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The Preparation of Buffers and Other Solutions: A Chemist's Perspective

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Editor's note: Many, perhaps most, molecular biology procedures don't require perfection in the handling of reagents and solution preparation. When procedures fail and logical thinking produces a dead end, it might be worthwhile to carefully review your experimental reagents and their preparation. The author of this discussion is an extremely meticulous analytical chemist, not a molecular biologist. He describes the most frequent mistakes and misconceptions observed during two decades of experimentation that requires excruciating accuracy and reproducibility in reagent preparation.

BUFFERS

Why Buffer?

The primary purpose of a buffer is to control the pH of the solution. Buffers can also play secondary roles in a system, such as controlling ionic strength or solvating species, perhaps even affecting protein or nucleic acid structure or activity. Buffers are used to stabilize nucleic acids, nucleic acid–protein complexes, proteins, and biochemical reactions (whose products might be used in subsequent biochemical reactions). Complex buffer systems are used in electrophoretic systems to control pH or establish pH gradients.

Can You Substitute One Buffer for Another?

It is rarely a good idea to change the buffer type—that is, an amine-type buffer (e.g., Tris) for an acid-type buffer (e.g., phosphate). Generally, this invites complications due to secondary effects of the buffer on the biomolecules in the system. If the purpose of the buffer is simply pH control, there is more latitude to substitute one buffer for another than if the buffer plays other important roles in the assay.

How Does a Buffer Control the pH of a Solution?

Buffers are solutions that contain mixtures of weak acids and bases that make them relatively resistant to pH change. Conceptually buffers provide a ready source of both acid and base to either provide additional H^+ if a reaction (process) consumes H^+, or combine with excess H^+ if a reaction generates acid.

The most common types of buffers are mixtures of weak acids and salts of their conjugate bases, for example, acetic acid/sodium acetate. In this system the dissociation of acetic acid can be written as

$$CH_3COOH \rightarrow CH_3COO^- + H^+$$

where the acid dissociation constant is defined as $K_a = [H^+][CH_3COO^-]/[H_3COOH]$.

Rearranging and taking the negative logarithm gives the more familiar form of the Henderson-Hasselbalch equation:

$$pH = pK + \log\frac{[CH_3COO^-]}{[CH_3COOH]}$$

Inspection of this equation provides several insights as to the functioning of a buffer.

When the concentrations of acid and conjugate base are equal, $\log(1) = 0$ and the pH of the resulting solution will be equal to the pK_a of the acid. The ratio of the concentrations of acid and conjugate base can differ by a factor of 10 in either direction, and the resulting pH will only change by 1 unit. This is how a buffer maintains pH stability in the solution.

To a first approximation, the pH of a buffer solution is independent of the absolute concentration of the buffer; the pH depends only on the ratio of the acid and conjugate base present. However, concentration of the buffer is important to buffer capacity, and is considered later in this chapter.

When Is a Buffer Not a Buffer?

Simply having a weak acid and the salt of its conjugate base present in a solution doesn't ensure that the buffer will act as a buffer. Buffers are most effective within ± 1 pH unit of their pK_a. Outside of that range the concentration of either the acid or its salt is so low as to provide little or no capacity for pH control. Common mistakes are to select buffers without regard to the pK_a of the buffer. Examples of this would be to try to use K_2HPO_4/KH_2PO_4 ($pK_a = 6.7$) to buffer a solution at pH 4, or to use acetic acid ($pK_a = 4.7$) to buffer near neutral pH.

What Are the Criteria to Consider When Selecting a Buffer?

Target pH

Of primary concern is the target pH of the solution. This narrows the possible choices to those buffers with pK_a values within 1 pH unit of the target pH.

Concentration or Buffer Capacity

Choosing the appropriate buffer concentration can be a little tricky depending on whether pH control is the only role of the buffer, or if ionic strength or other considerations also are important. When determining the appropriate concentration for pH control, the following rule of thumb can be used to estimate a reasonable starting concentration.

1. If the process or reaction in the system being buffered does not actively produce or consume protons (H^+), then choose a moderate buffer concentration of 50 to 100 mM.
2. If the process or reaction actively produces or consumes protons (H^+), then estimate the number of millimoles of H^+ that are involved in the process (if possible) and divide by the solution volume. Choose a buffer concentration at least 20× higher than the result of the estimation above.

The rationale behind these two steps is that a properly chosen buffer will have a 50:50 ratio of acid to base at the target pH, therefore you will have 10× the available capacity to consume or supply protons as needed. A 10% loss of acid (and corresponding increase in base species), and vice versa, results in a 20% change in the ratio ($[CH_3COO^-]/[CH_3COOH$ from the Henderson-Hasselbalch example above]) resulting in less than a 0.1 pH unit change, which is probably tolerable in the system. While most biomolecules can withstand the level of hydrolysis that might accompany such a change (especially near neutral pH), it is possible that the secondary and tertiary structures of bioactive molecules might be affected.

Chemical Compatibility

It is important to anticipate (or be able to diagnose) problems due to interaction of your buffer components with other solution components. Certain inorganic ions can form insoluble complexes with buffer components; for example, the presence of calcium will cause phosphate to precipitate as the insoluble calcium phosphate, and amines are known to strongly bind copper. The presence of significant levels of organic solvents can limit solubility of some inorganic buffers. Potassium phosphate, for example, is more readily soluble in some organic solutions than the corresponding sodium phosphate salt.

One classic example of a buffer precipitation problem occurred when a researcher was trying to prepare a sodium phosphate buffer for use with a tryptic digest, only to have the Ca^{2+} (a nec-

essary enzyme cofactor) precipitate as $Ca_3(PO_4)_2$. Incompatibilities can also arise when a buffer component interacts with a surface. One example is the binding of amine-type buffers (i.e., Tris) to a silica-based chromatography packing.

Biochemical Compatibility

Is the buffer applied at an early stage of a research project compatible with a downstream step? A protein isolated in a buffer containing 10 mM Mg^{2+} appears innocuous, but this cation concentration could significantly affect the interaction between a regulatory protein and its target DNA as monitored by band-shift assay (Hennighausen and Lubon, 1987; *BandShift Kit Instruction Manual*, Amersham Pharmacia Biotech, 1994). Incompatible salts can be removed by dialysis or chromatography, but each manipulation adds time, cost, and usually reduces yield. Better to avoid a problem than to eliminate it downstream.

What Can Generate an Incorrect or Unreliable Buffer?

Buffer Salts

All buffer salts are not created equal. Care must be exercised when selecting a salt to prepare a buffer. If the protocol calls for an anhydrous salt, and the hydrated salt is used instead, the buffer concentration will be too low by the fraction of water present in the salt. This will reduce your buffer capacity, ionic strength, and can lead to unreliable results.

Most buffer salts are anhydrous, but many are hygroscopic—they will pick up water from the atmosphere from repeated opening of the container. Poorly stored anhydrous salts also will produce lower than expected buffer concentrations and reduced buffering capacity. It is always wise to record the lot number of the salts used to prepare a buffer, so the offending bottle can be tracked down if an error is suspected.

If a major pH adjustment is needed to obtain the correct pH of your buffer, check that the correct buffer salts were used, the ratios of the two salts weren't switched, and finally verify the calculations of the proper buffer salt ratios by applying the Henderson-Hasselbalch equation. If both the acid and base components of the buffer are solids, you can use the Henderson-Hasselbalch equation to determine the proper mass ratios to blend and give your target pH and concentration. When this ratio is actually prepared, your pH will usually need some minor adjustment, which should be very minor compared to the overall concentration of the buffer.

pH Adjustment

Ionic strength differences can arise from the buffer preparation procedure. For example, when preparing a 0.1 M acetate buffer of pH 4.2, was 0.1 mole of sodium acetate added to 900 ml of water, and then titrated to pH 4.2 with acetic acid before bringing to 1 L volume? If so, the acetate concentration will be significantly higher than 0.1 M. Or, was the pH overshot, necessitating the addition of dilute NaOH to bring the pH back to target, increasing the ionic strength due to excess sodium? The 0.1 M acetate buffer might have been prepared by dissolving 0.1 mole sodium acetate in 1 liter of water, and the pH adjusted to 4.2 with acetic acid. Under these circumstances the final acetate concentration is anyone's guess but it will be different from the first example above.

The best way to avoid altering the ionic concentration of a buffer is to prepare the buffer by blending the acid and conjugate base in molar proportions based on Henderson-Hasselbalch calculations such that the pH will be very near the target pH. This solution will then require only minimal pH adjustment. Dilute to within 5% to 10% of final volume, make any final pH adjustment, then bring to volume.

Generally, select a strong acid containing a counter-ion already present in the system (e.g., Cl^-, PO_4^{3+}, and OAc^-) to adjust a basic buffer. The strength (concentration) of the acid should be chosen so that a minimum (but easily and reproducibly delivered) volume is used to accomplish the pH adjustment. If overshooting the pH target is a problem, reduce the concentration of the acid being used. Likewise, choose a base that contains the cations already present or known to be innocuous in the assay (Na^+, K^+, etc.)

Solutions of strong acids and bases used for final pH adjustment usually are stable for long periods of time, but not forever. Was the NaOH used for pH adjustment prepared during the last ice age? Was it stored properly to exclude atmospheric CO_2, whose presence can slowly neutralize the base, producing sodium bicarbonate ($NaHCO_3$) which further alters the buffer properties and ionic strength of the solution?

Buffers from Stock Solutions

Stock solutions can be a quick and accurate way to store "buffer precursors." Preparing 10× to 100× concentrated buffer salts can simplify buffer preparation, and these concentrated solutions can also retard or prevent bacterial growth, extending almost indefinitely the shelf stability of the solutions.

The pH of the stock solutions should not be adjusted prior to dilution; the pH is the negative log of the H^+ ion concentration, so dilution by definition will result in a pH change. Always adjust the pH at the final buffer concentrations unless the procedure explicitly indicates that the diluted buffer is at an acceptable pH and ionic concentation, as in the case with some hybridization and electrophoresis buffers (Gallagher, 1999).

Filtration

In many applications a buffer salt solution is filtered prior to mixing with the other buffer components. An inappropriate filter can alter your solution if it binds with high affinity to one of the solution components. This is usually not as problematic with polar buffer salts as it can be with cofactors, vitamins, and the like. This effect is very clearly demonstrated when a solution is prepared with low levels of riboflavin. After filtering through a PTFE filter, the filter becomes bright yellow and the riboflavin disappears from the solution.

Incomplete Procedural Information

If you ask one hundred chemists to write down how to adjust the pH of a buffer, you'll probably receive one hundred answers, and only two that you can reproduce. It is simply tedious to describe in detail exactly how buffer solutions are prepared. When reading procedures, read them with an eye for detail: Are all details of the procedure spelled out, or are important aspects left out? The poor soul who tries to follow in the footsteps of those who have gone before too often finds the footsteps lead to a cliff. Recognizing the cliff before one plunges headlong over it is a learned art. A few prototypical signposts that can alert you of an impending large first step follow:

- Which salts were used to prepare the "pH 4 acetate buffer"? Sodium or potassium? What was the final concentration?
- Was pH adjustment done before or after the solution was brought to final volume?
- If the solution was filtered, what type of filter was used?
- What grade of water was used? What was the pH of the starting water source?

