HAMILTON®

Liquid Handling Reference Guide

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1 About This Guide

This guide introduces the concept of liquid handling and explains the fundamentals of how to perform liquid handling on an automated liquid handler. It is great for both new learners, who need to understand liquid handling basics, as well as experienced liquid handlers, who can benefit from best practices and troubleshooting tips.

This guide offers insights into liquid properties and how different liquids can affect the results of your pipetting. Utilizing the information in this guide will ensure your liquid handling measurement, aspiration, and dispensing techniques are precise and accurate.

The concepts in this guide are intended for use on all Hamilton automated liquid handlers and their related software. As a result, no detailed steps are provided for individual platform and software combinations.

2 About Liquid Handling

Liquid handling is the act of transferring liquid from one location to another in a laboratory, usually for testing purposes.

This section introduces liquid handling, different ways liquid handling can be performed, and the important physical aspects of liquids.

2.1 How It's Used

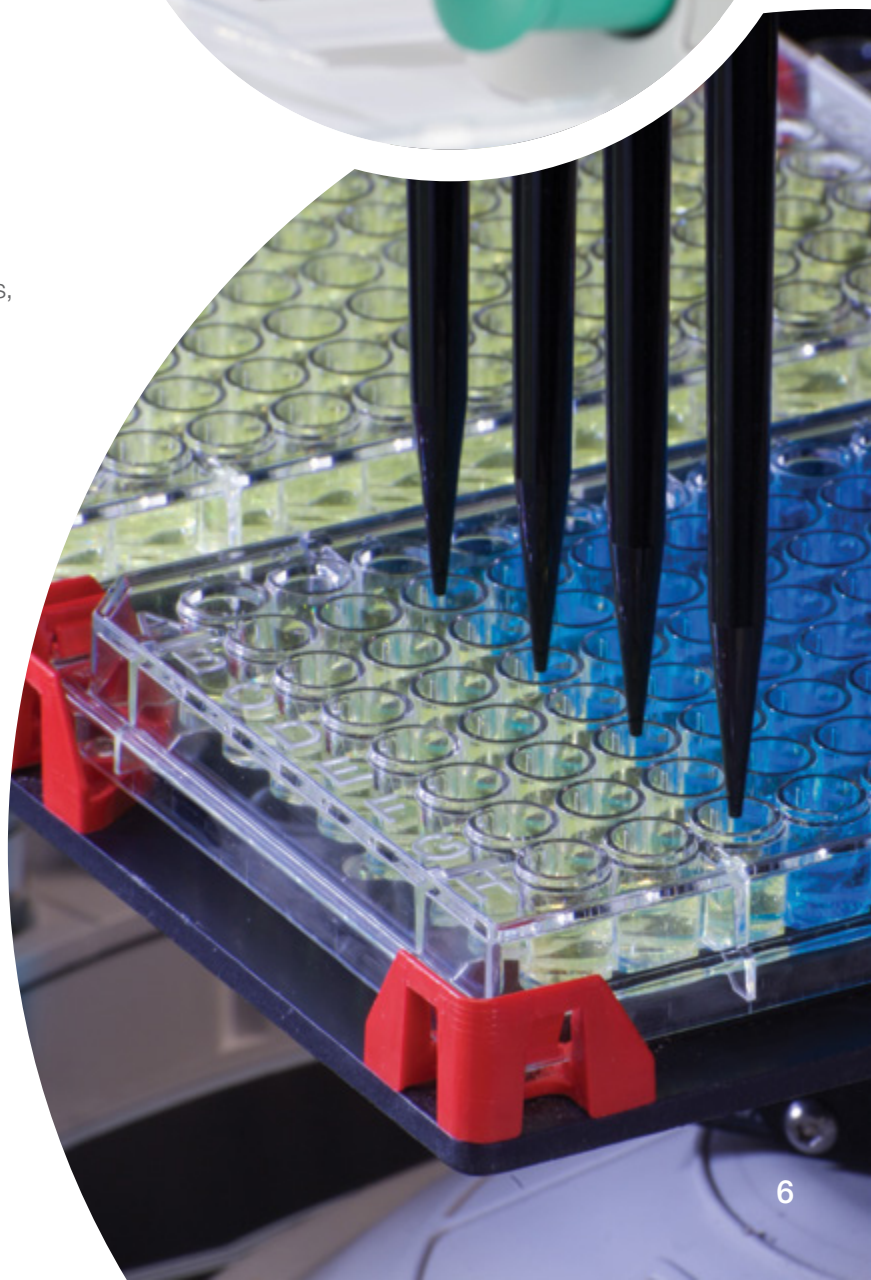
Simple though it seems, liquid handling is important to laboratories around the world. Most testing involves checking countless, tiny samples of liquid for certain attributes. Samples can be smaller than 1 microliter (µL) and still help the lab detect chemicals, screen for diseases, and multiply DNA for further testing.

However, liquid handling can be a time-consuming process for a lab. While a 1 µL sample may be an efficient use of a liquid for a single test, testing equipment usually provides room for dozens if not hundreds of samples, with each one requiring a transfer of liquid.

Imagine a lab technician using a hand pipettor to carefully transfer 96 1 µL samples onto a single microplate. Precision is critical to good test results, so each transfer of liquid needs to be exact. An exact liquid transfer has everything to do with the type of liquid that's being transferred and the technique of the technician as the liquid is dispensed. It's both a science and a technique.

To save time, a Hamilton automated liquid handler can automate the science and the technique. Instead of a lab tech carefully dispensing a sample into 96 individual wells, the automated liquid handler is programmed to use settings that facilitate proper and consistent transfers. Programming an automated liquid handler means taking everything that was done manually by the lab tech and defining it literally in a set of instructions for the automated liquid handler to follow.

Labs often use a mixture of hand and automated pipetting to achieve their goals involving liquid handling.



2.2 Manual Pipetting vs. Semi-Automation vs. Automation

Both hand pipetting and automation can be used effectively in a lab that is managing liquid samples. The method to choose depends on the application. See below for some key factors to help you decide which method is best for your application.

2.2.1 When to Use Manual Pipetting

Hand pipetting is fast in small applications and only requires the hand of a practiced lab tech instead of extra hardware. While manual pipetting is straightforward, it does introduce risks of technician-to-technician variability.

2.2.2 When to Use Semi-Automation

Semi-automation offers a way for labs to incrementally scale up production and increase reproducibility through liquid handlers that automate certain aspects of sample preparation. Instead of a technician manually setting volumes on a pipette or keeping track of steps in a method, semi-automated liquid handlers (like Hamilton's Microlab 600) automate those aspects of the work, only requiring the technician to move a hand probe from vessel to vessel.

2.2.3 When to Use Automation

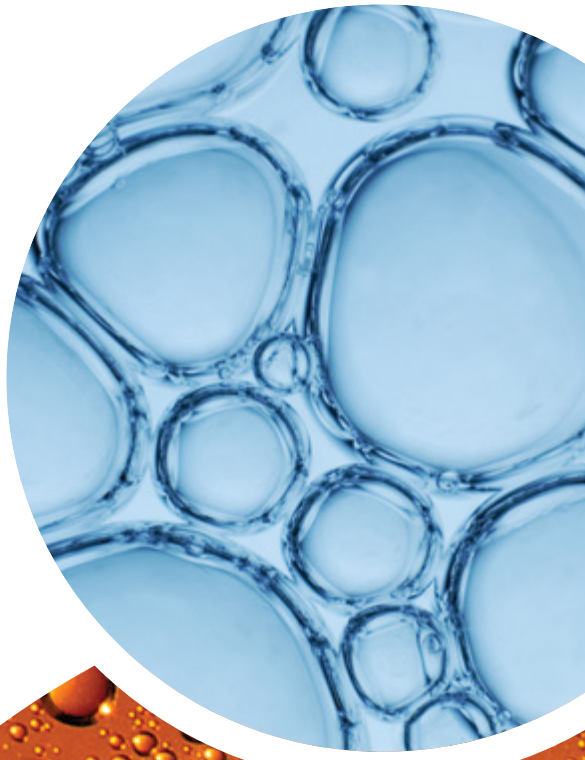
Full automation is most valuable in high-throughput applications that benefit from completely removing human movements. Liquid handling platforms process hundreds of samples at a time and follow highly complex methods without deviation.

When to Use Manual Pipetting vs. Semi-Automation vs. Automation

	Manual Pipetting	Semi-Automation	Automation
Sample Involved	Few	Dozens	Hundreds/Thousands
Throughput Needed	Low (5–10 samples per hour)	Moderate (11–100 samples per hour)	High (100+ samples per hour)
Dead Volume Allowed?	Small amount	Small to none	High Amount
High Reproducibility Needed?	No	Yes	Yes
Are labor costs a concern?	No	Moderately	Yes
Chance of repetitive stress injuries?	Yes	Some	No
Protection from hazardous/ infectious samples?	No	No	Yes

