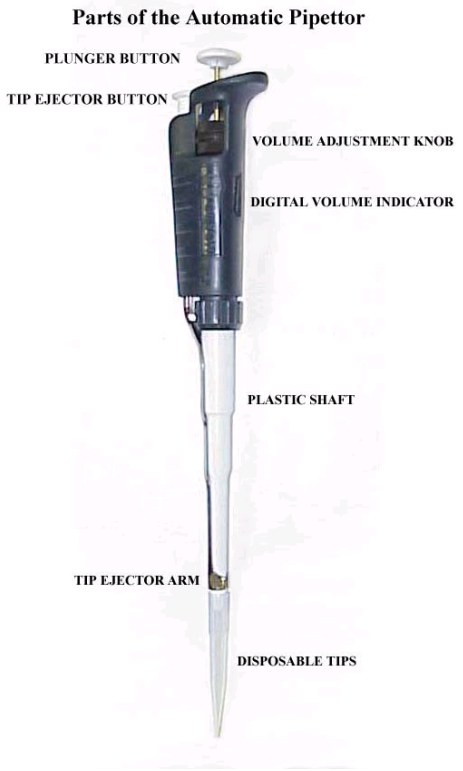
Materials and Reagents

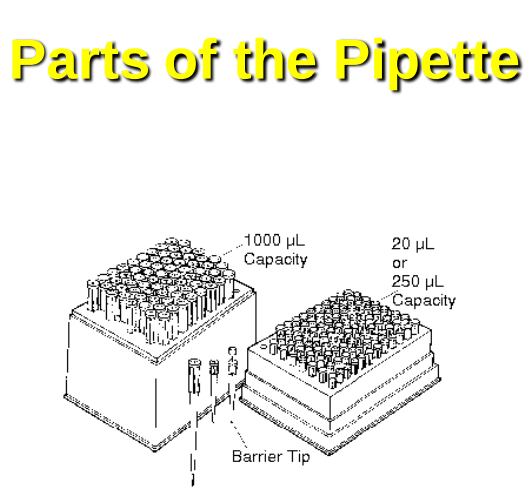
20 ul	Pipetman
100 ul	Pipetman
200 ul	Pipetman
1000 ul	Pipetman

Section 2

Protocol

The following series of images are provided courtesy of the CDC. These images provide a visual map of how to pipette small volumes. Following the photo guide is a text reprinted with permission from Tomtec, Inc. that provides an in depth review of small volume pippetting.





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Operating the Micropipette

Step 1: Set the Volume

Pipettors – 3 Volumes:

Volume Adjustment Knob:

Digital Volume Indicator:

Image is public domain courtesy of CDC
(*wwwn.cdc.gov/dls/ila/cd/zambia/files/Micropipettes.ppt)*

Operating the Micropipette

Step 1: (Continued) Read the Volume

How to Read the Volume Indicator:

		
(a): P-20 Model **6.86 µ l = 0.00686** **or 6.86 x 10^{-3} ml**	**(b): P-200 Model** **132.4 µ l = 0.1324** **or 1.324 x 10^{-1} ml**	**(c): P-1000 Model** **262 µ l= 0.262** **or 2.62 x 10^{-1} ml**

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Operating the Micropipette

Step 2: Attach the Disposable Tip

Attaching the disposable tip

Example of tip sizes:

Image is public domain courtesy of CDC (*wwwn.cdc.gov/dls/ila/cd/zambia/files/Micropipettes.ppt)*

Operating the Micropipette

Step 3: Depress the Plunger to the First Stop

Step 4: Immerse Tip in Sample

Step 5: Draw up the sample

To aspirate the sample into the tip, allow the pushbutton to return slowly and smoothly to the fully extended UP POSITION. NEVER LET THE PLUNGER SNAP UP! This draws the exact calibrated volume into the tip if the tip remains below the liquid surface during withdrawal.

Step 6: Pause

Wait a few seconds to ensure that the full volume of sample is drawn into the plastic tip. WAIT LONGER FOR LARGER VOLUMES. WAIT LONGER FOR MORE VISCOUS ("SYRUP-LIKE") SUBSTANCES.

Image is public domain courtesy of CDC (*wwwn.cdc.gov/dls/ila/cd/zambia/files/Micropipettes.ppt)*

Operating the Micropipette

Step 7: Dispense the Sample

To dispense the sample from the pipette:
a) Touch the tip end to the side wall of the receiving vessel and
b) Depress the plunger to the FIRST STOP.
c) Pause for at least one second-- 1-2 seconds for P-1000, 2-3 seconds for P-5000, or longer for viscous liquids.
d) Press the plunger to the SECOND STOP (the second point, of greater resistance, at the bottom of the stroke) to expel any residual liquid in the tip (like "blowing out" a glass pipette).