What Is the Storage Lifetime of a Buffer?

A stable buffer has the desired pH and buffer capacity intended when it was made. The most common causes of buffer failure are

pH changes due to absorption of basic (or acidic) materials in the storage environment, and bacterial growth. Commercially prepared buffers should be stored in their original containers. The storage of individually prepared buffers is discussed below. The importance of adequate labeling, including preparation date, composition, pH, the preparer's name, and ideally a notebook number or other reference to the exact procedure used for the preparation, cannot be overemphasized.

Absorption of Bases

The most common base absorbed by acidic buffers is ammonia. Most acidic buffers should be stored in glass vessels. The common indicator of buffer being neutralized by base is failure to achieve the target pH. In acidic buffers the pH would end up too high.

Absorption of Acids

Basic buffers can readily absorb CO_2 from the atmosphere, forming bicarbonate, resulting in neutralization of the base. This is very common with strong bases (NaOH, KOH), but often the effect will be negligible unless the system is sensitive to the presence of bicarbonate (as are some ion chromatography systems) or the base is very old. If high concentrations of acids (e.g., acetic acid) are present in the local environment, basic buffers can be neutralized by these as well. A similar common problem is improper storage of a basic solution in glass. Since silicic materials are acidic and will be attacked and dissolved by bases, long-term storage of basic buffers in glass can lead to etching of the glass and neutralization of the base.

Microbial Contamination

Buffers in the near-neutral pH range can often readily support microbial growth. This is particularly true for phosphate-containing buffers. Common indicators of bacterial contamination are cloudiness of the solution and contamination of assays or plates.

Strategies for avoiding microbial contamination include sterilizing buffers, manipulating them using sterile technique, refrigerated storage, and maintaining stock solutions of sufficiently high ionic concentration. A concentration of 0.5 M works well for phosphate buffers. For analytical chemistry procedures, phosphate buffers in target concentration ranges (typically 0.1–0.5 M) should be refrigerated and kept no more than one week. Other buffers could often be stored longer, but usually not more than two weeks.

REAGENTS

Which Grade of Reagent Does Your Experiment Require?

Does your application require top-of-the-line quality, or will technical grade suffice? A good rule of thumb is that it is safer to substitute a higher grade of reagent for a lower grade, rather than vice versa. If you want to apply a lower grade reagent, test the substitution against the validated grade in parallel experiments.

Should You Question the Purity of Your Reagents?

A certain level of paranoia and skepticism is a good thing in a scientist. But where to draw the line?

New from the Manufacturer

The major chemical manufacturers can usually be trusted when providing reagents as labeled in new, unopened bottles. Mistakes do happen, so if a carefully controlled procedure fails, and you eliminate all other sources of error, then consider the reagents as a possible source of the problem.

Opened Container

Here's where the fun begins. Once the bottle is opened, the manufacturer is not responsible for the purity or integrity of the chemical. The user must store the reagent properly, and use it correctly to avoid contamination, oxidation, hydration, or a host of other ills that can befall a stored reagent. How many times have you been tempted to use that reagent in the bottle with the faded label that is somewhere over 40 years old? A good rule of thumb is if the experiment is critical, use a new or nearly new bottle for which the history is known. If an experiment is easily repeated should a reagent turn out to be contaminated, then use your judgment when considering the use of an older reagent.

How can you maintain a reagent in nearly new condition? Respect the manufacturer's instructions. Storage conditions (freezer, refrigerator, dessicator, inert atmosphere, etc.) are often provided on the label or in the catalog. Improper handling is more likely than poor storage to lead to contamination of the reagent. It is rarely a good idea to pipette a liquid reagent directly from the original bottle; this invites contamination. Instead, pour a portion into a second container from which the pipetting will be done. Solids are less likely to be contaminated by removing them directly from the bottle, but that is not always the case. It's usually satisfactory to transfer buffer salts from a bottle, for instance, but use greater care handling a critical enzyme cofactor.

Reagents Prepared by Others

Never blindly trust a reagent prepared by someone other than yourself, especially for critical assays. It's a lot like packing your own parachute—it's your responsibility to prepare your important solutions. If you want to trust the outcome of an important experiment to something someone else may have prepared while thinking about an upcoming vacation, it's up to you. Prepare critical solutions yourself until you have a solid working relationship with whomever you plan to share solutions with. Even then, don't get offended if they don't trust your solutions!

Reagents Previously Prepared by You

How reliable are your solutions? Your solutions are probably fine to use if:

- Your labeling and record-keeping are contemporary and accurate.
- You don't share solutions with anyone who could have mishandled and contaminated them.
- Your material is within it's expected shelf life.

What Are Your Options for Storing Reagents?

Storage is half the battle (handling is the other half) in keeping reagents fit for use. Follow the manufacturer's recommendations.

Shelf (Room Temperature)

Solids, like buffer salts, are usually stored on the shelf in sealed bottles. Sometimes it is appropriate (e.g., for hygroscopic materials) to store them in a dessicator on a shelf. Many nonflammable liquid reagents can be also stored on a shelf. Care should be taken to store incompatible chemicals separately. For example, store acids and bases separated; store strong oxidizers away from other organics.

Vented Flammables Cabinet

Flammables or reagents with harmful vapors (e.g., methylene chloride) should be stored in ventilated cabinets designed for chemical storage. These cabinets are designed to minimize the chance of fire from flammable vapors; they often are designed to contain minor leaks, preventing wider contamination and possible fire. It is a good practice to use secondary spill containers (e.g., polypropylene or Teflon™ trays) in the flammables cabinet if they are not already built into the design.

Refrigerators

Many reagents require refrigeration for storage stability. Working buffers, particularly phosphates, will usually last a little longer if refrigerated between uses. Refrigerators used for storing chemicals must not be used to store foodstuffs.

Freezer

Check the label; many standards require freezer temperatures for long-term stability. Check that the freezer is functioning properly.

Are All Refrigerators Created Equal?

Household Refrigerator

It is cheap, stays cold, and is often perfectly fine for storing aqueous samples. It can have serious problems storing flammable organics, however, since the thermostat controls are usually located inside the refrigerator, which can spark and ignite flammable vapors.

Flammable Storage Refrigerator

The thermostat controls have been moved outside the cooled compartment. Unless a refrigerator is specifically labeled "Flammable Storage" by the manufacturer, don't assume it is appropriate for storing flammables.

Explosion-Proof Refrigerator

These units meet specific requirements regarding potential spark sources and can be used in hazardous environments. They are usually extremely expensive.

Safe and Unsafe Storage in Refrigerators

Volumetric Flasks and Graduated Cylinders

How tempting to prepare a fresh solution in a volumetric flask and store it in the refrigerator. Then, an hour later, you reach into the refrigerator to grab a sample prepared the previous week, and accidentally knock over the flask. Tall narrow vessels like volumetric flasks and graduated cylinders are unstable, especially if they sit on wire refrigerator shelves. Solutions should be transfered to a more stable bottle or flask before storing in the refrigerator.

The Shelf in the Door

A long time ago in a basement laboratory, reagents were stored within a shelf in a refrigerator door. The refrigerator was opened, the shelf broke, and bottles spilled onto the floor, breaking two of them. One was dimethyl sulfate, a strong alkylating reagent, and the other was hydrazine, which is pyrophoric. Upon exposure to the air, the hydrazine burst into flame, vaporizing the dimethyl sulfate. It was several days before it was clear that the people exposed to the vapors wouldn't die from pulmonary edema. It may be 20 years before they know whether they have been compromised in terms of lung cancer potential.

Hazardous reagents should not be stored on shelves in refrigerator doors.

Poorly Labeled Bottles

A heavily used, shared refrigerator quickly begins to resemble a dinosaur graveyard. Rummage around in back, and you find a jumble of old, poorly or unlabeled bottles for which nobody assumes responsibility. Ultimately someone gets assigned the task of sorting out and discarding the chemicals. It is much simpler to put strict refrigerator policies in place to avoid this situation, and conduct regular refrigerator purges, so no ancient chemicals accumulate.

What Grades of Water Are Commonly Available in the Lab?

Tap Water

Tap water is usually of uncontrolled quality, may have seasonal variations such as level of suspended sediment depending on the source (municipal reservoir, river, well), may contain other chemicals purposely added to drinking water (chlorine, fluoride), and is generally unsuitable for use in important experiments. Tap water is fine for washing glassware but should always be followed by a rinse with a higher-grade water (distilled, deionized, etc.).

Distilled Water

Distillation generally eliminates much of the inorganic contamination and particularly sediments present in tap water feedstock. It will also help reduce the level of some organic contaminants in the water. Double distilling simply gives a slightly higher grade distilled water, but cannot eliminate either inorganic or organic contaminants.

Distilled water is often produced in large stills that serve an entire department, or building. The quality of the water is depen-

dent on how well the equipment is maintained. A significant stir occurred within a large university's biochemistry department when the first mention of a problem with the house distilled water was a memo that came out from the maintenance department that stated: "We would like to inform you that the repairs have been made to the still serving the department. There is no longer any radium in the water." The next day, a follow-up memo was issued that stated: "Correction—there is no longer any sodium in the distilled water."

Deionized Water

Deionized water can vary greatly in quality depending on the type and efficiency of the deionizing cartridges used. Ion exchange beds used in home systems, for instance, are used primarily to reduce the "hardness" of the water usually due to high levels of divalent cations such as magnesium and calcium. The resin bed consists of a cation exchanger, usually in the sodium form, which releases sodium into the water in exchange for removing the divalent ions. (Remember that when you attempt to reduce your sodium intake!) These beds therefore do not reduce the ionic content of the water but rather exchange one type of ion for another.

Laboratory deionizing cartridges are usually mixed-bed cartridges designed to eliminate both anions and cations from the water. This is accomplished by preparing the anion-exchange bed in the hydroxide (OH^-) form and the cation-exchange resin in the acid (H^+) form. Anions or cations in the water (including monovalent) are exchanged for OH^- or H^+, respectively, which combine to form neutral water. Any imbalance in the removal of the ions can result in a pH change of the water. Typically water from deionizing beds is slightly acidic, often between pH 5.5 to 6.5.

The deionizing resins can themselves increase the organic contaminant level in the water by leaching of resin contaminants, monomer, and so on, and should always be followed by a bed of activated carbon to eliminate the organics so introduced.