2.3 Liquid Properties

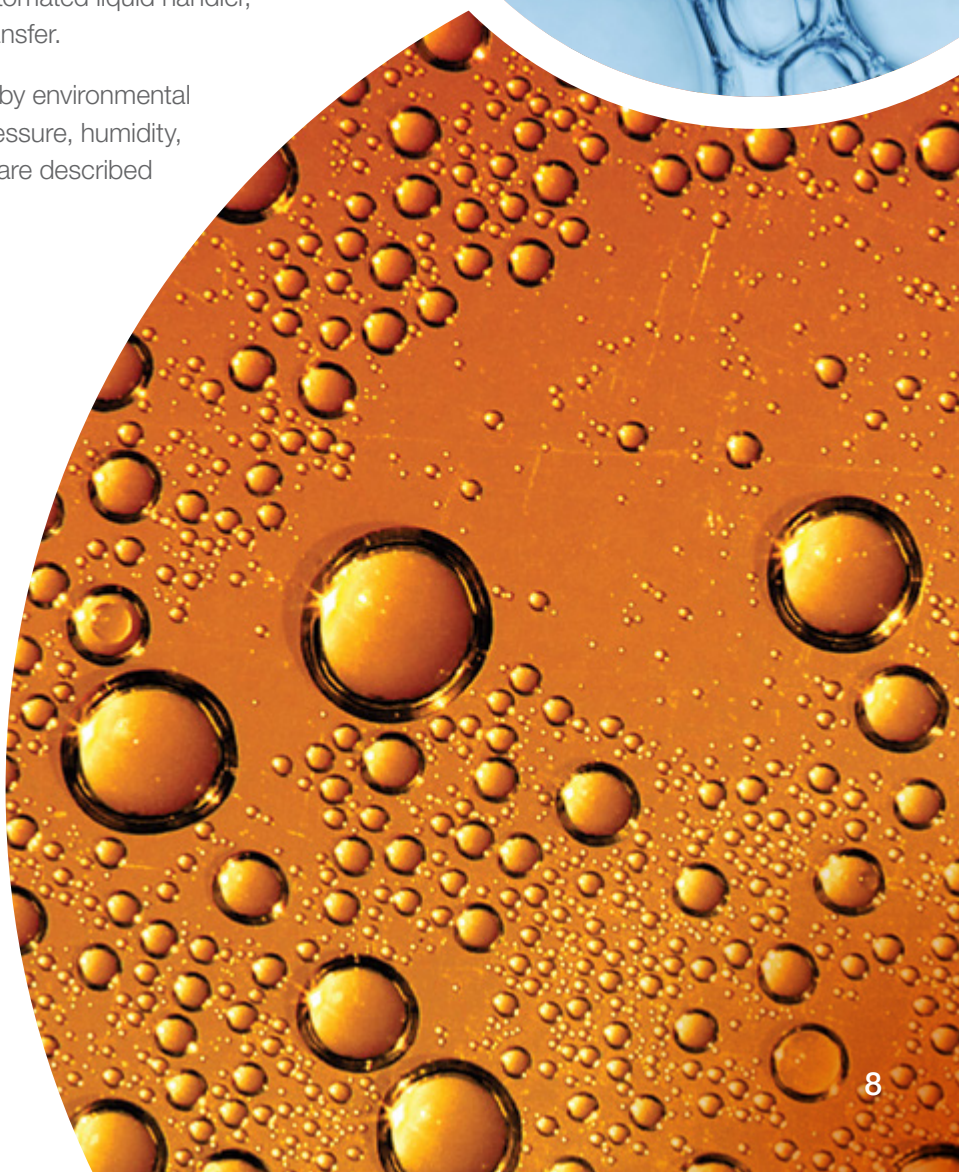
Liquids tested in labs are as varied as the industries they appear in—everything from sticky honey to a fast-flowing petroleum. Depending on what the lab needs to test, these varied liquid types can be transferred by either hand pipettors or Hamilton automated liquid handlers.



Successful liquid transfers involve knowing the type of liquid and accounting for its behavior in a particular environment. A lab tech can rely on experience and intuition to inform how each liquid should be treated. The lab tech learns where to position the tip to make sure there are no bubbles as the liquid dispenses, where to hold the tip to prevent contamination between samples, and how to position the tip and for how long to make sure the liquid dispenses completely.

While humans can readily assess the liquid's attributes and adapt accordingly, an automated liquid handler must know everything about the liquid in advance. The properties in this section all have an impact on the liquid handling information given to the automated liquid handler, which makes all the difference in a successful transfer.

All liquid properties are affected to some degree by environmental conditions such as temperature, atmospheric pressure, humidity, etc. These conditions along with their influences are described in the following sections:



2.3.1 Viscosity

What Is It?

Viscosity describes the flow behavior of a liquid.

High viscosity liquids have a thicker flow and are less fluid. Examples of high viscosity liquids are honey and glycerin.

Low viscosity liquids have a thinner flow and are more fluid. Examples of low viscosity liquids are water and petroleum.

Why It's Important

Viscosity impacts the way a liquid is transferred in very specific ways. The spectrum below shows two example liquids and how to convey the property to the automated liquid handler.

High Viscosity Liquid

Use a **lower flow rate**. If you try to aspirate too quickly, suction in the tip prevents the fluid from entering the tip, resulting in inaccurate volumes.

Use a **surface dispense mode**. Jet dispensing isn't as effective with high viscosity liquids.

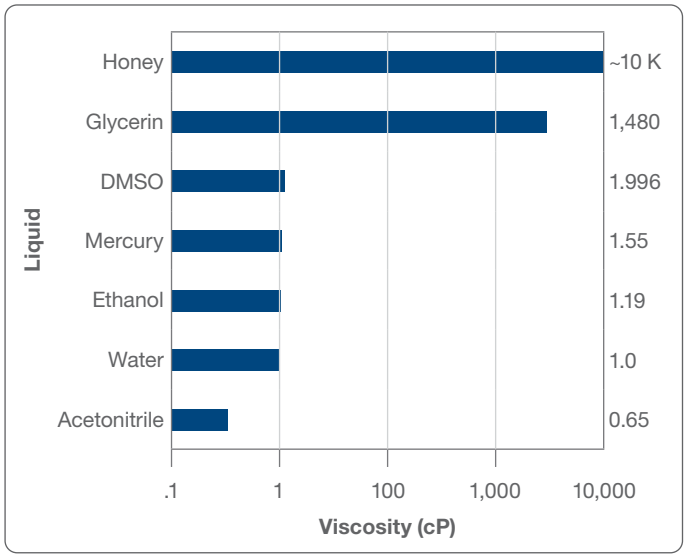
Low Viscosity Liquid

Use a **higher flow rate**. The goal is to get the liquid into the tip as fast and effectively as possible. Low viscosity liquids don't resist entering the tip, so the flow rate can be higher.

Spectrum of Viscosity

The following chart shows a spectrum of viscosity for a variety of liquids. Note that many liquids such as water and ethanol have similar values for viscosity, but when compared to liquids such as glycerin and honey, the scale of the difference is stark.

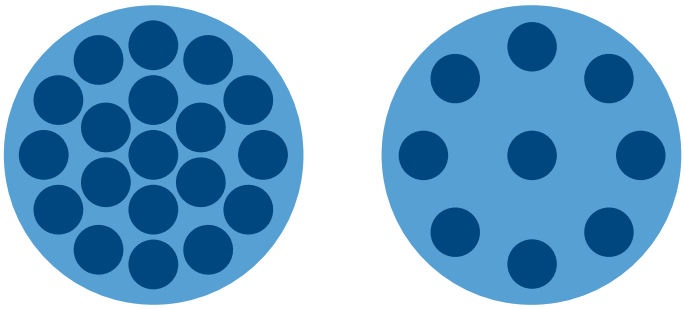
Viscosity of Sample Liquids at 20˚C / 86˚F



2.3.2 Density

What Is It?

Density is a measurement of how much of a substance can occupy a space. A material's density gives the impression of heaviness or lightness. Density is affected by temperature and atmospheric pressure.



High Density **Low Density**

Why It's Important

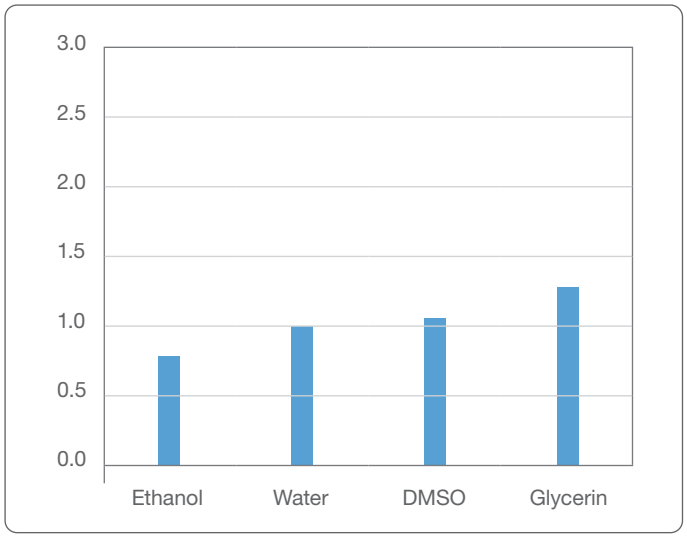
The density value for a liquid is required for measuring liquid transfers gravimetrically. The volumetric mass density of a substance is its mass per unit volume. If the weight of a liquid transfer is determined gravimetrically, then the transfer volume can be determined. Density is often provided on material safety data sheets, but can also be determined empirically.

Mixtures of liquids with different densities may not remain in suspension, which can make it hard to transfer a mixture properly. A suspension is a mixture of two or more substances that essentially become one homogenous liquid. However, some suspensions eventually settle and separate. Liquids with less density rise to the top, while the heavier density liquids fall to the bottom. Many applications require the addition of one liquid to another with the eventual transfer of a single solution. Awareness of the density of different liquids can guide you to mix your samples before aspirating, or aspirating quickly before settling can occur.

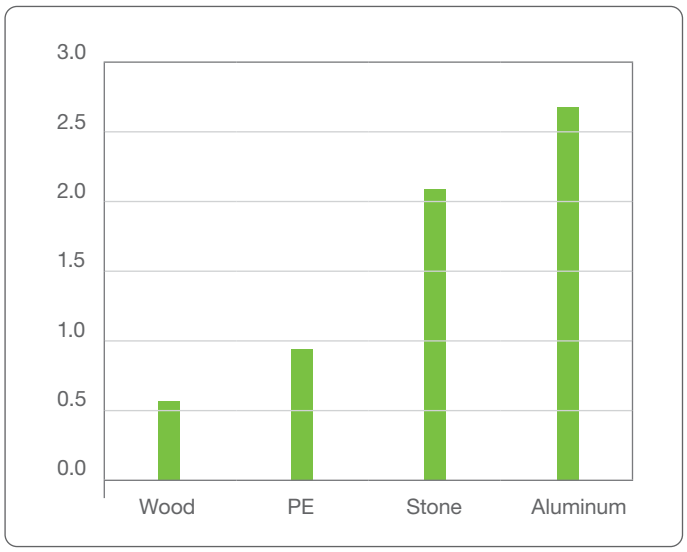
Density Ranges

The density of different liquids and solids is shown below to visualize the range (in g/cm3):

Density of Various Liquids



Density of Various Solids



2.3.3 Adhesion / Cohesion

What Is It?

Adhesion and cohesion are two different ways to describe the stickiness of a liquid. Liquids can be high in one or both with different impacts on the liquid transfer.

Adhesion is the measure of how much the liquid wants to stick to other substances. A high adhesive liquid like glue wants to bind to substances nearby, like tips and labware. A low adhesive liquid like mercury resists nearby substances.