(a) Start Dispensing	(b) 1st Stop = Dispense	(c) 2nd Stop = Expel

Image is public domain courtesy of CDC
(*wwwn.cdc.gov/dls/ila/cd/zambia/files/Micropipettes.ppt)*

Operating the Micropipette

Step 8: Withdraw the Pipette

With the plunger fully depressed, withdraw the pipet from the receiving vessel carefully, sliding the tip along the wall of the vessel. Holding the tip against the side of vessel is <u>especially important when transferring small volumes of liquid</u>.

Step 9: Release the Plunger

Gently allow the plunger to return to the UP position. DO NOT allow it to SPRING BACK!

Step 10: Discard the Tip

Discard the tip by <u>depressing the</u> tip ejector button, as shown below. A fresh tip should be used for each sample to prevent sample carryover.

Press ejector button to discard tip.

Image is public domain courtesy of CDC
(*wwwn.cdc.gov/dls/ila/cd/zambia/files/Micropipettes.ppt)*

Step-wise Operation of the Automatic Pipette

(1) Set the volume
(2) Attach disposable tip
(3) Depress the plunger to the first stop
(4) Immerse tip in sample
(5) Draw up the sample
(6) Pause
(7) Withdraw the tip
(8) Dispense the sample
(9) Withdraw the pipette
(10) Release plunger
(11) Discard the tip

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Pippetting Guidelines and Precautions

For optimal reproducibility, use the following pipetting procedures:

(1) Consistent SPEED and SMOOTHNESS when you press and release the PLUNGER

(2) Consistent pressure on the PLUNGER at the FIRST STOP

(3) Consistent and sufficient IMMERSION DEPTH

(4) Nearly VERTICAL POSITIONING of pipette

(5) AVOID ALL AIR BUBBLES: Since the plastic pipette shaft can be damaged if liquids are drawn beyond the tip into the shaft

(6) NEVER lay the pipette on its SIDE nor INVERT the pipette if liquid is in the tip

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A copy in pdf format may be found on the Tomtec web site:
http://www.tomtec.com/pages/applications/pdf/smallvolumepipetting.pdf

SMALL VOLUME PIPETTING

By: Thomas W. Astle, P.E.

INTRODUCTION

As pharmaceutical research continues the drive to screen higher numbers (FITS), economics dictate the use of smaller quantities of precious compounds and expensive reagents per assay, researchers are meeting this requirement with new assay developments. This impacts the use of liquid handling instrumentation, to meet the dimensional requirements of the higher density formats while maintaining the precision the industry has come to expect.

There are two types of liquid dispensing methods in use today - piston displacement and ink jet technology. Piston displacement has been the workhorse of the industry. Simply, a piston moving in a close fitting cylindrical space that is sealed at one end and open at the other. It is exemplified by the myriad of hand held and automated pipettors that are currently on the market, in a variety of forms.

Ink jet technology derives its name from its early adaptation in the printing industry. Ink jet technology uses some means of creating pressure in a liquid against a small orifice. As the orifice opens, the back pressure imparts sufficient linear velocity to the liquid to eject it, in a fast moving stream. However, by closing the orifice quickly, the stream can become an ejected drop. The size of the drop is primarily controlled by the orifice size, back pressure and length of time the orifice is open. An informative article on ink jet dispensing by Don Rose appeared in *LAN (Vol. 2 No. 4, Sept 1997).*

With piston displacement, if the piston moves fast enough against a small outlet orifice, it also will deliver as a stream. The normal method of delivery however, controls the piston movement, so as to measure a specific amount. As a result, there is usually insufficient stream velocity to overcome the effects of surface tension and the associated capillary action at the dispensing tip orifice. These parameters become a greater factor in small volume liquid handling. In general terms, piston displacement becomes limited in the sub-microliter range of delivery. With current technology, it is difficult to achieve the accepted limits of precision below 0.2 to 0.5 microliters (200 to 500 nanoliters). Even at these limits special precautions are required.