18 MΩ Water (Reverse Osmosis/MilliQ™)

The highest grade of water available is generally referred to as 18 MΩ water. This is because when the inorganic ions are completely removed, the ability of the water to conduct electric current decreases dramatically, giving a resistance of 18 MΩ. Commercial systems that produce this grade of water usually apply a multiple-step cleanup process including reverse osmosis, mixed-

bed ion exchangers, carbon beds, and filter disks for particulates. Some may include filters that exclude microorganisms, resulting in a sterile water stream. High-grade 18 MΩ water tends to be fairly acidic—near pH 5. Necessary pH adjustments of dilute buffer solutions prepared using 18 MΩ water could cause discrepancies in the final ionic concentration of the buffer salts relative to buffers prepared using other water sources.

When Is 18 MΩ Water Not 18 MΩ Water?

Suppose that your research requires 18 MΩ water, and you purchased the system that produces 500 ml/min instead of the 2 L/min version. If your research doesn't require a constant flow of water, you can connect a 20 L carboy to your system to store your pristine water. Bad Move.

18 MΩ is not the most inert solvent; in practice, it is very aggressive. Water prefers the presence of some ions so as your 18 mΩ water enters the plastic carboy, it starts leaching anything it can out of the plastic, contaminating the quality of the water. The same thing happens if you try to store the water in glass. 18 mΩ water loves to attack glass, leaching silicates and other ions from the container. If you need the highest purity water, it's best not to store large quantities, but rather prepare it fresh.

For the same reason, the tubing used to transfer your high-grade water should always be the most inert available, typically Teflon™ or similar materials. Never use highly plasticized flexible plastic tubing. Absolutely avoid metals such as copper or stainless steel, as these almost always guarantee some level of contaminants in your water.

What Is the Initial pH of the Water?

As mentioned above, the initial pH of typical laboratory-grade distilled and deionized water is often between 5.5 and 6.5. Check your water supply from time to time, particularly when deionizing beds are changed to ensure that no major change in pH has occurred because of seasonal variation or improperly conditioned resin beds.

Although the initial pH of laboratory water may be slightly acidic, the good news is deionized water should have little or no buffer capacity, so your normal pH adjustment procedures should not be affected much. Pay particular attention if your buffer concentrations are very low (<10 mM) resulting in low buffer capacity.

What Organics Can Be Present in the Water?

The answer to this important question depends on the upstream processing of the water and the initial water source. Municipal water drawn from lakes or streams can have a whole host of organics in them to start with, ranging from petroleum products to pesticides to humic substances from decaying plant material to chlorinated species like chloroform resulting from the chlorination process. Well water may have lower levels of these contaminants (since the water has been filtered through lots of soil and rock, but even groundwater may contain pesticides and chlorinated species like trichloroethylene depending on land use near the aquifer.

Municipal processing will remove many organic contaminants from the tap water, but your in-lab water purifier is responsible for polishing the water to a grade fit for experimental use. Most commercial systems do a good job of that, but as mentioned previously, care must be taken to not introduce contaminants after the water has been polished. Plasticizers from tubing or plastic storage tanks, monomer or resin components from deionizer beds, and surfactants or lubricants on filters or other system components are the most common type of organic to be found in a newly installed system.

Another common, yet often overlooked source, is microbial contamination. In one case, a high-grade water purifier mounted on a wall near a window suddenly started showing evidence of organic background. Changing the carbon cartridge did not help the situation. Close inspection of the system showed the translucent plastic tubing connecting the reverse osmosis holding tank to the deionizer beds, and ultimately the lines that delivered the polished water to the spigot, had been contaminated by microbial growth. It was surmised that the intense sunlight during part of the day was providing a more hospitable environment for microorganisms to gain a foothold in the system. The clear tubing was replaced with opaque tubing and the problem disappeared.

In a second instance, a facility changed its water source from wells to a river draw-off. This drastically changed the stability of the incoming water quality. During periods of heavy rain, silt levels in the incoming water increased dramatically, quickly destroying expensive reverse osmosis cartridges in the water purifier system. The solution was to install two pre-filters of decreasing porosity in line ahead of the reverse osmosis unit. The first

filter needed replacing monthly, but the second filter was good for three to six months. The system functioned properly for a while, but then problems reappeared in the reverse osmosis unit. Inspection showed heavy microbial contamination in the second prefilter which had a clear housing, admitting sunlight. After cleaning and sterilizing the filter unit, the outside of the housing was covered with black electrical tape, and the microbial contamination problem never returned.

As discussed in Chapter 12, dispensing hoses from water reservoirs resting in sinks can also lead to microbial contamination.

What Other Problems Occur in Water Systems?

Leaks

Leaks are sometimes one of the most serious problems that can occur with in-lab water purification systems. Leaks come in three kinds, typically. Leaks of the first kind start as slow drips, and can be spotted and corrected before developing into big unfriendly leaks.

Leaks of the second kind are generally caused by a catastrophic failure of a system component (tubing, valve, automatic shutoff switch, or backflush drain). Although highly uncommon, they usually occur around midnight on Fridays so as to maximize the amount of water that can escape from the system, therefore maximizing the resulting flooding in the lab. The likelihood of a leak of the second kind seems to increase exponentially with the cost of instrumentation in laboratories on floors directly below the lab with the water purifier system.

Leaks of the third kind result when a person places a relatively large vessel beneath the water system, begins filling, and walks away to tend to a few minor tasks or is otherwise distracted. The vessel overflows, flooding the lab with the extent of the flood depending on the duration of the distraction.

Leaks of the third kind are by far the most common type of leak, and are also the most preventable. Locating the water purification system immediately above a sink, so that any vessel being filled can be placed in the sink, usually prevents this type of catastrophe. If placement above a sink is not possible, locating the water purification system in a (relatively) high-traffic or well-used location in the lab can also minimize or eliminate the possibility of major spills, since someone is likely to notice a spill or leak.

Leaks of the first or second type are highly uncommon, but do occur. The best prevention is to have the system periodically inspected and maintained by qualified personnel, and never have

major servicing done on a Friday. Problems seem to be most likely after the system has been poked and prodded, so best to do that early in the week. Then the system can be closly watched for a few days afterward before leaving it unattended.

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How to Properly Use and Maintain Laboratory Equipment

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Data on the performance characteristics of different protein concentration assays were generously provided by Bio Rad Inc.

BALANCES AND SCALES (Trevor Troutman)

How Are Balances and Scales Characterized?

Balances are classified into several categories. Top-loaders are balances with 0.001 g or 1 mg readability and above, where readability is the lowest possible digit that is seen on the display. Analytical balances are instruments that read 0.1 mg. Semimicro balances are those that are 0.01 mg. Microbalances are 1 μg. Finally, the ultramicrobalances are 0.1 μg.

How Can the Characteristics of a Sample and the Immediate Environment Affect Weighing Reproducibility?

Moisture

Condensation forms on reagents that are not kept airtight while they equilibrate to room temperature. Similarly moisture is generated if the sample is not allowed to reach the temperature of the weighing instrument. This is especially problematic when weighing very small samples. You the researcher are also a source of moisture that can be transmitted quite easily to the sample in the form of fingerprints and body oils.

Air Buoyancy

Akin to water keeping something afloat, samples can be "lifted" by air, artificially decreasing their apparent weight. This air buoyancy can have a significant effect on smaller samples.

Electrostatic Forces

Electrostatic charges are almost always present in any environment, particularly in areas with very low humidity. If there are considerable charges present in a sample to be weighed on a high-precision instrument, it will manifest itself in the form of drifting, constant increase or decrease of weight readings, or nonreproducible results. Variability occurs when these electrical forces build up on the sample and the fixed parts of the balance that are not connected to the weighing pan. Substances with low electrical conductivity (e.g., glass, plastics, filter materials, and certain powders and liquids) lose these charges slowly, prolonging the drift during weighing.

The charges most likely originate when the sample is being transported or processed. Examples include friction with air in a convection oven, friction between filters and the surface they contact, internal friction between powders and liquids during transportation, and direct transfer of charged particles by persons. This charge accumulation is best prevented by use of a Faraday cage, which entails shielding a space in metallic walls. This frees the inside area from electrostatic fields. A metallic item can serve the same purpose. Surrounding the container that houses the reagent in foil can also reduce charge accumulation. For nonhygroscopic samples, adding water to increase the humidity inside the draft chamber can reduce static electricity. Accomplish this by placing a beaker with as much water as possible into the draft chamber. An alternative is to bombard the sample with ions of the opposite charge, as generated by expensive ionizing blowers and polonium radiators. A simple and effective solution is to place an inverted beaker onto the weigh pan, and then place the sample to be weighed onto the inverted beaker. This strategy increases the distance between the sample and the weigh pan, thus weakening any charge effects.

Temperature

Airing out a laboratory or turning the heat on for the first time with the change of seasons has a profound effect on an analytical balance. The components of a weighing system are of different size and material composition, and adapt to temperature changes at

different rates. When weighing a sample, this variable response to temperature produces unreliable data. It is recommended to keep a constant temperature at all times in an environment where weighing instruments are kept. When room temperature changes, allow the instrument to equilibrate for 24 hours.

Air Currents or Drafts

The flow rate of ambient air should be minimized to get quick and stable results with weighing equipment. For balances with a readability of 1 mg, an open draft shield (glass cylinder) will suffice. Below 0.1 mg, a closed draft chamber is needed.

These shields or chambers should be as small as possible to eliminate convection currents within the chamber to minimize temperature variation and internal draft problems.

Magnetic Forces and Magnetic Samples

Magnetic forces are produced when a sample is magnetized or magnetizable, which means it contains a percentage of iron, cobalt, or nickel. Magnetic effects manifest themselves in the sample's loss of reproducibility. But unlike electrostatic forces, magnetic forces can yield a stable measurement. Changing the orientation of the magnetic field (moving the reagent sample) relative to the weigh system causes the irreproducible results.

Magnetic effects are thus difficult to detect unless the same sample is weighed more than once. Placing an inverted beaker or a piece of wood between the sample and the pan can counteract the magnetic force. Some instruments allow for *below balance weighing*, in which a hook used to attach magnetic samples lies underneath the weigh pan at a safe distance in order to eliminate magnetic effects.

Gravitational Tilt

A balance must be level when performing measurements on the weighing pan. Gravity operates in a direction that points straight to the center of the earth. Thus, if the weigh cell in not directly in this path, the weight will end up somewhat less. For example, say we weigh a 200 g sample that is 0.2865° (angle = α) out of parallel. We have

$$\text{Apparent weight} = \text{weight} * \cos\alpha$$

$$\text{Apparent weight} = 200 * \cos 0.2865 = 199.9975\,\text{g}$$

This result represents a 2.5 mg deviation. This is a significant quantity when working with analytical samples.

By What Criteria Could You Select a Weighing Instrument?

Capacity and Readability

Focus on determining your true readability needs. Cost increases significantly with greater readability. Also bear in mind that a quality balance usually has internal resolutions that are better than the displayed resolution. Stability is a more desired trait in analytical balances due to the small sample size.

Calibration

Most high-end lab balances will calibrate themselves or will have some internal weight that the user can activate.