Cohesion is the measure of how much the liquid wants to stick to itself. A high cohesive liquid like mercury wants to bind to itself and remain together. A low cohesive liquid like methane disperses and moves away from itself.

The adhesive and cohesive properties of a liquid are affected by the chemical compatibility of the tips and labware used to make a liquid transfer. Some liquids and materials are attracted to one another while others are inert when placed together. Chemical compatibility information is important to understanding how a liquid will interact with the various parts of the automated liquid handler and labware involved in the transfer.

The pressure of adhesion and cohesion results in capillary action, which causes the liquid to work against gravity. Capillary action occurs when the adhesion of the liquid to the tips or labware is stronger than the cohesive forces between the liquid molecules.

Why It's Important

Adhesion and cohesion impact the way a liquid is transferred in very specific ways. The spectrum below shows two example liquids and how to convey the property to the automated liquid handler.

High Adhesion Liquid

Increase the blowout volume. More force is needed to get a sticky substance out of the tip.

Low Adhesion Liquid

Decrease the blowout volume. The liquid doesn't resist being removed from the tip, so not as much force is needed to remove the liquid from tip.

High Cohesion Liquid

Decrease the air transport volume. Since the liquid wants to stick to itself, less force is needed to hold the liquid in the tip.

May need to increase the blowout volume.

Low Cohesion Liquid

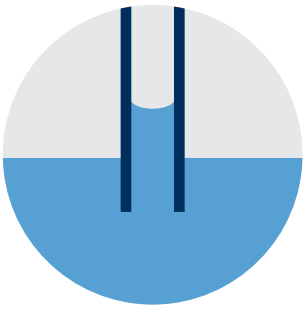
Increase the air transport volume to protect from drips. The increased air transport volume ensures that the liquid has more of an air buffer inside the tip during transport.

2.3.4 Capillary Action

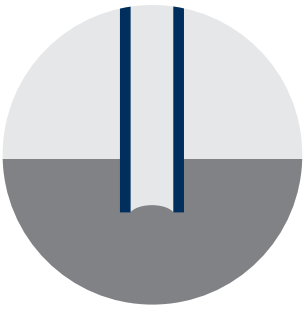
What Is It?

Capillary action is the tendency of a liquid to work against gravity and atmospheric pressure. Liquids with high capillary action can actually climb the tip in small volumes. Capillary action occurs when the adhesion and cohesion qualities of a liquid work together to move the liquid upward. Other properties can impact capillary action, including vapor pressure and environmental variables.

Liquids with low capillary action or any liquid in larger volumes generally are drawn downward by gravity and atmospheric pressure.



In this image, the capillary action of **water** is shown. For water, the adhesion is greater than the cohesion, which results in a meniscus that turns downward and could cause the liquid to move up inside a tip.



Mercury, on the other hand, has high cohesion and low adhesion. It results in a meniscus that turns downward and will not climb into the tip without help from the channel.

Why It's Important

Capillary action impacts the way a liquid is transferred in very specific ways. The spectrum below shows two example liquids and how to convey the property to the automated liquid handler.

High Capillary Action Liquid

Be careful with low volumes. Especially when working with 10 or 50 µL tips, capillary action can have a bigger effect. More liquid can be aspirated than intended.

Low Capillary Action Liquid

No changes to make. Since the liquid has no tendency to climb the tip, nothing extra needs to be taken into account for this factor.

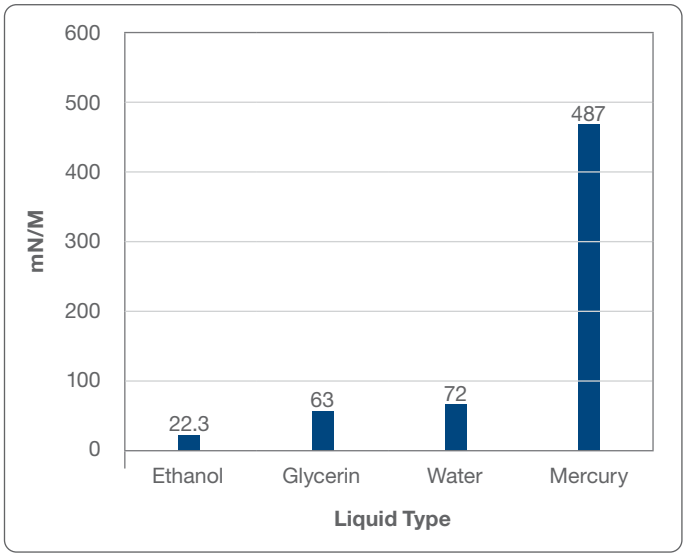
2.3.5 Surface Tension

What Is It?

Surface tension is the elastic tendency of liquids that makes the liquid acquire the least surface area possible. In terms of liquid handling, the surface tension affects how well the molecules at the surface of the liquid cohere to each other and to the walls of the tip.

The chart shows the surface tension of various liquids. The higher the number, the higher the surface tension and the more likely the liquid is to form a strong cohesion at the surface of the liquid.

Surface Tension of Various Liquids



Why It's Important

Surface tension impacts the way a liquid is transferred in very specific ways. The spectrum below shows two example liquids and how to convey the property to the automated liquid handler.

High Surface Tension Liquid

Use a **lower air transport volume**. Liquids with high surface tension stay in the tip and don't require a large air buffer to hold the liquid in.

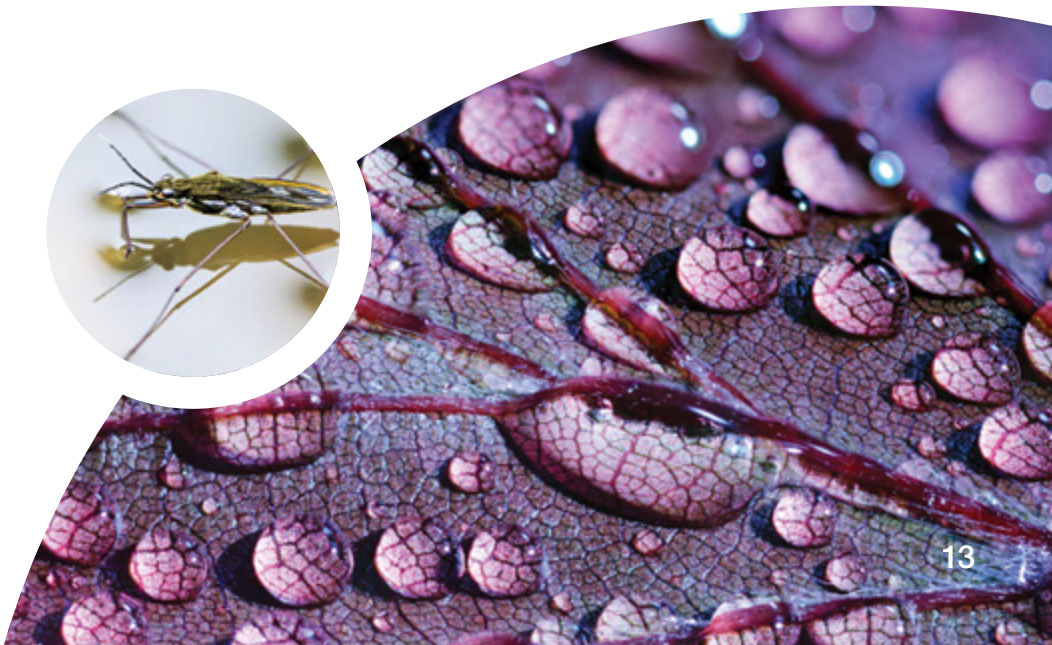
Use a **high swap speed** to break the connection between the liquid in the well or tube and the tip.

Use **side touch** or **minimize the distance from the end of the tip and the labware** to allow the liquid to be more easily removed from the tip.

Low Surface Tension Liquid

Increase the air transport volume. Since the liquid wants to leave the tip, increasing the transport volume provides a greater air buffer in the tip.

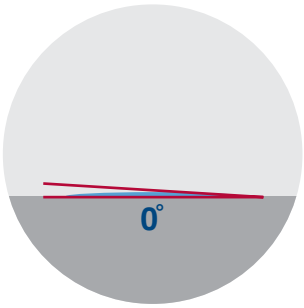
Decrease the settling time to minimize the amount of time the tip is in contact with the liquid and prevent time for droplet formation.



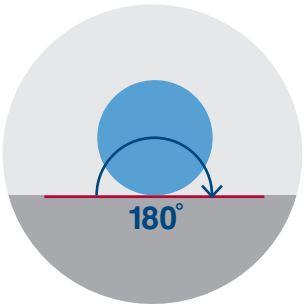
2.3.6 Contact Angle

What Is It?

The contact angle is the angle the liquid makes to the surface of the labware.



A low contact angle like 0° makes it harder for low volumes to be transferred, since the liquid spreads out very thinly.

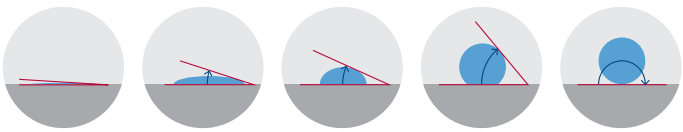


A high contact angle like 180° causes the liquid to stand taller in smaller volumes, but can make it difficult to aspirate and dispense since the cohesion can be repellent.

The contact angle is also affected by the type of surface where the liquid sits. See below for how different surfaces are characterized:

- On a hydrophilic surface, the contact angle will be low (0°).
- On a hydrophobic surface, the contact angle will be high (90°).
- On a super hydrophobic surface, a contact angle will be higher than 160°.

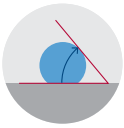
The figure below shows a variety of contact angles, ranging from 0° to 180°.



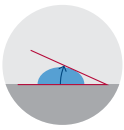
0° Angle **180° Angle**

Examples of Contact Angles

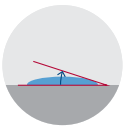
The below images show varying contact angles on different surfaces.



100 µL water on hydrophobic coated plastic has a contact angle of ~130°.



100 µL water on untreated plastic has a contact angle of ~90°.



Water on aluminum surface.

Ethanol on aluminum surface. The contact angle is lower than water.

Why It's Important

The contact angle impacts the way a liquid is transferred in very specific ways. The spectrum below shows two example liquids and how to convey the property to the automated liquid handler.