ACCURACY VS. PRECISION

Accuracy in pipetting is defined as the relationship between the volume that is set and the volume that is actually delivered. If the dial or display is set at 100 microliters and 98 microliters is actually delivered, the pipettor has an accuracy of 98% or a 2% error. Precision is a measure of repeatability. It is expressed as a non-dimensional coefficient of variation (C_v). The C_v is the standard deviation of a number of events (dispenses), divided by the mean value of those events. In biological assays, it is a greater concern that multiple samples are handled alike. Thus, precision normally has a higher priority than accuracy.

AIR DISPLACEMENT VS. POSITIVE DISPLACEMENT PIPETTING

Air displacement is the most common method of pipetting liquids. A piston is moved within its' cylindrical housing, displacing a volume of air. This creates a vacuum in the pipette tip, causing liquid to move into the tip to displace the vacuum that was created. There is always a dead air cushion between the piston and the liquid in the tip.

In a positive displacement pipettor there is no compressible air cushion between the piston and the liquid in the pipette tip. Normally, the piston goes to the bottom of the tip and is in contact with the liquid in the tip that is being pipetted. Contaminating the end of the piston, with the liquid being pipetted, is not desirable in most cases. An alternative is to back fill, what would be the air space between the piston and the end of the pipette tip,

with a non compressible fluid such as water. Then a slight air gap is drawn to separate this backfill liquid and the liquid being pipetted. This is commonly done with syringe pump pipettors.

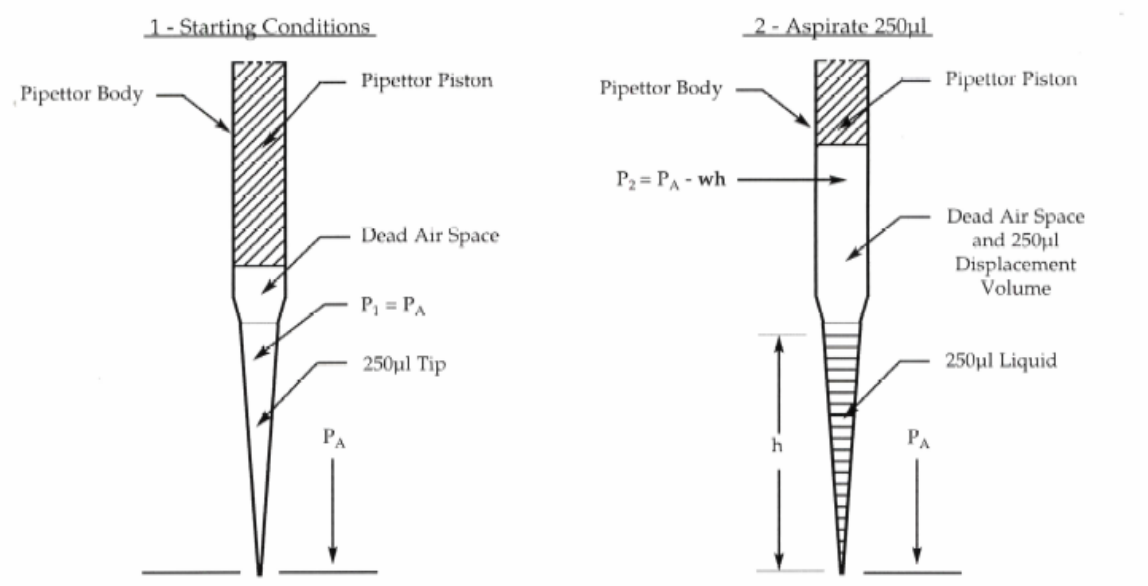

Figure 1. Pipetting

Air displacement pipetting, while easier to accomplish, does not have the accuracy or precision, particularly at small volumes, that is available with positive displacement pipettors. This is due to the compressibility of the dead air column in the pipettor. The explanation is as follows:

Assume a manual 250µL air displacement pipettor is used with a 250µL pipette tip. The dead air volume will be a function of the mechanical design plus the maximum volume of the tip being used. At the start, the air pressure in the tip was atmospheric (i.e. $P_1 = P_A$). As the piston moves up into the body of the pipette it displaces air, creating a negative pressure in the tip. The liquid flows into the tip orifice due to atmospheric pressure acting on the liquid reservoir. At condition #2, in the ideal condition, the air pressure in the tip would be equal to atmospheric P_A and V_1 would equal V_2. This is defined by the ideal gas law PV = RT. Since it is the same gas (air) at the same temperature ($T_1 = T_2$) then $P_1V_1 = P_2V_2$. However, the column of liquid has a pressure head, defined as **wh** (**w** = specific gravity of the liquid and **h** = the column height). At the tip orifice the pressure is atmospheric. If the liquid is to remain in the tip then this pressure at the orifice must be offset by a negative pressure (vacuum), within the closed or dead air portion of the tip. Thus, P_2 must be less than P_A by the value of **wh**.