Applications

Most lab applications require straight weighing (or "weigh only"). Analytical balances may have an air buoyancy correction application, or determination of filament diameters. Other functions include:

- *Checkweighing*. Sets a target weight or desired weight; then weighs samples to see if they hit the target weight.
- *Accumulation*. Calculates how much of a pre-set formula has been filled by the material being weighed.
- *Counting*. Calculates the number of samples present based on the reference weight of one sample.
- *Factor calculations*. Applies a weighed sample into a formula to calculate a final result.
- *Percent weighing*. A measured sample is represented as a percentage of a pre-set desired amount.
- *Printout of sample information and weights*.

Computer Interface

Some instruments can share data with a computer.

How Can You Generate the Most Reliable and Reproducible Measurements?

Vessel Size

Use the smallest container appropriate for the weighing task to reduce surface and buoyancy effects.

Sample Conditioning

The sample temperature should be in equilibrium with the ambient temperature and that of the balance. This will prevent

convection currents at the surface. Cold samples will appear heavier, and hot samples will be lighter.

Humidity

If there is low humidity in the weighing environment, plastic vessels should not be used, mainly for their propensity to gain electrostatic charges. Vessels comprised of 100% glass are preferred because they are nonconductive.

Sample Handling

If high resolution is required (1 mg or less), the sample should not make contact with the user's skin. Traces of sweat add weight and attract moisture (up to 400 micrograms [μg]). The body will also transfer heat to the sample, creating problems addressed above.

Sample Location

The sample should be centered as much as possible on the weighing pan. Off-center loading creates torque that cannot be completely counterbalanced by the instrument. This problem is called the off-center load error.

Hygroscopic Samples

Weigh these samples in a closed container.

How Can You Minimize Service Calls?

Ideally weighing equipment should be calibrated daily, and a certified technician should occasionally clean the balance and the internal calibration weights so that they keep their accuracy. More involved problems can be averted by minimizing the handling of the instruments.

If instruments must be handled or moved, apply great care. A drop of inches can cause thousands of dollars of damage to an analytical balance. For moves, use the original shipping packaging, which was specially designed for your particular instrument. Avoid any jarring movements, which can ruin an instrument's calibration.

CENTRIFUGATION (Kristin A. Prasauckas)

Theory and Strategy

Do All Centrifugation Strategies Purify via One Mechanism?

Zonal, or rate-zonal, centrifugation separates particles based on mass, which reflects the particle's sedimentation coefficient. The

greater the migration distance of the sample, the better is the resolution of separation. The average size of synthetic nucleic acid polymers are frequently determined by zonal centrifugation.

Isopycnic or equilibrium density centrifugation separates based on particle density, not size. Particles migrate to a location where the density of the particle matches the density of the centrifugation medium. Purification of plasmid DNA on cesium chloride is an example of isopycnic centrifugation. Voet and Voet (1995) and Rickwood (1984) discuss the techniques and applications of isopycnic separations.

Pelleting exploits differences in solubility, size, or density in order to concentrate material at the bottom of a centrifuge tube (Figure 4.1). The rotors recommended for these procedures are described in Table 4.1.

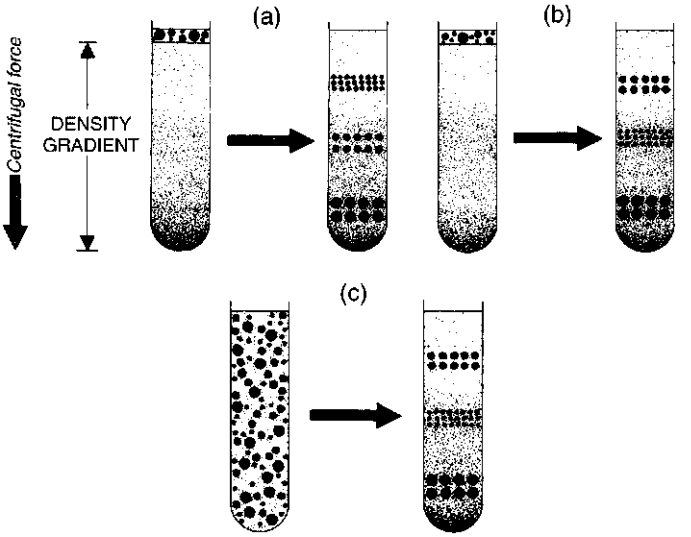

Figure 4.1 Types of density gradient centrifugation. (*a*) Rate-zonal centrifugation. The sample is loaded onto the top of a preformed density gradient (*left*), and centrifugation results in a series of zones of particles sedimenting at different rates depending upon the particle sizes (*right*). (*b*) Isopycnic centrifugation using a preformed density gradient. The sample is loaded on top of the gradient (*left*), and each sample particle sediments until it reaches a density in the gradient equal to its own density (*right*). Therefore the final position of each type of particle in the gradient (the isopycnic position) is determined by the particle density. (*c*) Isopycnic centrifugation using a self-forming gradient. The sample is mixed with the gradient medium to give a mixture of uniform density (*left*). During subsequent centrifugation, the gradient medium re-distributes to form a density gradient and the sample particles then band at their isopycnic positions (*right*). From Centifugation: A Practical Approach (2nd Ed.). 1984. Rickwood, D., ed. Reprinted by permission of Oxford University Press.

What Are the Strategies for Selecting a Purification Strategy?

Procedures for nucleic acid purification are abundant and reproducible (Ausubel et al., 1998). Methods to isolate cells and subcellular components are provided in the research literature and by manufacturers of centrifugation media. But frequently they require optimization, especially when the origin of your sample differs from that cited in your protocol.

If you can't locate a protocol in the research literature and texts, contact manufacturers of centrifugation media. If a reference isn't found for your exact sample, consider a protocol for a related sample. If all else fails, search for the published density of your sample. Manufacturers of centrifuge equipment and media can provide guidance in the construction of gradients based on your sample's density. Rickwood (1984) also provides excellent instructions on gradient construction.

Which Centrifuge Is Most Appropriate for Your Work?

Your purification strategy will dictate the choice of centrifuge, but these general guidelines provide a starting point. An example is provided in Table 4.2.

Table 4.1 Rotor Application Guide

Rotor Type	Swinging-Bucket	Fixed-Angle	Vertical
Pelleting	Good	Best	Not recommended
Rate-zonal	Best	Not recommended	Good
Isopycnic	Good	Better	Best

Table 4.2 Centrifuge Application Guide

Application for Pelleting	Low Speed (7000 rpm/ 7000 × *g*)	High Speed (30,000 rpm/ 100,000 × *g*)	Ultra (100,000 rpm/ 1,000,000 × *g*)
Cells	Yes	Yes	Feasible, but not recommended
Nuclei	Yes	Yes	Feasible, but not recommended
Membranous organelles	Some	Yes	Yes
Membrane fractions	Some	Some	Yes
Ribosomes	No	No	Yes
Macromolecules	No	No	Yes

Source: Data from Rickwood (1984).

Practice

Can You Use Your Existing Rotor Inventory?

Most rotors are compatible only with centrifuges produced by the same manufacturer. Confirm rotor compatibility with the manufacturer of your centrifuge.

What Are Your Options If You Don't Have Access to the Same Rotor Cited in a Procedure?

Ideally you should use a rotor with the angle and radius identical to that cited in your protocol. If you must work with alternative equipment, consider the *g* force effect of the rotor format when adapting your centrifugation strategy. The *g* force is a universal unit of measure, so selecting a rotor based on a similar *g* force, or RCF, will yield more reproducible results than selecting a rotor based on rpm characteristics.

Conversion between RCF and rpm

$$g \text{ force} = 11.18 \times R\,(\text{rpm}/1000)^2$$

Rotor Format

Protocols designed for a swinging-bucket rotor cannot be easily converted for use in a fixed-angle rotor. The converse is also true.

Rotor Angle

The shallower the rotor angle (the closer to vertical), the shorter the distance traveled by the sample, and the faster centrifugation proceeds. This parameter also alters the shape of a gradient generated during centrifugation, and the location of pelleted materials. The closer the rotor is to horizontal, the closer the pellet will form to the bottom of the tube (Figure 4.2).

Radius

The radius exerts several influences on fixed-angle and horizontal rotors. The *g* force is calculated as follows, and holds true for standard and microcentrifuges:

$$g \text{ force} = 1.12 \times 10^{-5} \times r \times \text{rpm}$$

$$r = \text{radius in cm}$$

Centrifugation force can be described as a maximum (*g*-max), a miminum (*g*-min), or an average *g* force (*g*-average). If no suffix is given, the convention is to assume that the procedure refers to *g*-max. These various *g* forces are defined for each rotor by the manufacturer.

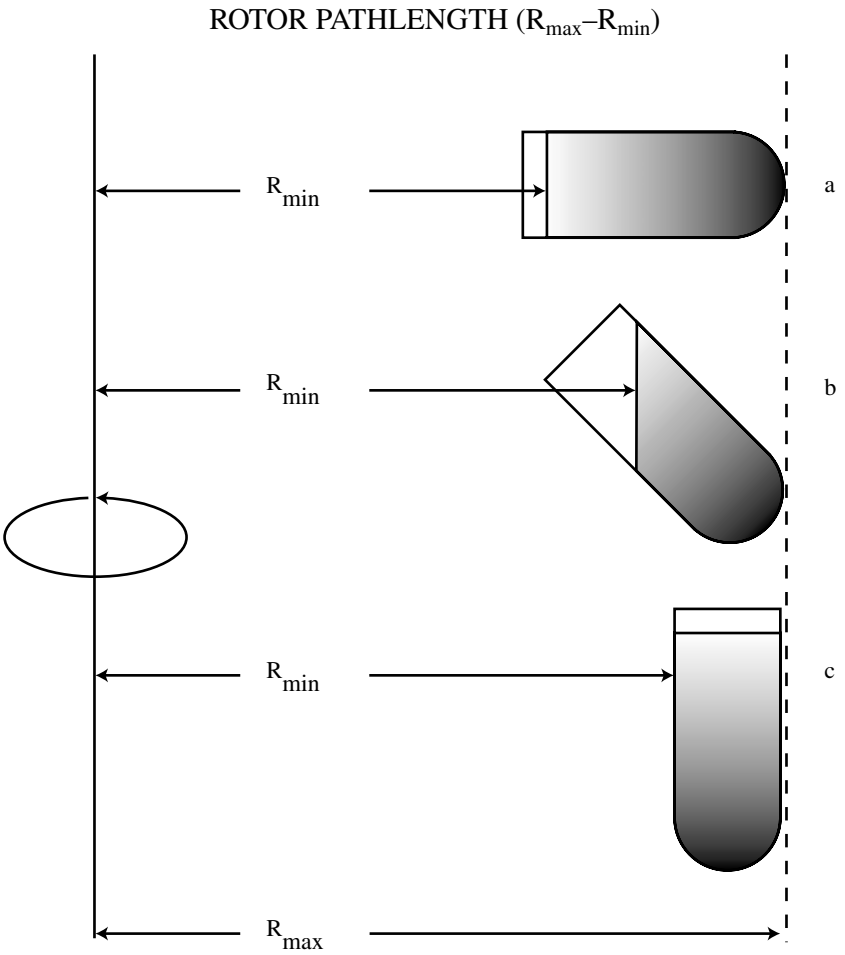

Figure 4.2 Effect of rotor angle on centrifugation experiments. Reproduced with permission of Kendro Laboratory Products. Artwork by Murray Levine.