High Contact Angle

Make sure the tip enters the liquid properly. If the contact angle is high, the liquid might deform around the tip and cause issues with the volume.

Low Contact Angle

Be careful with low volumes. A liquid with a low contact angle may be hard to aspirate in low volumes because the height of the liquid is minimal.

Use **bottom touch** off during aspiration and dispense to reach the very bottom of the well or tube.

Consider using **side touch** during dispense.

2.3.7 Vapor Pressure

What Is It?

Vapor pressure is the pressure created by vapor above a liquid's surface. As atmospheric pressure pushes downward, the liquid's vapor pressure is pushing upward.

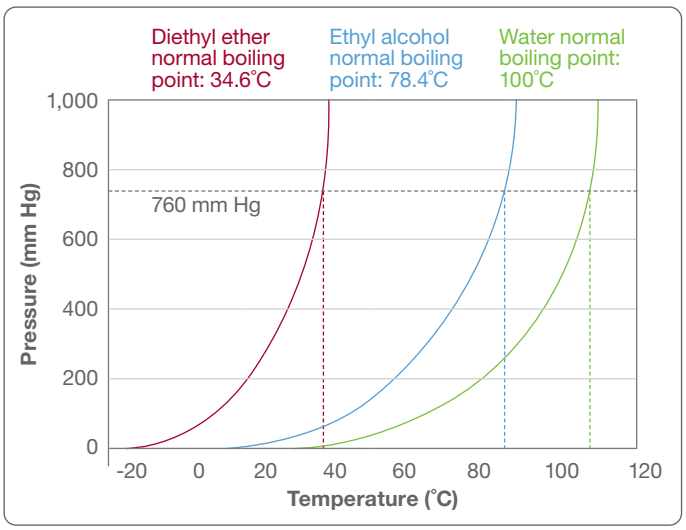
Liquids with **high vapor pressure** off-gas since the atmospheric pressure isn't enough to hold them down.

Liquids with **low vapor pressure** don't off-gas or try to escape into the air.

Liquids with high vapor pressure also tend to evaporate quickly. A substance with a high vapor pressure at normal temperatures is referred to as volatile. Liquids with high vapor pressure include alcohols and ethers.

The chart below shows a temperature and pressure comparison for three liquids. Note that 760 mmHg is atmospheric pressure.

Vapor Pressure Curves



Environmental properties in the lab can have a large impact on vapor pressure. Changes in the elevation alter the atmospheric pressure, which can in turn change when the liquid begins to off-gas.

Additionally, if the temperature of the liquid increases, the vapor pressure also increases, just as water boils and turns to steam.

Why It's Important

Vapor pressure impacts the way a liquid is transferred in very specific ways. The spectrum below shows two example liquids and how to convey the property to the automated liquid handler.

High Vapor Pressure Liquid

Use **Anti-Droplet Control**. Liquids with high vapor pressure are more likely to drip.

Pre-wet the tip. The processing of pre-wetting involves aspirating and immediately dispensing some liquid to allow the tip's vapor pressure to adjust, then aspirating enough volume for the liquid transfer.

Use a **larger blowout volume** to allow room for the vapor.

Low Vapor Pressure Liquid

No changes to make. Since the liquid isn't likely to drip based on off-gassing, nothing extra needs to be taken into account for this property.

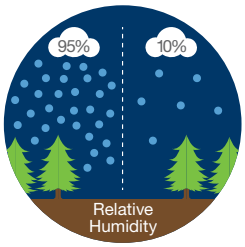
2.3.8 Environmental Influences

What Is It?

Environmental influences are properties of the space around the liquid that impact the behavior of the liquid itself.

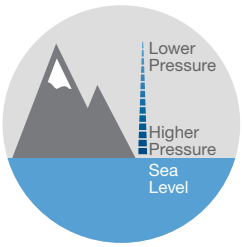
Here are some of the important environmental influences to consider along with their effects on liquids:

Humidity



- Low humidity results in a higher evaporation rate since there is less liquid in the air. There may also be higher instances of static electricity, which could impact low volume pipetting.
- High humidity results in a lower evaporation rate since the air is already saturated.

Atmospheric Pressure

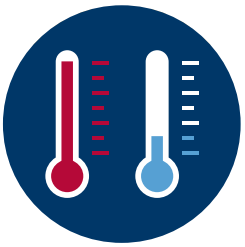


- A decrease in pressure increases the rate of evaporation.
- Closely linked to vapor pressure.
- Atmospheric pressure changes with the elevation. For example, if creating a liquid class at sea level, test the liquid class again before using it at a lab at 6,000 feet elevation.

Radiation

- Changes the chemical properties of a substance.

Temperature



- All liquid properties can shift with temperature, even when the change is just a few degrees: vapor pressure, atmospheric pressure, density, viscosity.
- It is recommended to control for temperature in the lab.
- Always develop liquid classes at common lab temperatures. Record the temperature during development.

Vibrations

- Vibrations can cause drops to form.
- Ideal to have no vibrations, especially during gravimetric measurements.

Why It's Important

The automated liquid handler can only execute a programmed sequence of activities for a set liquid class. Any changes in the environment that alter the liquid properties without the automated liquid handler knowing jeopardize the quality of the liquid transfer.

For example, if the temperature in a lab is usually controlled, but one day spikes due to an issue with the air conditioner, there could be a change in the liquid transfer due to the increased heat.

If the environmental conditions change, the performance of the liquid transfers must be confirmed again.

3 Automated Liquid Handling Methodology

The same general process applies when developing liquid handling methods across all labs, industries, and applications. Even though the details change, the liquids fundamentally need to be defined and optimized for use on the automated liquid handler.

The methodology explored in the subsequent pages walks you through the high level steps needed to develop optimized liquid handling performance, including best practices for common liquid handling activities.

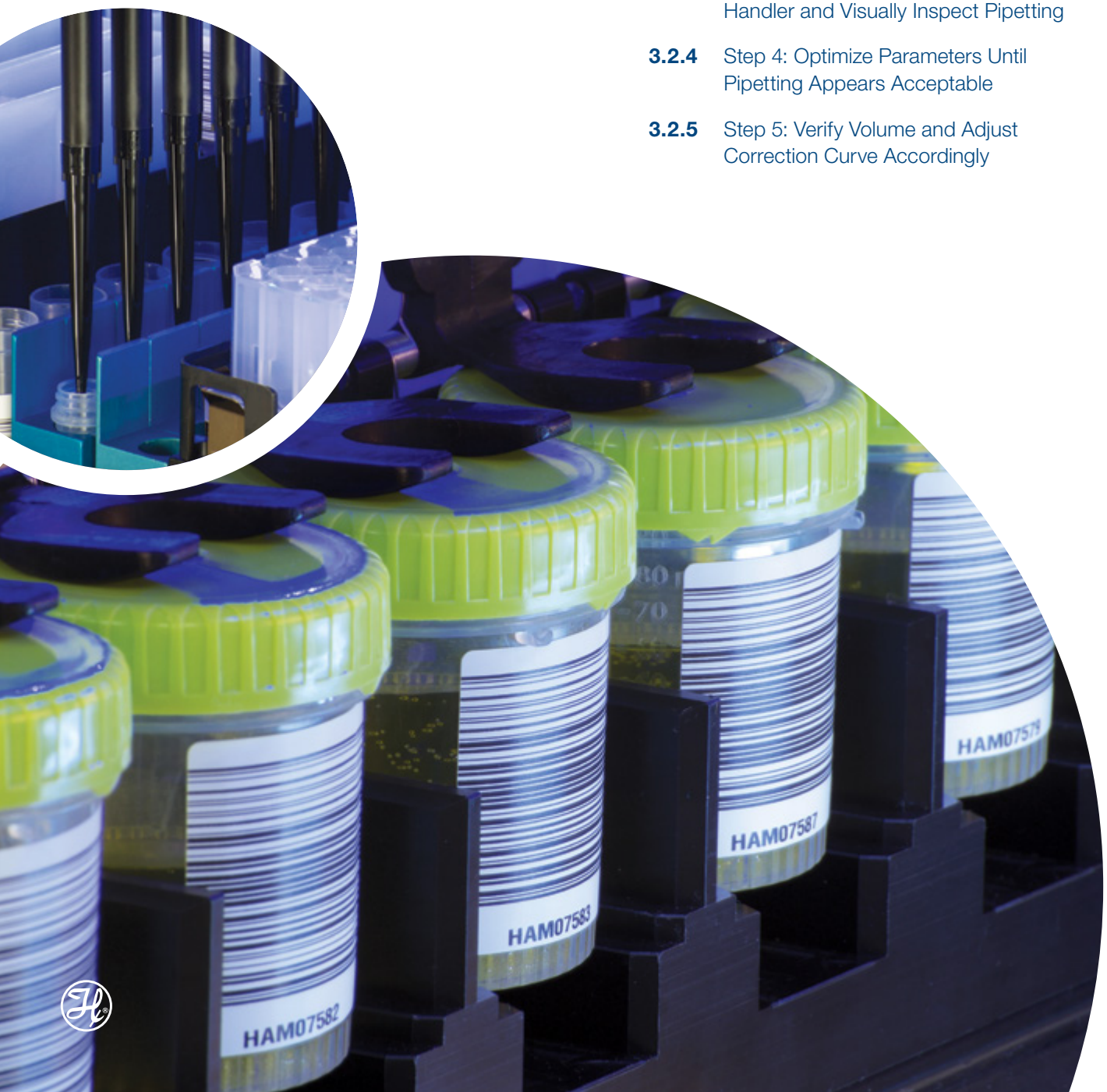
3.1 Before You Begin

Make sure that the following items are ready to go before getting started:

- The automated liquid handler needs to be installed, set up, and ready for use.
- The liquids for the application should be known, available, and brought to the desired temperature for the application.
- The tips and labware for the application should be known and available.

3.2 Steps for Setting Up Automated Liquid Handling

See the next sub-sections for a more in depth look at these steps for setting up automated liquid handling.



3.2.1 Step 1: Understand Properties of Liquid

The first step in the methodology is a careful examination of the physical properties of the liquid to be transferred. The purpose of the examination is to determine how the liquid behaves so you can get a sense for how to teach the automated liquid handler to handle it. For example, you could use a hand pipettor to pipette the liquid and get a feel for how it transfers before testing it in the automated liquid handler.