$$P_1V_1 = P_2V_2$$

since $P_1 = P_A$ and $P_2 = P_A - \mathbf{wh}$

$$\frac{P_AV_1}{P_A - \mathbf{wh}} = V_2$$

- V_1 = Dead Air Volume in Pipettor Body and Tip Volume, assume 500µl
- Atmospheric Pressure = 406.8 inches H_2O
- Assume the water in the tip = 2.0 inches

Equation 'A':

$$\frac{406.8 \text{ inches } H_2O \times 500\mu l}{406.8 \text{ inches } H_2O - 2.0 \text{ inches } H_2O} = V_2$$

$$1.0049 \times 500\mu l = V_2$$

$$V_2 = 502.47\mu l$$

$$V_2 - V_1 = 2.47\mu l$$

Figure 2 Pipetting Volume

Thus, V_2 the displaced volume must be greater than the original volume V_1 by the factor **wh**. In other words, the pipettor piston must move back not only the desired volume of 250µL (V_1) but an additional volume to create enough vacuum to offset the pressure head in the tip. To put it in actual terms, assume water is being pipetted.

Thus, the piston had to displace an additional 2.47µL to aspirate and hold the 250µL sample, or 1% accuracy error. The equation at 'A' also demonstrates the effect of the volume of dead air. If the 500µL figure is reduced to 250µL then the difference between V_2 and V_1 is cut in half. If taken to the limit (i.e. no dead air), then $V_2 = V_1$, which is the case in positive displacement pipettors. This equation has been simplified to demonstrate the relationship of dead air volume and liquid level in the tip. It ignores other factors, such as surface tension and its associated capillary action at the tip orifice. These factors must also be overcome by a related increase or decrease in pressure, within the pipettors enclosed space, and its associated volume change.

In small volume pipetting, the inside diameter of the tip orifice must be reduced. The proximity of the inner walls of the tip increases the surface tension forces at the orifice. These additional forces must be overcome, both on the aspirate (flow in) and dispense (flow out) function. On the aspirate function the surface tension forces tend to impede flow in. Thus, the speed of the aspirating piston may have to be slower with a small orifice and particularly if the liquid has any viscosity (i.e. a 1% solution of BSA will not flow as fast as water). On the dispense stroke, it is beneficial to have some amount of blow out air following the sample. Even then, surface tension will still hold some amount

at the tip orifice. A drop of 0.1 microliters is insignificant when pipetting 100µL or even 10µL, but at 0.5 microliters it represents 20% of the sample volume.

SUGGESTED PRACTICES FOR SMALL VOLUME PIPETTING WITH TOMTEC'S POSITIVE DISPLACEMENT PIPPETOR HEADS

Positive displacement pipettors can provide better precision at small volumes than air displacement. This can be accomplished with backfilled systems or with pipettors that bring the piston in close proximity to the tip orifice. The later poses the problem of piston contamination between samples. Unless the piston is to be replaced each time, some method of washing the tip and piston is required.

The use of Teflon® coatings has become an accepted method of making tip surfaces hydrophobic (repel water). Augmenting these coatings with tip washing methods, such as the use of ultrasonics, provides a solution. The action of the ultrasonics agitates the tip and piston surface at a high frequency, which in effect breaks down the surface tension forces. There is a trade off concerning the use of ultrasonics. An ultrasonic bath has a transducer attached to the metal container of the bath which causes it to vibrate at ultrasonic frequencies. This vibration is transmitted to the tips by means of the water in the bath. If the water has entrained air, the efficiency of this transfer is diminished. The ultrasonic action will remove entrained air. While it is desirable to have fresh water in the bath it is more efficient to gradually change it, thus reaching a compromise between deacrated water and clean water.

A small pipette tip orifice is required, on the order of 0.015 inch diameter. This presents a problem with respect to particulate matter. Of equal importance the small orifice must be kept clean and free of dried salt solutions. Both are easier said than done. Thus, it is desirable to have the small orifice only at the tip opening and not a long narrow passage to the orifice. Cleaning is facilitated by simply inserting a needle or cleaning wire at the orifice.