The radius of a swinging bucket rotor is the distance between the center of the rotor and the bottom of the bucket when it is fully horizontal (Figure 4.2*a*). The greater the rotor radius, the greater is the *g* force.

The distance between the center of a fixed angle rotor and the bottom of the tube cavity determines the radius of a fixed-angle rotor (Figure 4.2*b*). Again the *g* force increases directly as the radius increases.

The greater the rotor angle, the greater is the distance the sample must travel before it pellets (Figure 4.2*b*). This travel distance also affects the shape of a density gradient (Figure 4.3).

The k-factor of a fixed-angle rotor provides a method to predict the required time of centrifugation for different fixed-angle rotors.

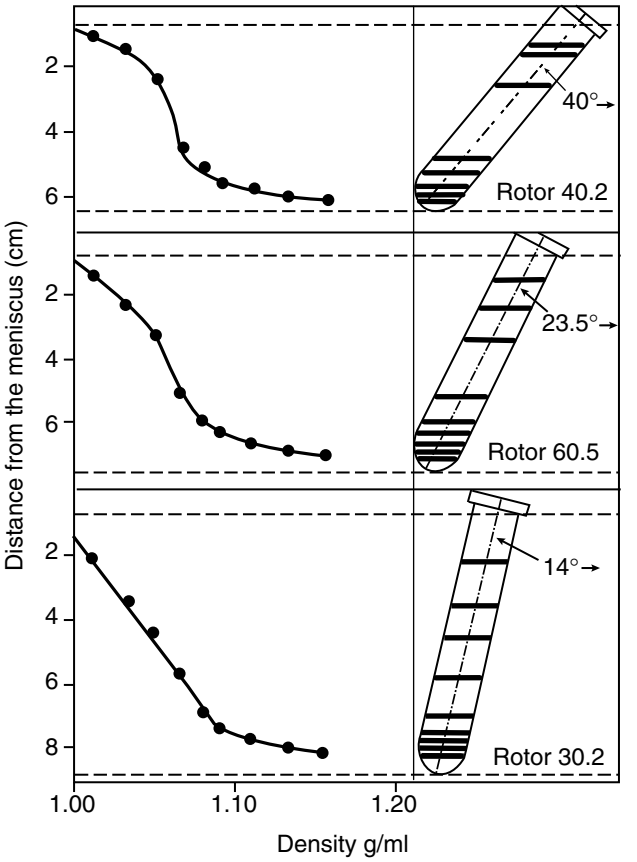

Figure 4.3 Effect of rotor angle on gradient formation. Reproduced with permission of Amersham Pharmacia Biotech.

The k-factor is a measure of pelleting efficiency; rotors with smaller k-factors (smaller fixed angles or vertical angles) pellet more efficiently, requiring shorter run times. The k-factor can also calculate the time required to generate a gradient when switching between different rotors. The k-factor can be determined by

$$\mathrm{k} = \left(\frac{2.53 \times 10^{11}}{(\mathrm{rpm})^2} \right) \ln(r_{\max} / r_{\min}) \tag{1}$$

Equation (2) uses the k-factor to predict the time required for centrifugation for different fixed-angle rotors:

$$\frac{T_1}{k_1} = \frac{T_2}{k_2} \tag{2}$$

T_1 is the run time in minutes for the established protocol. First, calculate the k_1 factor at the appropriate speed for the rotor that is referenced. Next, calculate the k_2 factor at the chosen speed for your rotor. Finally, solve for T_2. This strategy is not appropriate to

convert protocols between rotor types (fixed-angle, horizontal, and vertical).

Should Flamables, Explosive, or Biohazardous Materials Be Centrifuged in Standard Centrifugation Equipment?

Centrifuge manufacturers strongly recommend that standard laboratory centrifuges should not be exposed to any materials capable of producing flammable or explosive vapors, or extreme exothermic reactions. Specialized equipment exists for centrifuging dangerous substances.

Which Centrifuge Tube Is Appropriate for Your Application?

A broken or leaking sample container can seriously damage a centrifuge by knocking the rotor out of balance or exposing mechanical and electrical components to harsh chemicals. Damage can occur at any speed. Use only the tubes that are recommended for centrifugation use. If unsure, contact the tube manufacturer to assess compatability.

With the trend toward smaller sample size and greater throughput, microplates have become very popular. Other protocols call for vials and slides. Never attempt to create your own adapter for these containers; ask the rotor manufacturer about the availability of specialized equipment.

Fit

Correct tube fit is critical, especially at higher *g* forces. Tubes or containers that are too large can get trapped in rotors, while tubes that fit loosely can leak or break. Never use homemade adapters. While a broken tube doesn't sound costly, poorly fitted containers can lead to costly repairs.

g Force

Many tubes are not suitable for high stress centrifugation. When in doubt about *g* force limitations, contact the tube's manufacturer. If this isn't feasible, you can test the tube by filling it with water, centrifuging at low rpm's, and inspecting the tube for damage or indications of stress while slowly increasing the speed.

Chemical Compatibility

Confirm the tube's resistance with the manufacturer. Containers that are not resistant to the sample might survive one or more centrifugations but will surely be weakened. Chemically resistant containers should always be inspected for signs of stress before using them. Repeated centrifugation can damage any container.

A Checklist for Centrifuge Use

Inspect the centrifuge for frost on the inside chamber. Accumulated frost must be removed because it can prevent proper temperature control. Previous spills should also be cleaned before starting the centrifuge.

If your instrument uses rotor identification codes, does your instrument have the appropriate software to recognize and operate your rotor? Don't apply the identification code of one rotor for a second rotor that does not possess it's own code. Manually confirm the speed limitations of your rotor if identification codes aren't relevant.

Inspect the rotor for signs of corrosion and wear-and-tear. If you see any pitting or stress marks in the rotor cavities, do not use the rotor. If it is difficult to lock the rotor lid down or lock the rotor to the centrifuge, don't use the rotor. Check that all O-rings on the rotor and sample holders are present, clean, in good physical condition, and well lubricated. Many fixed-angle rotors have a cover O-ring, while many rotors that get locked to the drive have a drive spindle O-ring. If you have concerns about the rotor's condition, don't use it. Request an inspection from the manufacturer.

All the buckets and/or carriers within a swinging bucket rotor must be in place, even when these positions are empty. Utilize the proper adaptors and tubes, as described above. Balance your tubes or bottles. Refer to the manufacturer's instructions for balance tolerance, which vary with different rotors. Place the rotor onto the drive shaft and check that it is seated properly. Many rotors must be secured to the drive. Gently try to lift the rotor off the drive as a final check that the rotor is properly installed.

Begin centrifugation. Even though most imbalances occur at lower speed, monitor the centrifuge until it approaches final speed. If an imbalance occurs, reinspect the balancing of the tubes and the placement of the rotor.

Should the Brake Be Applied, and If So, to What Degree?

If a brake is turned completely off, it could take hours for the rotor to stop, depending on the top speed and instrument conditions (i.e., vacuum run). The stiffer the brake setting, the greater the jolt to the sample, so take note that the default setting of most instruments is the hardest, quickest brake rate. A reduced brake rate is recommended when separating samples of similar densities, when high resolution gradients and layers are required, and when fluffy, noncompacted pellets are produced. The degree of jolting, the braking technique, and the terminology varies among

manufacturers, so consult your operating manual or contact the manufacturer to discuss the most appropriate brake setting for your application. [The reverse is true when looking at the deceleration.) If you have an option as to where to control to (or from), 1000 to 1500 rpm is recommended.

Centrifugation of DNA and RNA

How Does a Deration Curve Affect Your Purification Strategy?

Deration describes the situation where a rotor should not attain its maximum speed because a high-density solution is used in the separation. For example, centrifuged at high *g* force, dense solutions of CsCl will precipitate at a density of about 1.9 g/ml, a situation that can blow out the bottom of the rotor. The deration curve supplied by the rotor's manufacturer will indicate those speeds that could cause centrifugation media to precipitate and potentially damage the rotor and or centrifuge (Figure 4.4).

Is a Vertical Rotor the Right Angle for You?

Vertical rotors can purify DNA via cesium chloride centrifugation in three hours, as compared to the overnight runs using fixed-angle rotors. Vertical rotors re-orient your sample (Figure 4.5), so there is the small possibility that the RNA that pelleted against the outer wall of the tube will contaminate the DNA as the gradient re-orientes.

A near-vertical rotor from Beckman-Coulter eliminates this problem (Figure 4.6). The 9° angle of this rotor allows the RNA to pellet to the bottom of the tube without contaminating the DNA. The closeness to vertical keeps centrifugation times short. Triton X-100 was applied in this near-vertical system to improve the separation of RNA from plasmid DNA, although the impact of the detergent on the later applications of the DNA was not tested (Application Note A-1790A, Beckman-Coulter Corporation).

Vertical rotors also allow for the tube to be pulled out straight without the worry of disrupting the gradient. Fixed-angle rotors produce bands at an angle, requiring greater care when removing samples from the rotor.

What Can You Do to Improve the Separation of Supercoiled DNA from Relaxed Plasmid?