You can also refer to related reference documentation to get the specifics. Common resources include the MSDS (Material Safety Data Sheets) and online resources like wolframalpha.com.

For a detailed list of physical properties to analyze and their implications, refer to Section 2.3 Liquid Properties.

3.2.2 Step 2: Select a Predefined Liquid Class

All Hamilton software platforms come with predefined liquid classes. These liquid classes can be implemented in methods or used as a starting point for developing a more precise liquid class for a specific application.

When selecting a liquid class for use in a method or developing a new liquid class, always choose the liquid class with the closest match to the liquid. A suitable liquid class allows the automated liquid handler to know how to interact with the liquid and prevent mishandling.

For example, if developing a new liquid class for Master Mix, use the existing water definition as a starting point (green row) since both liquids are part aqueous and will have similar properties.

Examples of Synonymous Liquid Classes

Aqueous Solution	Volatile Organic Solvents	Involatile Organic Solvents	Viscous Liquid	Blood Products
Water	*Ethanol*	*DMSO*	*Glycerol*	*Serum*
PBS	Isopropanol	Alkane	Beads	Plasma
Master Mix	Acetone		Oil	Whole Blood
TRIS	Formaldehyde			Red Blood Cell

3.2.3 Step 3: Run Test and Visually Inspect Pipetting

Once the predefined liquid class is selected, it's time to test the liquid transfer on the robot. The predefined liquid class is used as a baseline to speed up the process of testing.

1. Set up the automated liquid handler with the appropriate source and destination labware.
2. Fill the labware with the liquid that you intend to transfer. If the liquid in question is costly or in short supply, use water for initial testing and optimization.
3. In the automated liquid handler's software, build a simple method to transfer the liquid that will mimic the step in the actual process. Focus on one transfer step in the process before testing the next. Make sure that the method settings are defined as you want them for the actual transfer.
4. Run the method and observe the transfer. Look for the following:
    a. Is the aspirate height or liquid level submerge depth too high/low?
    b. Are there any droplets on the end of the tips after aspiration?
    c. Is the dispense height or liquid level submerge depth too high/low?
    d. Are there any droplets on the end of the tips after dispense?
    e. Are the channels properly following the liquid level during aspiration and dispense? Should following be turned off?

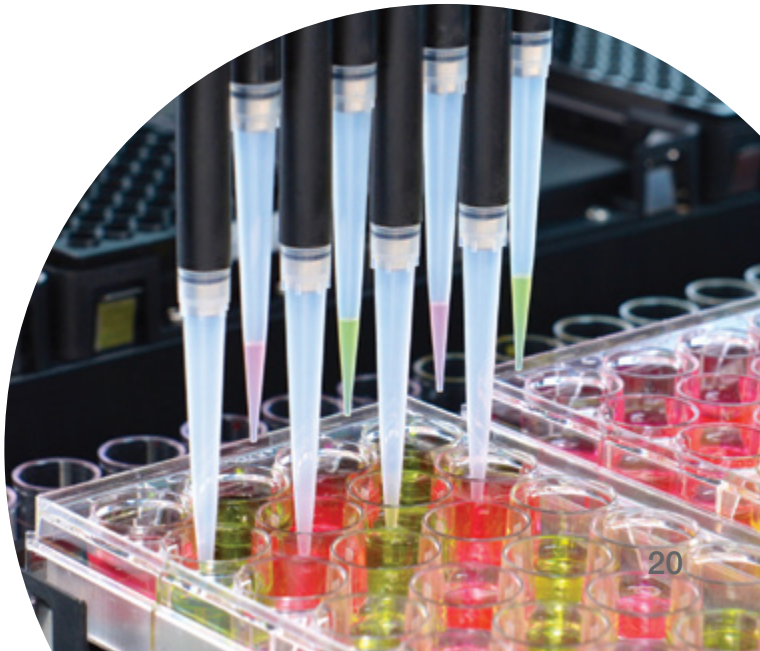
3.2.4 Step 4: Optimize Parameters Until Pipetting Appears Acceptable

Continue to run the simple method, observe the pipetting, and make adjustments based on what you see. The goal is to make sure that the pipetting looks correct. For example, there should be no dripping from the tip and no bubbles on dispense.

Start by making modifications to method settings such as enabling cLLD on the aspirate or adjusting the fixed height.

If the method settings are optimized, but the transfer still appears inconsistent, you can then focus on modifying the liquid class settings to improve performance. Follow these steps to adjust the liquid class:

1. Save the liquid class under another name. Now it can be modified to work for the specific application.
2. Change one liquid class parameter at a time to see its effect on the liquid transfer.
3. Inspect transfers.
    a. Visually inspect for consistent transfers.
    b. Spot check with a handheld pipette to give an indication of consistency and if the transferred volume is short or in excess
4. Once the transfers look consistent, move on to step 5.



3.2.5 Step 5: Verify Volumes and Adjust Correction Curve Accordingly

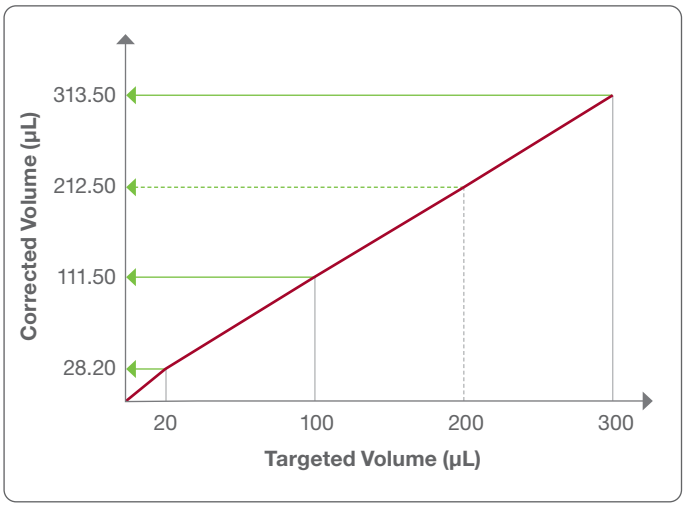
Once the pipetting appears to be acceptable, the transferred volumes can be quantified to determine the precision and the trueness of the liquid transfers.

Before measuring, it is important to know your application's pipetting requirements to make sure that the final optimizations meet the need. If the requirements are unknown and you want to minimize the amount of variability that pipetting contributes to your application, you can strive to match the specifications set for the pipetting device you are using.

You might measure volumes gravimetrically to make sure the transfers are both accurate and precise. Then, you can continue to adjust liquid class settings until optimal precision is achieved.

When precision is achieved, the correction curve of the liquid class can be adjusted to ensure trueness of all volumes of interest for your application. For example, if you are transferring a volume of 300 µL, but are measuring a value of 295 µL, then you can increase the corrected value in the liquid class by an additional 5 µL. Continue to adjust until the proper target volume is achieved.

Corrected Volume and Targeted Volume



Once verification is complete, make sure the updated liquid class is implemented in your method. The liquid class can then be used by lab technicians running liquid transfers for experiments. Periodically check the performance of the liquid transfers to make sure that no changes are needed.

Keep in mind that the correction curve for any default liquid classes cannot be modified.

4 Common Examples and Best Practices

Certain types of liquid handling activities are common across most labs and can be a challenge to resolve. This section contains best practices and troubleshooting advice for these types of common liquid handling activities.

4.1 Best Practices for Pipetting Low Volumes Using 1,000 µL Channels

Low volume pipetting using the 1,000 µL channels is defined as any volume between 0.5 and 20 µL.

Method Settings

- Use 50 µL tips
- Single dispense if possible
- Use surface empty mode
- Keep distance between tip and pipetting surface minimal without blocking the tip
- Liquid following turned off
- Use new tips for each transfer

Liquid Class Parameters

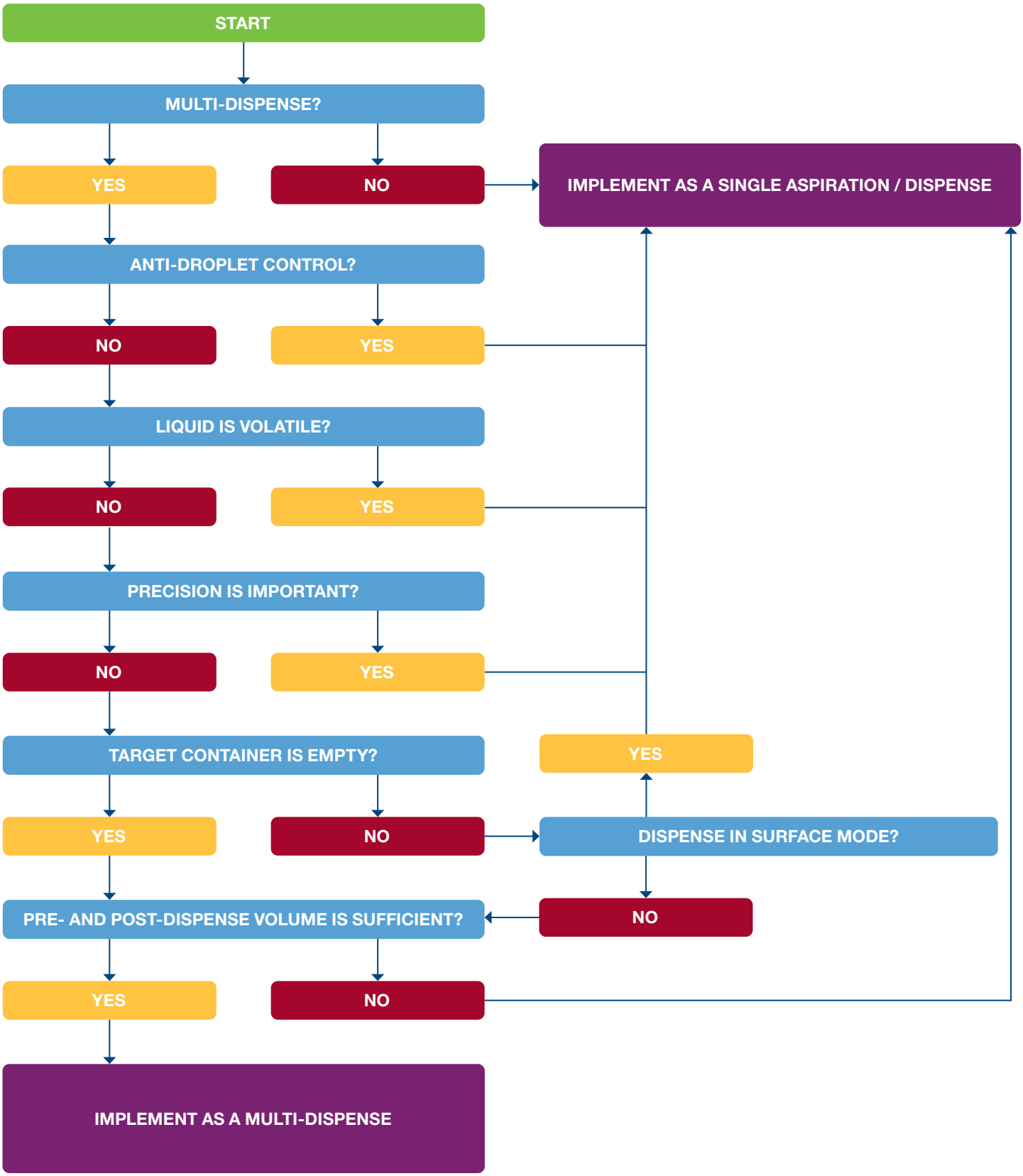
- May require an increase in settling time
- Slow swap speeds
- Increase blowout volume on aspiration, but minimize on dispense. Prevents capillary action by creating an overpressure