Several techniques are available to improve precision at the 0.5µL level. Aspirating 0.5µL is not the problem, dispensing 0.5µL at the desired location is. To dispense 0.5µL the tips must touch off to a surface, either liquid or solid. Liquid touch off is not a problem. On a multiple tip pipettor, such as 96 or 384 well, it is questionable whether all tips will mate with each well surface to permit touching off, without sealing tight. It can be done with reasonable confidence in a 'V' bottom well, since the 'V' bottom tends to guide the tip to a surface. The use of 96 flat bottom wells provides marginal results.

A more reliable technique is pipeline pipetting. With this method multiple reagents are aspirated into the tip sequentially, using an air gap for separation between reagents. In addition to speed in processing, it allows the higher volume of the diluent to wash out the small 0.5µL volumes. The small trace amount left in the tip will be diluent, not the 0.5µL test volume.

Experience has further refined that procedure. Assume a 384 well assay requires 20µL of reagent A, 10µL of reagent B and 0.5µL of compound and 0.5µL of standards and

controls. Repeated testing with different assays provides the following recommended protocol.

- Aspirate 5µL air to serve as final blow out
- Aspirate 20µL of reagent A
- Aspirate 5µL of air gap separation
- Aspirate 10µL of reagent B
- Aspirate 0.5µL of compound
- Aspirate 0.5µL of standards & controls
- Aspirate 5µL of air
- Rinse tip exterior in ultrasonic bath
- Dispense 46µL total volume into 384 well plate
- Mix three times by aspirating from the bottom of the well and dispensing at the top

Note: The initial dispense should be made above the final well volume, so as not to inject air bubbles into the well contents. Air bubbles, if created, are difficult to remove in the 384 well format. They may be detrimental to the readout.

The logic in the above sequence is to use the 20µL volume of reagent A to wash out the 0.5µL volume of compound. The air gap between A and B prevents their interaction until dispensed into the assay plate. Testing has shown that by keeping the 0.5µL volume in contact with reagent B (no separating air gap) surface tension between the two liquids keep the 0.5µL volume intact. On a colorimetric assay C_v of 2 to 3% at the 0.5µL level are routine. Using an air gap between the 0.5µL volume and the adjacent volume gave lesser precision.

The last step of aspirating a 5µL air gap is used to move the reagents away from the end of the tip. The next step is simply dipping the tips in a water bath to rinse any reagent from the exterior. If the objective is to transfer only 0.5µL on the inside of the tip a 50 nanoliter (0.05µL) trace amount on the tip exterior contributes a 10% error.

CONCLUSION

- Positive displacement pipettors provide better precision at small volumes than air displacement pipettors.
- A small exit orifice is required at the tip. If it is a short pathway periodic cleaning is facilitated.
- Pipeline pipetting, using the larger volumes of reagents with air gap separations, to wash out small volumes improves precision.
- Volumes of 0.5µL and 1.0. µL should not use air gap separation must be kept in contact with previously pipetted volume.

- An exterior tip rinse is beneficial when pipetting small 0.5µL to 1.0µL volumes. An air gap should be use to move tip contents away from the exit orifice, prior to rinsing.
- Air gaps should be dispensed at the top of the well to prevent injecting air bubbles in the final test volume.
- Touch off all dispenses to some surface, liquid or solid.

- **Set pipette volume only within the range specified for that micropipette.** Do not attempt to set a volume beyond the pipette's minimum or maximum values. This will damage the micrometer gears!
- **When using a micropipette, first apply a tip**. Forgetting to do this would ruin the precision piston that measures fluid volume.
- **Always keep a micropipette in a vertical position when there is fluid in the tip.** Do not allow liquid to accidentally run back into the piston.
- **Use your thumb to control the speed at which the plunger rises after taking up or ejecting fluid.** Letting it snap back damages the piston!

Metric Conversions Involving Small Volumes

Familiarize yourself with metric units of measurements and their conversions. We will use the volume measurement (base unit: liter) but the prefixes we learn would also apply to mass (base unit: gram) or linear measurement (base unit: meter). The two most prevalent units of liquid measurement in molecular biology are the milliliter (mL) and the microliter (µL).

1 mL = 0.001 liter or 1/1,000 liter 1,000 mL = 1 liter, 1000 µl = 1 ml
1 µL = 0.000001 liter or 1/1,000,000 liter 1,000,000 µL = 1 liter

Section 3 References

Short Protocols in Molecular Biology. 4th Edition. Editors Frederick M. Ausubel, Roger Brent, Robert E. Kingston, David D. Moore, J.G. Seidman, John A Smith and Kevin Struhl. John Wiley & Sons, Inc. 1999

http://www.tomtec.com/pages/applications/pdf/smallvolumepipetting.pdf

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