Centrifugation at lower *g* force will increase the resolution of supercoiled and relaxed DNA. Apply a step-run gradient, where high speed establishes the gradient, followed by lower speeds and *g* forces to better separate supercoiled and relaxed DNA. Rotor

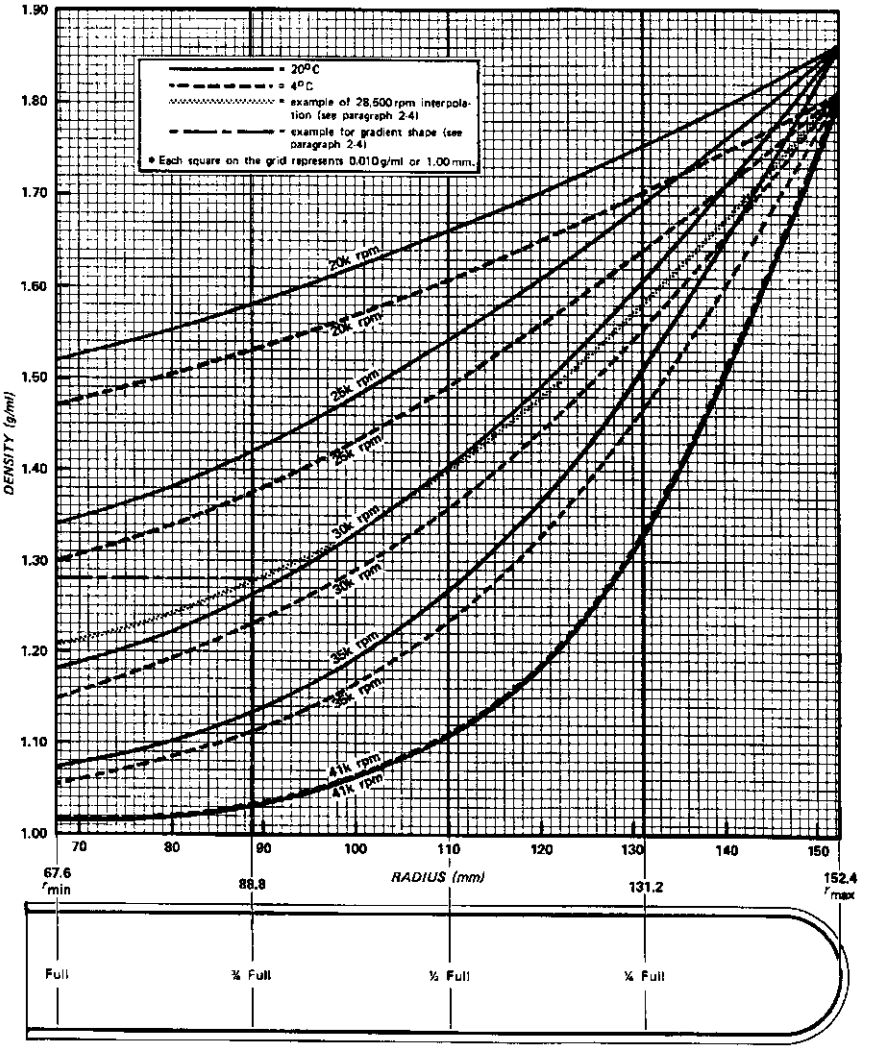

Figure 4.4 Deration Curve. Reproduced with permission of Kendro Laboratory Products.

manufacturers can provide the appropriate conditions for step-runs on your rotor-centrifuge combination.

Troubleshooting

How Can You Best Avoid Service Calls?

Too often operating manuals are buried in unmarked drawers, never to be seen again. This is a costly error because manuals contain information that can often solve problems without the expense and delay of a service call.

Older instruments may have brush motors. The more frequently

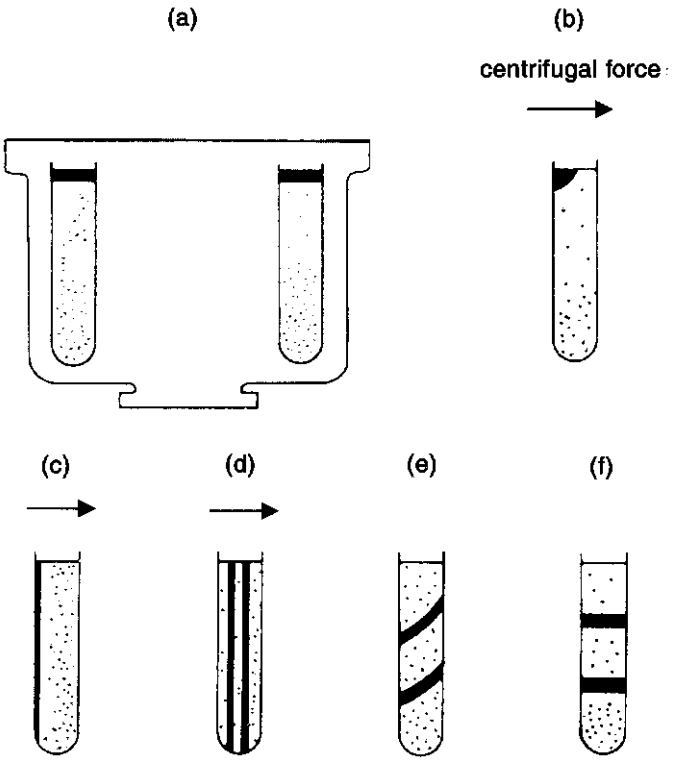

Figure 4.5 Operation of vertical rotors. (*a*) The gradient is prepared, the sample is layered on top, and the centrifuge tubes are placed in the pockets of the vertical rotor. (*b*) Both sample and gradient begin to reorient as rotor accelerates. (*c*) Reorientation of the sample and gradient is now complete. (*d*) Bands form as the particles sediment. (*e*) Bands and gradient reorient as the rotor decelerates. (*f*) Bands and gradient both fully reoriented; rotor at rest. From Centifugation: A Practical Approach (2nd Ed.). 1984. Rickwood, D., ed. Reprinted by permission of Oxford University Press.

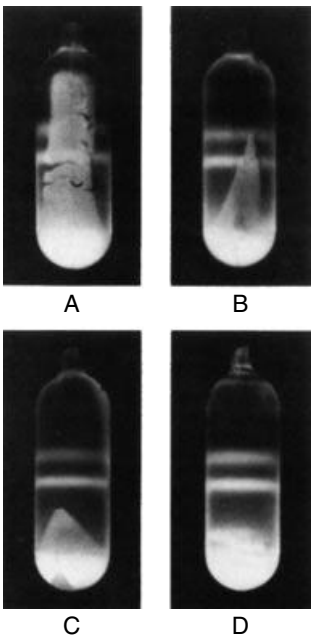

Figure 4.6 Effect of Triton X-100 on RNA pellet in the TLN-100 rotor. The final concentration of Triton X-100 in these tubes is (*a*) 0%, (*b*) 0.0001%, (*c*) 0.001%, and (*d*) 1%. As the Triton X-100 concentration increases, the pellet adhesion decreases. High Triton X-100 levels (0.1–1%) produce a visible reddish band at the top of the tube. Courtesy, Beckman Coulter, Inc., Fullerton, CA, application note A-1790A.

the instrument is used, the more frequently these brushes need to be changed. This is a procedure that you can do yourself, since brushes ordered form the manufacturer usually provide detailed instructions. Most instruments are equipped with an indicator light that signals a worn brush. As with any attempt to repair an instrument, disconnect any power source and consult the manufacturer for warnings on any hazards.

Cleanliness matters. Dirt and spilled materials can enter the motor compartment and cause failures. Clean any spills that occur inside the instrument as soon as possible.

When not in use, turn off the power to a refrigerated centrifuge, and open the chamber door to allow moisture to evaporate. If the instrument must be maintained with the power on, keep the chamber door shut as much as possible and check for frost before use.

Check the level of an instrument after it has been moved. An unleveled instrument can cause damage to the drive mechanism. A post-move preventative maintenance call can prevent problems and ensure that the machine is performing accurately.

What Is the Best Way to Clean a Spill within a Centrifuge?

Spills should be immediately cleaned using a manufacturer-approved detergent. Mild detergents are usually recommended and described in operating manuals. Avoid harsh solvents such as bleach and phenolics.

How Should You Deal with a Walking Centrifuge?

Older units are more prone to walking when imbalanced. If the instrument vibrates mildly, hit the stop button on the instrument. If there is major shaking, cut the power to the instrument by a means other than the instrument's power switch. You can't predict when or how the instrument is going to jump. An ultracentrifuge might require hours to come to a complete stop. Clear an area around the instrument and allow it to move if necessary. Vibration is going to be the greatest at a rotor-dependent critical speed, usually below 2000 rpm. Never attempt to open the chamber door while the rotor is spinning, and don't attempt to enter the rotor area even if the door is open. Don't attempt to use physical force to restrict the movement of the machine. Keep your hands off the instrument until it comes to a complete stop.

How Can You Improve Pellet Formation?

Fluffy pellets that form on the side of a wall are easily dislodged during attempts to remove the supernate. To form tighter pellets,

switch to a rotor with a steeper angle, or spin harder and longer at the existing angle.

PIPETTORS (Michele A. Kennedy)

The accurate delivery of a solution is critical to almost all aspects of laboratory work. If the volume delivered is incorrect, the results can be compounded throughout the entire experiment. This section will discuss issues ranging from selecting the correct pipette from the start to ensuring that the pipette is working properly.

Which Pipette Is Most Appropriate for Your Application?

Different applications will require the use of different types of pipettes or different methods of pipetting. Prior to purchasing a pipette, one should decide which type of pipette will be required to address the needs of the lab. There are two main types of pipettes: air displacement and positive displacement. The air displacement pipette is the most commonly used pipette in the lab. In this type of pipette, a disposable pipette tip is used in conjunction with a pipette that has an internal piston. An air space, which is moved by the internal pipette piston, allows for the aspiration and dispensing of sample. This type of pipette is ideal for use with aqueous solutions.

The second type of pipette is the positive displacement pipette. In this pipette, the piston is contained within the disposable tip and comes in direct contact with the sample solution. Positive displacement pipettes are recommended for use with solutions that have a high vapor pressure or are very viscous. When pipetting solutions with a high vapor pressure, it is recommended to pre-wet the tip. This allows the small air space within a positive displacement system to become saturated with the vapors of the solution. Pre-wetting increases the accuracy of the pipetting because the sample will not evaporate into the saturated environment, which would normally cause a pipette to leak.

Once you have decided if an air or positive displacement pipette is the right choice for your lab, then the next thing to choose is the proper volume range. Determine what will be the most frequently used volume. This will then help you to decide on the style of pipette that will achieve the best accuracy. Fixed-volume pipettes provide the highest accuracy of manual pipettes, but they are limited to one volume. Adjustable-volume pipettes are slightly less accurate, but they allow for the pipetting of multiple volumes with one pipette. For example, an Eppendorf® Series 2100 10 to 100 μl

adjustable pipette, set at 100 µl has an inaccuracy specification of ±0.8%, whereas a Series 2100 100 µl fixed-volume pipettes has an inaccuracy specification of ±0.6%. When choosing an adjustable-volume pipette, remember that all adjustable pipettes provide greater accuracy at the high end of their volume range. An Eppendorf Series 2100 10 to 100 µl adjustable pipette, set at 100 µl has an inaccuracy specification of ±0.8%, whereas a 100 to 1000 µl pipette set at 100 µl has an inaccuracy specification of ±3.0%.

What Are the Elements of Proper Pipetting Technique?

Once you have selected the correct pipette for your application, then you must ensure that the pipette is used correctly. Improper use of a pipette can lead to variations in the volume being dispensed. When working with a pipette, check that all movements of the piston are smooth and not abrupt. Aspirating a sample too quickly can cause the sample to vortex, possibly overaspirating the sample.

When aspirating a sample, it is important to make sure that the following guidelines are adhered to. First, the pipette tip should only be immersed a few mm into the sample to be aspirated (Figure 4.7).