4.2 Best Practices for Multi-Dispensing (Aliquot)

Multi-dispensing, also known as aliquotting, is the act of aspirating enough total volume to dispense more than once from the same tip. While multi-dispensing can save time and tips, it can take more effort to control for the trueness and precision for each liquid transfer.

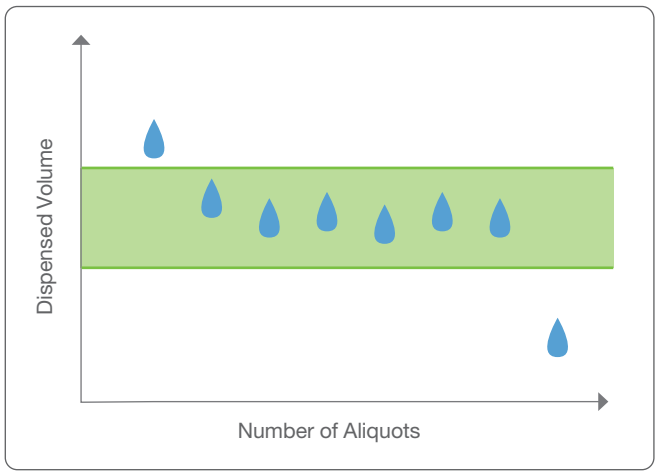
To decide if pipetting should be implemented as a multi-dispense, refer to the chart on the next page.

Determine if Pipetting Should Be Implemented as a Multi-Dispense



Method Settings

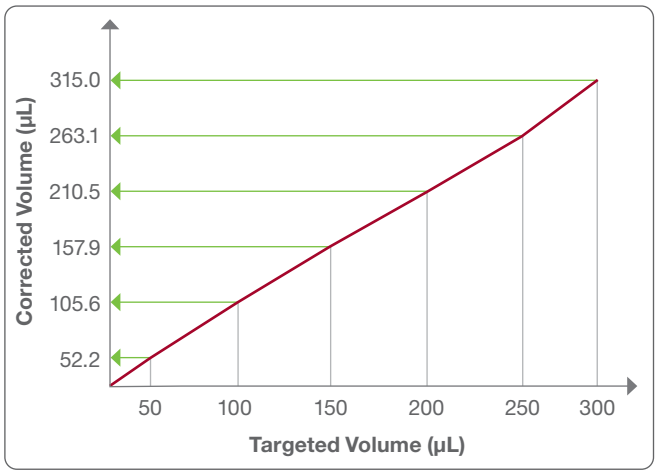
- Only use 50 µL tips or higher.
- The first and last aliquots are often inaccurate and should be discarded in order to maintain good precision overall amongst the transfers. Some liquids may require 2 pre-dispenses. See the image below for an example of a pre- and post-aliquot transfer that are discarded, ensuring that the transfer in between are within an acceptable range.



- Limit total number of aliquots per cycle.
  - The limit can vary based on transfer volume and tip size, but in general, it is recommended to keep the number of transfers to a minimum for easier control and more consistent liquid handling.
  - Recommend restricting the number of transfers for ease of method programming. For example, if pipetting to a 96-well format plate with an 8-channel automated liquid handler, limit the number of transfers steps to 6 (half the plate) or 12 (one whole plate).
- Pre-wet the tip on first aspirate. This action can nullify the effects of the correction curve and allow for consistent transfers.
- Use “minimize z-move step” function to prevent the pipettes from moving to a default traverse height in between transfers, increasing the speed of the transfers.
  - Set dispense height equal to the clearance height.
- To maximize speed, use “dispense on the fly” function to prevent the pipettes from stopping during the x and z-movement in between transfers. This function can be challenging to optimize.

Liquid Class Parameters

- Increase stop back flow rate to match dispense flow rate.
- Use a small stop back volume (dry dispense only).
- For the correction curve, it is important to note the total volume aspirated and then use a step down approach to determine the correction for each transfer.
  - For example, to transfer six aliquots of 50 µL using 300 µL tips, do not adjust for trueness at the 50 µL target volume like one would for a single transfer. Instead, adjust the target volume of 300 µL for the first transfer, 250 µL for the next, and so on. Refer to the table below.
  - Use more correction curve points to increase the trueness of every individual liquid transfer. The overall precision amongst all transfers would then improve.



Correction Curve

Target in (µL)	Corrected in (µL)
<New Entry>	
0.00	0.00
50.00	52.2
100.00	105.6
150.00	157.9
200.00	210.5
250.00	263.1
300.00	309.0

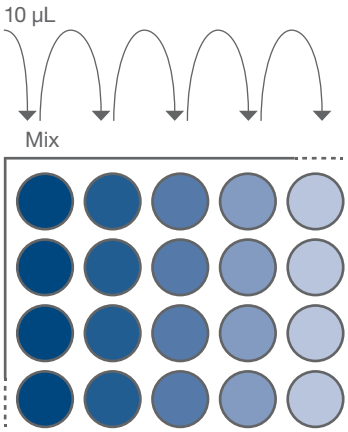
250.0 | 263.1 µL

Remove | New | Change

4.3 Best Practices for Serial Dilution

A dilution is one of the more common liquid transfers performed and occurs when a sample liquid is added to another liquid, decreasing its concentration. A serial dilution repeats this process over multiple steps in order to test samples at a variety of concentrations and/or to arrive at very low final concentration.

For example, 90 µL of buffer solution is added to all of the wells in a 96-well plate. 10 µL of sample is then added to column 1, mixed, and then 10 µL of that mixture is transferred to column 2, mixed, and so on. Each step dilutes the sample by a factor of 10.



Method Settings

- Use a new tip for each transfer.
- Dispense to surface if possible.
- Mix very well before and/or after each transfer. Start with many mix cycles and decrease only to optimize for speed if performance allows.
- Mix at a fixed position to optimize the dispense and mix heights.
- If using LLD and liquid following, properly defined labware is critical to ensure proper following and mixing.
- Mix volume should be less than 80% of total volume in the well. If the mix volume is larger than 80%, it increases the chance of aspirating air and creating bubbles.
- Be mindful of retract distance for air transport if dispensing at fixed height. If the retract distance and the fixed height are set too low, then it is possible that the tip may not be removed from the liquid before it aspirates the air transport volume. This results in the possibility of extra liquid being aspirated instead of air.

Liquid Class Parameters

- Depending upon dilution factors, different liquid classes for each step may be required.
- Remove the over-aspirate volume parameter, because it could lead to excess carryover after subsequent dispense and mixing.

5 Measure Liquid Transfers

Once the liquid handling is complete, it's valuable to be able to double check the automated liquid handler and make sure that the liquid was transferred at the correct volume. This Section describes how to measure the liquid transfer to ensure success. Liquids can be measured through a wide range of tests, from simple visual checks to more complex measurements that involve dyes or special scales.

Keep in mind that measurement described in this Section only provides you with a measure of the volume transferred.

Visual — A qualitative approach and not a trustworthy measure.

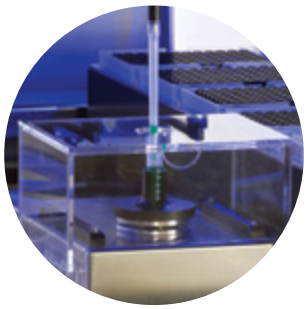
Using just your eyes, confirm that the transfers appear to be uniform.

Manual — A qualitative approach and not always a trustworthy source.

Using a hand pipette, aspirate what was dispensed by the instrument. Visually inspect for confirmation.

Photometric / Fluorometric — A valid and quantitative approach.

Add dye to the liquid and verify using a plate reader. Testing can generally be done in the actual assay labware (plates).



Gravimetric — A valid and quantitative approach.

Use an analytic balance to measure the mass of material dispensed.

The purpose of measuring liquid transfers is to check for a "true" and "precise" result. Each automated liquid handler has expected performance at various liquid volumes and tip sizes. These specifications can be used as a reference during verification, but keep in mind that differences in laboratory conditions, pipetting approaches, and liquid types can result in different performance. It is a good practice to consider the pipetting specifications to be the best case scenario and to consider your own method's pipetting performance requirements when evaluating verification results.

Trueness $\%R = 100 \times \left(\frac{V_M - V_T}{V_T} \right)$

Trueness is defined as Yield (% trueness / bias). It is a percent error between the average volume of solution measured and the expected or accepted value. A result of high trueness is equivalent to a small percent error.