This ensures that the hydrostatic pressure is similar during aspiration and dispensing. Next, the pipette should always be in a completely vertical position during aspiration. The result of holding a pipette at an angle of 30° could create a maximum of +0.5% inaccuracy (*Products and Applications for the Laboratory 2000*, Eppendorf® catalog, p. 161).

When dispensing the sample, the pipette tip should touch the side of the receiving vessel. This will ensure the even flow of the sample from the tip, without forming droplets. If a droplet remains inside the tip, the volume dispensed will not be correct.

Preventing and Solving Problems

A good maintenance and calibration schedule is the key to ensuring that your pipette is working properly. More often than not, the factory-set calibration on a pipette is changed before a proper inspection and cleaning has been performed. A good maintenance program can prevent unnecessary changes in the calibration, which saves money over the life of the pipette. A few maintenance suggestions are listed below:

- Always store pipettes in an upright position, preferably in a stand. This prevents the nose cones or pistons from being bent when placed in a drawer.

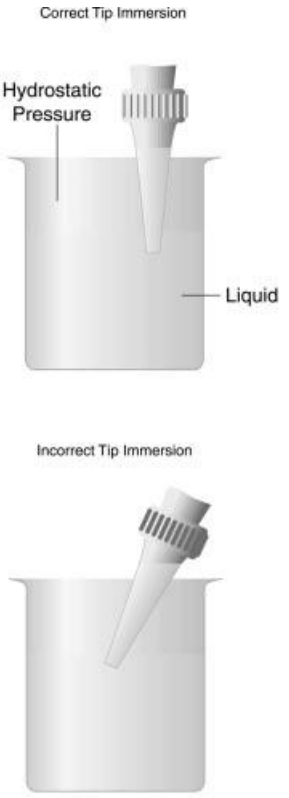

Figure 4.7 Proper placement of pipette tip. Reproduced with permission from Brinkmann™ Instruments, Inc.

- Always make sure that the pipette is clean. Dust or dirt on the nose cone can prevent the pipette tip from sealing properly, which in turn will affect the volume that is being aspirated and dispensed.
- Check that the nose cone of the pipette is not clogged or bent. If the nose cone is clogged or bent, it may change the air column that is aspirating the sample volume. This in turn will affect the volume that is aspirated.
- Make sure that the nose cone is securely fastened on the pipette. The process of placing tips on the pipette should not require the pipette to be twisted. This twisting method is often employed when tips that do not fit properly are used. This can loosen the nose cone and cause the pipette to aspirate incorrect volumes.
- It is strongly recommended that the leak tests described below are performed before you recalibrate a pipette. If a pipette is leaking, a new O-ring or seal might be all that is required to return the pipette to the original factory calibration. The last thing that you want to do is to change the factory calibration of a pipette.

How Should You Clean a Pipette?

Pipette cleaning should be conducted in a very methodical manner. Start from the outside and work your way in.

- Clean the external parts of the pipette with a soap solution. In most cases the pipette can also be cleaned with isopropanol, but check with the manufacturer. Next, remove the tip ejector to expose the nose cone. Ensure that the tip ejector contains no debris or residue. Thoroughly clean the external portion of the nose cone.
- Remove the nose cone, which is usually screwed into the pipette handle. In most cases this will expose the piston, seals, and O-rings. Clean the orifice at the tip of the nose cone to ensure that it is completely free of debris.
- Check the condition of the piston, O-ring, and seal. The piston should be free of any debris. Ceramic pistons (Eppendorf pipettes) should be cleaned and then lightly lubricated with silicone grease provided by the manufacturer. Stainless steel pistons (Rainin® pipettes) should be clean and corrosion-free, and should not be greased. The O-ring should fit on the seal and move freely on the piston.

Overall pipette design is similar from one brand to the next (Figure 4.8), but each pipette has a few slightly different features. Always refer to the manufacturer's instruction manual for recommended cleaning procedures.

How Frequently Should a Pipette Be Tested for Accuracy?

Protocols for testing a pipette may be dictated by several standards. The general market, which includes academic and research labs, follows ASTM, ISO, DIN, GMP, GLP, or FDA guidelines. Clinical laboratories may follow specific guidelines such as NCCLS, CAP, CLIA, and JCAHO.

Pipettes should be inspected and tested on a regular basis. The time interval between checks depends on the established guidelines in the facility and the frequency of use, but in general, a minimum of a quarterly evaluation is recommended.

The best way to establish the time interval between evaluations is to look at the work that you are conducting and determine how far back you would like to go to repeat your research if a problem is detected. For example, if a pharmaceutical company was at the halfway point of developing a new drug and had the calibration of their pipettes checked, only to find that they were all out of calibration—the course of action would be to repeat all of the work that was conducted using these out-of-calibration pipettes. By reducing the amount of time between calibrations,

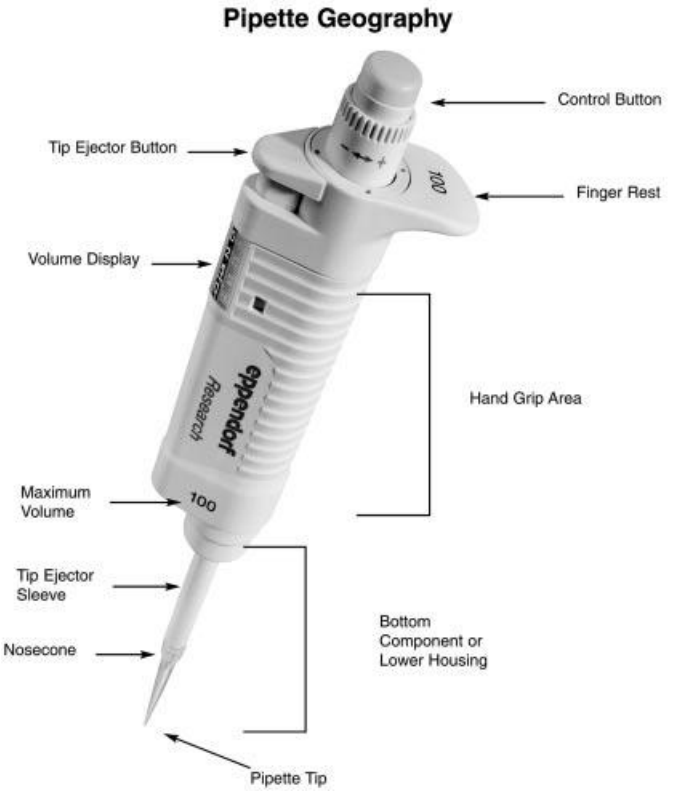

Figure 4.8 Pipette anatomy. Reproduced with permission from Brinkmann™ Instruments, Inc.

you can reduce the amount of time redoing experiments, if a problem is found.

How Can You Monitor the Performance of a Pipette?

All of the internal components of the pipette must be tested to determine that they are fully functional. The first thing that should be checked is the free movement of the piston. The piston should move up and down very smoothly. Next, verify that the internal parts are working properly by performing a leak test. Although this test does not measure accuracy and reproducibility, it is a quick and easy determination of the proper functioning of the internal parts. Please remember that this type of test will only ensure that the internal parts of the pipette are not contributing to a leak in the pipette or tip system. It does not test if the pipette is delivering the specified volume set on the volume display. Two methods to detect leaks are described below. These tests are appropriate for pipetted volumes greater than 10 μl; smaller volumes do not displace a sufficient amount of air to visually check the performance of the pipette.

The first, and easiest, approach is to set the pipette to the maximum rated volume, attach a pipette tip, aspirate liquid into the pipette tip, and hold the pipette in a vertical position for 15 seconds. If the liquid does not drip out, the fit of the seals and O-rings around the piston is good and there is no need to replace them. A leak will appear as a droplet on the pipette tip, which indicates that the pipette needs to be serviced.

A second method is to monitor the stability of a column of liquid in an attached 20 cm segment of PVC tubing. Hold the pipette vertically, aspirate the liquid (colored liquid can be used) and mark the meniscus level on the tubing. Wait one minute, then check if the meniscus level has changed. If a change in the level occurs, a leak exists and the pipette should be serviced. (*Eppendorf SOP Manual*, p. 26.) If the tubing is connected directly to the pipette, the internal parts are tested but not the interaction of the pipette and tip. Testing the direct connection to the pipette and then testing the pipette and tip connection ensures that internal parts are not leaking and that the tip is not causing any leaks. The size of the tubing depends upon the volume of the pipette to be checked.

Pipette Volume	Tubing Inner Diameter (mm)
10–100 µl	0.5–1
100–500 µl	1.5–2
>500 µl	5

How Can You Check If a Pipette Is Dispensing Accurate Volumes?

Gravimetric testing of pipettes refers to the technique of weighing a dispensed amount of liquid, changing the weight to a volume, and then determining if the volume is within the manufacturer's stated specifications. This is the most accepted form of testing the volume delivery of a pipette.

According to the Eppendorf standard operating procedure for pipette calibration, the following information details the equipment, the actual procedure, and the mathematical calculations needed to determine if the pipette is within the factory stated calibration specifications.

The following components are required for a measuring station for calibrating or adjusting pipettes:

1. Fine balance (tested by Board of Weights and Measures; e.g., Sartorius®, Mettler®, Ohaus®, or AnD). The resolution of the

balance depends on the volume of the pipette that is to be tested. The lower the volume, the better the resolution of the balance needs to be. The balance should be located in an area that is free of drafts and vibrations.

Nominal Volume of Pipette (µl)	Error Limits of Device to Be Tested (µl)	Required Accuracy to Be Tested (g)
1–50*	0.1–1.0	0.00001
100–1000	1.0–10	0.0001
>1000	>10	0.001

2. Evaporation protection. A moisture trap or other equipment that prevents evaporation, such as a narrow volumetric flask, are recommended for use. In addition to the narrow weighing vessel, it is advisable to use a moisture trap within the balance. This can be as simple as placing a dish filled with approximately 10 ml of distilled water within the balance. For pipettes with a maximum volume of 5000 µl and above, a moisture trap is not needed.

3. Room Temperature. Ambient temperature should be 20° to 25°C, ±0.5°C during measurement. Factors that affect the temperature of the pipettes and measuring station (e.g., direct sunlight) should be avoided. The ambient temperature and the temperature of the test liquid and pipettes must be the same as the temperature of the pipette tip. For example, if the sample is at 4°C and the pipette is at room temperature (22°C), this could result in a maximum error of –5.4% (Eppendorf catalog 2000, p. 161). It is advisable to equilibrate all components for approximately three to four hours prior to calibration.

4. Test Liquid. Degassed, bi-distilled, or deionized water which is at room temperature (20–25°C) should be used. The water in the liquid supply or in the weighing vessel must be changed every hour and must not be reused. The air humidity over the liquid surface of the weighing vessel should be maintained at a uniform value between 60% and 90% of the relative humidity.

**For volumes <1 µl, the balance is set with six decimal places, or where appropriate, a photometric test may be used.*

5. Instruction Manual. In view of the many different types of volume measuring devices, it is particularly important to refer to the manufacturer's instruction manual during testing.