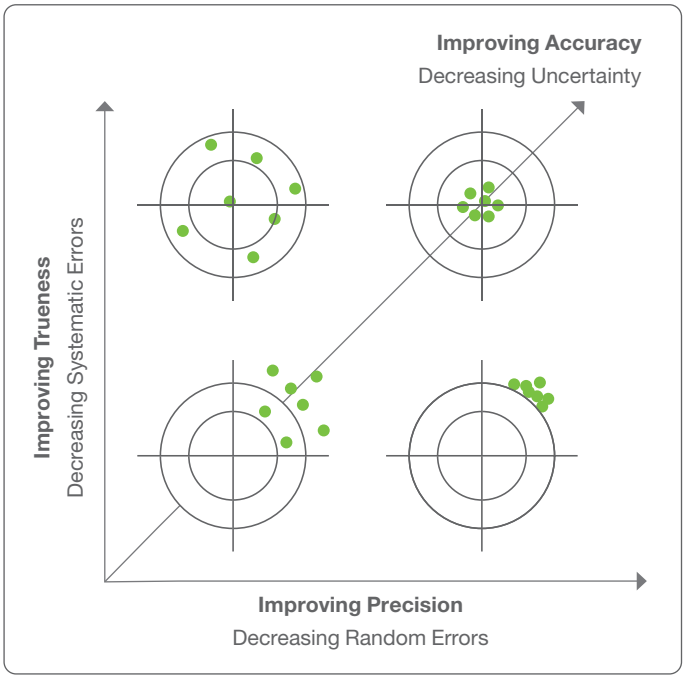
Precision $\%CV = 100 \times \frac{SD}{Mean}$

Precision is defined as Reproducibility (% CV). It is the closeness of a set of values obtained from identical measurements of the same volume. A high amount of precision is equivalent to a small percent coefficient of variation.

In addition to trueness and precision, the term "accuracy" is often used in discussions about automated liquid handlers. In 2015, liquid handler manufacturers defined and standardized the terms to be used across the industry. The manufacturers also standardized the volumetric performance determination for Automated Liquid Handling Systems (ALHS). For reference, the standardization decisions are logged in ISO International Workshop Agreement (IWA) 15 titled "Specification and method for the determination of performance of automated liquid handling systems."

ISO IWA 15 describes accuracy as the relationship between trueness and precision. Definitions from the ISO document include the following:

- **Trueness** — An average which is very close to the true value. As trueness improves, there is a decrease in systematic errors.
- **Precision** — Highly consistent results. As precision improves, random errors decrease.
- **Accuracy** — Knowing that each measurement correctly represents what is present in the sample. As accuracy is obtained, uncertainty is decreased.



5.1 Photometric Measurement

A photometric approach uses a dye and a plate reader to analyze results. Add dye to the liquid and verify using a plate reader. Testing can generally be done in the actual assay labware (plates). Keep in mind that it can be difficult to choose an appropriate dye for the specific test, and dyes can alter physical characteristics of the solutions being tested.

Advantages

- Allows testing to be done in the actual labware used in the process, which makes for a truer comparison.
- Is good at gathering large amounts of data. For example, photometric measurement is useful for testing with a 96 or 384 multi-probe head since all transfers can be performed and measured at the same time. Each well in a 96- or 384-well plate corresponds to a channel on the multi-probe head. Since an absorbance value is collected per well, the transfer volume can be determined per channel.
- Is the only method that works for liquid filled systems because of a potential dilution effect that cannot be detected by weighing the sample. In contrast to the gravimetric approach, the plate reader absorbance read could detect unwanted dilution relative to reference standard or manually pipetted transfer of the sample. Since the photometric approach is required for liquid filled systems, it is therefore a popular and accepted standard of measurement for any type of automated liquid handling system.
- Works with off-the-shelf products and readily available laboratory equipment. Options exist to purchase a ready-made solution or create your own at less cost.

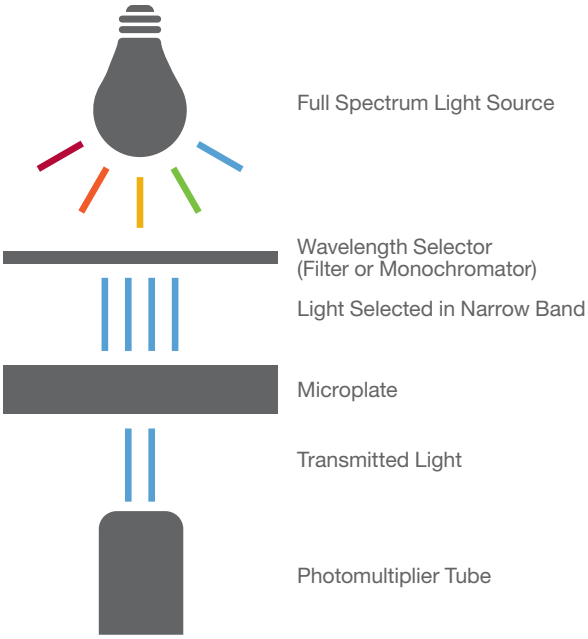
Disadvantages

- Can be challenging to develop a test from scratch, since it requires identifying the optimal wavelength, using and/or acquiring an appropriate reader, and setting up the necessary calculations. For reference, the photometric measurement process is described in detail below.
- Can be difficult to choose an appropriate dye for the specific test. The addition of dyes to the liquid can alter the physical characteristics. This makes the liquid type not truly representative of the one you are trying to measure.
- Can limit the amount volume to be tested. The transfer volume may be limited to the labware required for analysis in the plate reader. A microplate typically holds about 300 µL of liquid. If more volume is required, additional testing procedures must be used that are different from the liquid transfer settings in the method.
- Requires the use of a plate reader and further calculations in order to obtain results. Data collection can therefore be slower using this approach.
- Not ideal for small volumes under 10 µL. The path length for the light to travel through the liquid would be minimal and the approach may not be sensitive enough for reliable measurement. This depends especially upon well geometry. To avoid this issue, small volumes may require additional buffer, which creates additional variables. Such variables include the potential for uneven path lengths and variable path length and concentration which complicates the application or Beer's Law.

Alternatives to the photometric measurement include the fluorometric approach, which can help address some of these disadvantages.

How to Perform Photometric Measurement

1. Obtain a dye and known optimal wavelength and concentration for use. If unknown, determine it using spectral analysis of the color. Measure with scanning spectrometer (i.e. Molecular Devices Spectramax®) when possible to determine optimal wavelength and concentration. If not possible, guess based on dye color: (Yellow dye transmits yellow light, so it absorbs blue (450 nm)).
2. Use a flat bottom plate for testing. For example, a round well plate can cause issues with the test due to light reflection and refraction on the curved part of the well. If needed, other plate types can be accommodated, but it takes modification in the reader software and equation.
3. Test several concentrations to determine reader saturation point and aim for a test concentration 50% to 75% of saturation.



4. Apply Beer's Law to determine volume: Absorbance (Abs) = eLc. Refer to the instructions below for details on the application of Beer's Law. Use it to create a standard curve around the value of interest.

Beer's Law

Abs = eLc
e = extinction coefficient
L = pathlength (depth of liquid in well)
c = concentration of dye

5. Create a standard curve with same dye solution that is being used for unknown volumes. This allows constants to cancel out. The curve is obtained by collecting data below and above the volume of interest and fitting a linear curve to determine the slope and offset.

6. Additional detail on Beer's Law:

    1. Because e and c are constants that do not change from well to well, they can be viewed as a single proportionality constant, k, so Beer's Law is simplified to: **Abs = k* Volume**

    2. Using the curve fit parameters, the slope is the value for k.

    3. There also exists an offset due to absorbance within the plate material itself that is the y-intercept.

7. Apply the curve to calculate unknown data.

    1. Read a plate containing wells of liquid transfers of the volume to be tested. These are considered the unknowns in this test.

    2. Apply curve values to calculate unknown data.

Example Where the Transfer Volume of Interest is 50 µL:

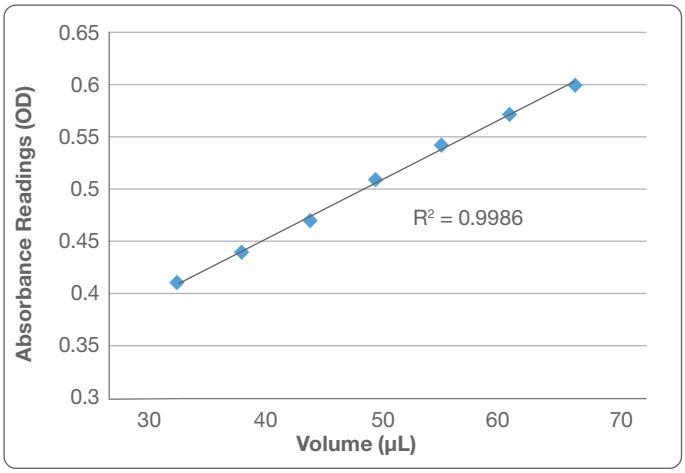
1. First, absorbance data is collected for several volumes of dye transfers below and above 50 µL. Specifically, 30, 40, 50, 60, and 70 µL.

Raw Data From Reader and Average of Replicates

Volume	Row	Rep 1	Rep 2	Rep 3	Average
35	A	0.4076	0.4121	0.4113	0.41033
40	B	0.4381	0.4412	0.4421	0.44046
45	C	0.4701	0.4693	0.4692	0.46953
50	D	0.5072	0.5102	0.5093	0.50890
55	E	0.5418	0.5406	0.5399	0.54076
60	F	0.5721	0.5693	0.5704	0.57060
65	G	0.6013	0.6004	0.5989	0.60020

2. A linear curve is fitted to determine the slope and offset.

    y = 0.0064x + 0.184 where **y** is the Absorbance and **x** is the Volume



3. A plate of unknown 50µL liquid dye transfers is read collecting absorbance values for each transfer.

Raw Data From Reader

Row	1	2	3	4	5	6	7	8	9	10	11	12
A	0.5176	0.5153	0.5040	0.5045	0.5029	0.5102	0.5015	0.5090	0.5133	0.5164	0.5143	0.5048
B	0.5050	0.5087	0.5042	0.5146	0.5015	0.5078	0.5144	0.5151	0.5162	0.5011	0.5006	0.5017
C	0.5075	0.5145	0.5142	0.5091	0.5096	0.5051	0.5176	0.5169	0.5064	0.5076	0.5133	0.5143
D	0.5178	0.5047	0.5035	0.5130	0.5014	0.5033	0.5117	0.5095	0.5058	0.5032	0.5013	0.5113
E	0.5035	0.5180	0.5073	0.5131	0.5120	0.5158	0.5128	0.5066	0.5127	0.5055	0.5088	0.5096
F	0.5181	0.5099	0.5176	0.5140	0.5074	0.5035	0.5138	0.5152	0.5120	0.5110	0.5058	0.5108
G	0.5030	0.5177	0.5090	0.5079	0.5176	0.5048	0.5176	0.5172	0.5101	0.5021	0.5171	0.5025
H	0.5142	0.5149	0.5059	0.5022	0.5170	0.5057	0.5057	0.5005	0.5046	0.5044	0.5119	0.5126

4. The curve values are applied to the unknown absorbance values to determine the volume for each transfer.

Average	50.76435
STDEV	0.841395
% CV	1.657453
% Trueness	1.5287

Calculated Data and Summary Statistics

Row	1	2	3	4	5	6	7	8	9	10	11	12
A	50.08	49.96	51.73	51.55	51.56	50.92	50.71	51.10	51.85	52.26	50.26	51.88
B	50.50	51.82	50.36	51.40	51.49	51.61	51.02	49.74	52.26	52.13	50.50	50.57
C	49.97	49.65	50.08	50.11	51.23	49.55	49.80	49.97	51.20	50.84	51.49	50.67
D	51.19	51.39	52.18	49.65	49.48	50.33	51.08	49.77	51.66	51.40	49.71	52.03
E	51.42	51.54	50.62	51.62	50.79	52.03	49.62	50.37	51.53	49.53	49.97	50.04
F	50.44	50.48	50.81	49.49	50.34	51.79	50.23	51.39	50.41	51.61	51.16	50.48
G	49.62	50.52	52.09	49.80	51.57	51.66	50.49	50.43	50.48	50.81	49.72	52.20
H	51.99	49.44	49.66	49.87	50.01	49.85	49.61	52.10	51.38	49.87	50.25	50.56

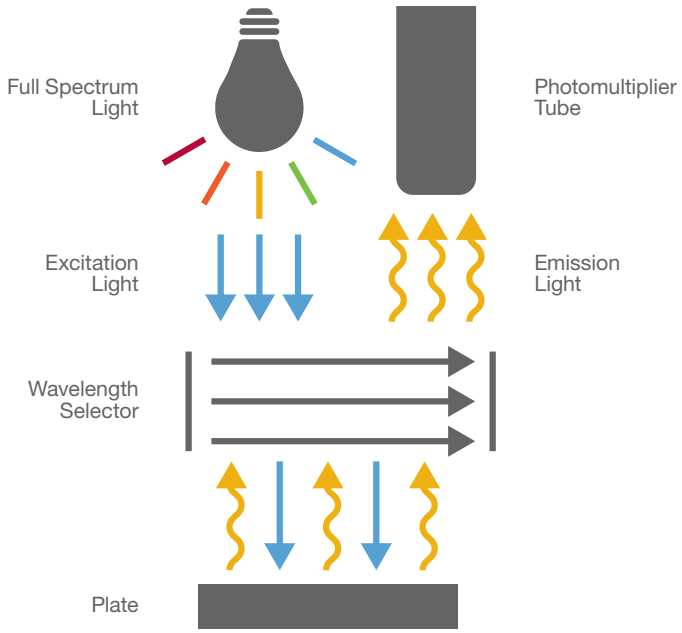
5.2 Fluorometric Measurement

Fluorometric measurement is similar to photometric approaches, because both types of measurement require light to be directed onto the sample well and then measured using a photomultiplier tube. However, there are some significant differences in the approach that can lead to one being a better option than the other depending on the circumstances.

In photometric measurement, the amount of light transmitted is subtracted from the amount of light generated at the source. This allows you to determine the total amount of light absorbed.

In contrast, when using fluorometric measurement, the dye is illuminated at a specific wavelength and absorbs light energy. The light energy then emits at a different wavelength. Since the light emits directly from the dye molecule, it emits in all directions equally. This means that a variety of plates can be used, for example black, opaque plates without an optically clear bottom. In fact, using black plates during testing reduces background interference, because they absorb scattered light.

To perform fluorometric measurement, follow the same general steps and data handling as used for photometric measurement.



Advantages

- Allows testing to be done in the actual labware used in the process, which makes for a truer comparison.
- Is good at gathering large amounts of data and is the only method besides photometric that works for liquid filled systems.
- Is not effected by well geometry because the measurement is only dependent on the total amount of fluorescent molecule residing in the area illuminated by the light source.
- Is effective for small volumes, but may still need a diluent. Fluorescent dyes tend to be sensitive and often require substantial dilutions from a stock solution to remain in the dynamic read range for a plate reader. This ensures accurate measurements of low volumes. Fluorescent measurement examines the total amount of fluorophore in the area that is excited, eliminating the effect of varying diluent volumes.

Disadvantages

- Can be difficult to choose an appropriate dye for the specific test. The addition of dyes to the liquid can alter the physical characteristics. This makes the liquid type not truly representative of the one you are trying to measure. Fluorescent dyes are more expensive than the dyes used for photometry and also tend to be sensitive to light and sometimes temperature. In addition, fluorescent dyes do generally come with known excitation and emission wavelengths.
- Can limit the volume to be tested. The transfer volume may be limited to the labware required for analysis in the plate reader. A microplate typically holds about 300 µL of liquid. If more volume is required, additional testing procedures must be used that are different from the liquid transfer settings in the method.
- Requires the use of a plate reader and further calculations in order to obtain results. Data collection can therefore be slower using this approach. Plate readers for fluorescence measurements tend to be more expensive than those that just read absorbance.

5.3 Gravimetric Measurement

The gravimetric approach uses an analytical balance integrated directly on the automated liquid handler. Once the liquid is weighed, the known density of the liquid can be used to calculate the volume.

Advantages

- Uses only the liquid of interest with no dyes or other additives.
- Covers a large range of pipette channel volume. It can cover a range of volumes used by all Hamilton pipetting devices from 0.5 µL to 5 mL.
- Provides immediate feedback from the balance which allows for quicker optimization of liquid classes.

Disadvantages

- Doesn't use the actual labware used in the assay. Instead, you may be pipetting to a large tube on a balance instead of a plate or specific tube type.
- Requires you to know the density of the liquid in order to correlate the weight to a volume. While it is easy to look up the density of common liquid types, it may be unknown for less common types or for mixtures.
- Affected by temperature. The temperature can impact the density of the liquid, so the temperature needs to be monitored to properly check for trueness. See Section 2.3.2 for more information.
- Allows only one transfer at a time to be weighed. This prevents the testing of multiple channels at the same time to collect individual measurements. Specifically, this limitation prohibits testing of all channels in a multi-probe head (96- and 384-) at the same time.
- With small volumes, the balance quality and readability sensitivity can play a factor. It may not be possible to obtain significant digits with low volume transfers given the sensitivity of the balance and laboratory conditions.

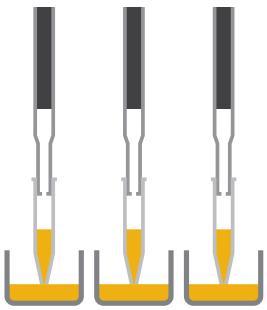
Hamilton makes a gravimetric measurement system, known as the Liquid Verification Kit (LVK). The LVK is made up of a graphical user interface that directs liquid handling tests on an analytical balance that is placed on the automated liquid handler's deck. The same Mettler Toledo WXS 205S analytical balance is used by customers who purchase the LVK and Hamilton field service engineers to conduct liquid testing. During execution, the LVK program displays data in real-time and creates reports on the pipetting performance. It is also possible to program custom methods using the Hamilton software that control the balance to verify liquid transfers without the use of the LVK interface.

Industry standard processes for gravimetric testing are defined in ISO 8655-6.

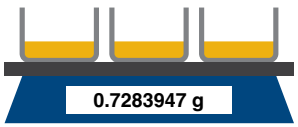
5.4 Combined Photometric and Gravimetric Measurement

It is also possible to combine the photometric and gravimetric approaches. The first liquid transfer containing dye can be weighed, then later be analyzed on a plate reader to determine the volume per well. This approach is used by Hamilton service engineers to perform installation and operational qualification testing of the automated liquid handlers as part of the Field Verification Kit 2. This process allows both the independent pipetting channels and the multi-probe heads (96- and 384) to be verified in a similar fashion.

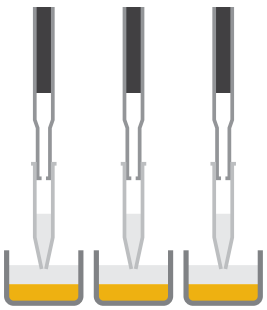
With the weight measurement and the obtained optical density (OD) values, the volume of each well can be determined.



1. Pipette dye liquid in a microplate.



2. Weigh the plate.



3. Add clear liquid like Borate Buffer, to make sure that the liquid sufficiently covers the bottom of the well. Mix the clear liquid and dye together.

4. Measure the optical density by reading the plate with a photometric reader.

6 Hamilton Parameter Glossary

Hamilton Parameter	Definition	Other Industry Terms
Flow Rate	Liquid flow rates in µL/s, corresponding to plunger speed	Aspiration Speed Dispense Speed
Mix Flow Rate	Liquid flow rates in µL/s, corresponding to plunger speed for mixing	Aspiration Speed Dispense Speed
Air Transport Volume	Volume of air in µL is aspirated at the end of the aspiration and dispense step	Trailing Air Gap (TAG)
Blowout Volume	Volume of air in µL that is aspirated first during the aspiration step. If dispensing in empty tip mode, part or all of the air is dispensed	System Trailing Air Gap (STAG)
Swap Speed	Speed in mm/s which the pipette head is moved out of the liquid	Retract Speed
Settling Time	Time in seconds that the pipette head remains in the liquid after aspiration or dispense	Delay
Over-Aspirate Volume	After aspirating the required volume an additional volume in µL is aspirated and dispensed again immediately	Conditioning Volume
Clot Retract Height	A parameter for clot detection that determines how high the pipette head is allowed to move out of the liquid while there is still a liquid detection signal after aspiration	Exit Signal Detection
Stop Flow Rate	Dispense flow rate in µL/s at which the dispense step terminates abruptly	Dispense Speed Break-off Speed
Stop Back Volume	Volume in µL which is aspirated again immediately after the dispense	No equivalent, could be done but would need to be done as a new aspiration action immediately following the dispense into vessel with the z-travel set at the dispense location

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