6. Test Points. The number of test points is determined by the standard that is used. As a rule of thumb, a quick check involves 4 test points, a standard check involves approximately 8 test points, and a full calibration can involve 20 or more test points at each volume.

7. Test Volumes. Most standards test adjustable-volume pipettes at the following three increments:

a. The nominal volume (the largest volume of the pipette)
b. Approximately 50% of the nominal volume
c. The smallest adjustable volume, which should not be less than 10% of the nominal volume

When testing fixed-volume pipettes, only the nominal volume is tested. When testing multiple-channel pipettes, the same volumes are tested for each channel.

Perform The Gravimetric Test

1. Weigh the samples:

- Tare the balance.
- Pre-wet the tip.
- Aspirate and then dispense the set volume three times. Execute blow-out.

2. Aspirate the volume that is to be tested from the liquid supply as follows:

- Hold the pipette vertically in the liquid supply.
- Immerse the tip approximately 2 to 3 mm into the test liquid.
- Aspirate the test volume slowly and uniformly. Observe the waiting period of one to three seconds.
- Remove the pipette tip from the test liquid slowly and uniformly. Remove any remaining liquid by placing the pipette tip against the inside of the vessel.

3. Dispense the test volume into the weighing vessel as follows:

- Place the filled tip at an angle of 30° against the inside of the weighing vessel
- Dispense the test volume slowly and uniformly up to the first stop (measuring stroke) and wait for one to three seconds. (This applies to manual pipettes only.)

- Press the control button to the second stop (blowout) and dispense any liquid remaining in the tip
- Hold down the control button and pull the tip up along the inside of the weighing vessel. Release the control button.

4. Document the value that appears in the display of the balance immediately after the display has come to rest. Record the values from a measurement series as described above. Evaluate the inaccuracy and the imprecision as described below.

Determine Calibration Accuracy

In order to determine if the pipette is with in the factory calibration range, the mean volume, standard deviation, coefficient of variation, % inaccuracy and % imprecision must be determined. This involves completing the following calculations.

1. Mean Volume ($\bar{x}$). This is the sum of the number of weights (at one volume setting) divided by the number of test points.

$$\bar{x} = \frac{X_1 + X_2 + X_3 + X_4}{\text{Number of weighings}}$$

where X_1, X_2, X_3, X_4, etc. are the actual measured weights

2. Adjustment for Z Factor. The Z factor accounts for the temperature and barometric pressure conditions during testing. (See Table 4.3.)

$$V = \bar{x} * Z$$

where $Z = Z$ factor
$\bar{x}$ = mean of measured volume in µl
V = adjusted mean volume

3. Calculation of (In)Accuracy (A). Accuracy points to the amount of scatter that a pipette varies from its set point:

$$A = \frac{V - SV}{SV} * 100$$

where A = accuracy
V = adjusted mean volume
SV = set volume of pipette

4. Calculation of Standard Deviation (sd). The sd calculation points to the scatter of volume around the mean value:

Table 4.3 Factor *Z* (μl/mg) as a Function of Temperature and Air Pressure for Distilled Water (ISO DIS 8655/3)

Temperature (°C)	hPa(mbar)					
	800	853	907	960	1013	1067
15	1.0018	1.0018	1.0019	1.0019	1.0020	1.0020
15.5	1.0018	1.0018	1.0019	1.0020	1.0020	1.0020
16	1.0019	1.0020	1.0020	1.0021	1.0021	1.0022
16.5	1.0020	1.0020	1.0021	1.0022	1.0022	1.0023
17	1.0021	1.0021	1.0022	1.0022	1.0023	1.0023
17.5	1.0022	1.0022	1.0023	1.0023	1.0024	1.0024
18	1.0022	1.0023	1.0024	1.0024	1.0025	1.0025
18.5	1.0023	1.0024	1.0025	1.0025	1.0026	1.0026
19	1.0024	1.0025	1.0025	1.0026	1.0027	1.0027
19.5	1.0025	1.0026	1.0026	1.0027	1.0028	1.0028
20	1.0026	1.0027	1.0027	1.0028	1.0029	1.0029
20.5	1.0027	1.0028	1.0028	1.0029	1.0030	1.0030
21	1.0028	1.0029	1.0030	1.0030	1.0031	1.0031
21.5	1.0030	1.0030	1.0031	1.0031	1.0032	1.0032
22	1.0031	1.0031	1.0032	1.0032	1.0033	1.0033
22.5	1.0032	1.0032	1.0033	1.0033	1.0034	1.0035
23	1.0033	1.0033	1.0034	1.0035	1.0035	1.0036
23.5	1.0034	1.0035	1.0035	1.0036	1.0036	1.0037
24	1.0035	1.0036	1.0036	1.0037	1.0038	1.0038
24.5	1.0037	1.0037	1.0038	1.0038	1.0039	1.0039
25	1.0038	1.0038	1.0039	1.0039	1.0040	1.0041
25.5	1.0039	1.0040	1.0040	1.0041	1.0041	1.0042
26	1.0040	1.0041	1.0042	1.0042	1.0043	1.0043
26.5	1.0042	1.0042	1.0043	1.0043	1.0044	1.0045
27	1.0043	1.0044	1.0044	1.0045	1.0045	1.0046
27.5	1.0044	1.0045	1.0044	1.0045	1.0045	1.0046
28	1.0046	1.0046	1.0047	1.0048	1.0048	1.0049
28.5	1.0047	1.0048	1.0048	1.0049	1.0050	1.0050
29	1.0049	1.0049	1.0050	1.0050	1.0051	1.0052
29.5	1.0050	1.0051	1.0051	1.0052	1.0052	1.0053
30	1.0052	1.0052	1.0053	1.0053	1.0054	1.0055

$$sd = Z * \sqrt{\frac{(X_1 - SV)^2 + (X_2 - SV)^2 + (X_3 - SV)^2 + (X_4 - SV)^2}{\text{Number of weighings} - 1}}$$

where $Z = Z$ factor
X_1, X_2, X_3, X_4, etc. are the actual measured weights
SV = set volume of pipette

5. Calculation of (Im) Precision with the Coefficient of Variation (CV). Calculate the standard deviation in percent:

$$CV = \frac{sd}{V} * 100$$

where sd = standard deviation
V = adjusted mean volume

After obtaining all of the preceding information, the results should be compared to the manufacturer's stated specifications. If the pipette is within the stated calibration specifications, it has passed the calibration test.

If the pipette does not meet the specifications, the calibration on the pipette must be changed. This can be accomplished in two different ways, depending on the brand and style of the pipette. In some pipettes, to change the calibration, you adjust the piston stroke length. This basically changes the amount of movement that the piston has during an aspiration/dispensing step, thus changing the volume that is aspirated to match the volume that should be aspirated. The other way to change the calibration of a pipette is to change the volume display to match the volume that was actually dispensed. Please refer to the manufacturer's instruction manual of your pipette to determine the correct way to adjust your pipette.

Once the pipette has been adjusted, the pipette should be retested to ensure that the pipette is now in proper working order.

Troubleshooting

Table 4.4 describes commonly found problems and possible solutions.

pH METERS (Jane Stevens)

What Are the Components of a pH Meter?

Sensing Electrode

This is described in greater detail later in the section "Which pH electrode is most appropriate for your analysis?"

Reference Electrode

The "reference" is the electrochemical industry term for the half-cell electrode whose constant potential is measured as E_0 in the Nernst equation (Figure 4.9). This half-cell is held under stable conditions generating a fixed voltage to which the pH-sensing electrode is compared. There are several types of reference electrode systems. Some such as the standard hydrogen electrode are important theoretically but not practical for actual use. The most commonly used reference electrode system is silver/silver chloride (Ag/AgCl). A silver wire is suspended in a solution of potassium chloride that has been saturated with silver to replenish the wire with silver ions. The calomel reference system uses mercury

Table 4.4 Pipette Troubleshooting Guide

Problem	Possible Cause	Solution
Pipette drips or leaks	Tip is loose or does not fit correctly	Use manufacturer recommended tips Use more force when putting the tip on the pipette
	Nose cone is scratched	Replace the nose cone
	Seal of the nose cone leaks	Replace the nose cone
	Piston is contaminated by reagent deposits	Clean and lubricate the piston (if recommended) Replace the seal
	Piston is damaged	Replace the piston and the piston seal
	Piston seal is damaged	Replace the piston seal and lubricate the piston (if recommended)
	Nose cone has been loosened	Retighten nose cone
Push button does not move smoothly	Piston is scraping due to contamination	Clean and lubricate the piston
	Seal is swollen due to reagent vapors	Open pipette and allow it to ventilate Lubricate the piston only if necessary
	Piston is visibly damaged or coated with insoluble solution	Replace piston seal and piston
Inaccurate volumes	Pipette is leaking	Verify that all of the above situations have been checked
	Pipette's calibration has been changed incorrectly	Recalibrate according to manufacturer's specifications
	Poor pipetting technique	Refer to section on pipetting technique

instead of silver; manufacturers also provide reference systems that lack metal ions altogether.

Junction

The junction is the means for the sample and electrode to contact electrically. The internal filling solution and the sample mix at the junction. The electrode should have a sufficient flow of filling solution that passes through the junction so that the sample and filling solution meet on the sample's side of the junction. This better protects the electrode from backflow of sample components. An electrical potential (the junction potential) due to the ion movement develops at the junction contributing a small elec-

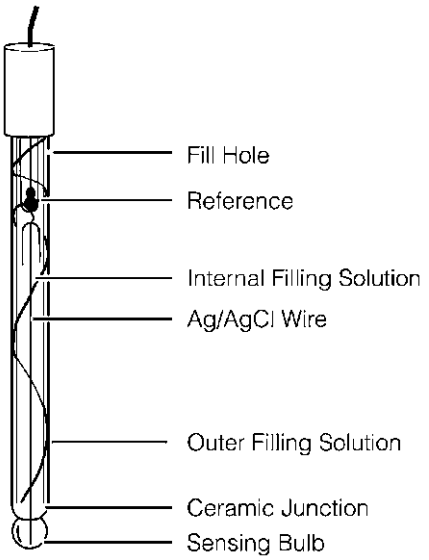

Figure 4.9 Double-junction combination electrode. Reproduced with permission from Thermo Orion Inc.

trical voltage to the overall measurement system. Generally this is a minor error. If the flow of filling solution is not adequate, backflow can cause this error to increase as ions moving at different rates cause an accumulation of charges. The filling solution should be equitransferent (the positive and negative ions can pass freely through the junction), thus minimizing charge accumulation and junction potential error. It is difficult to tell if the flow is adequate in some electrode junctions. A faster flowing junction such as the annular, flushable style (Figure 4.10) will reduce the chances of a poor junction between the sample and electrode. A poor junction will give erratic readings and thus erratic pH values as the additional charges are created in the dynamic solution. A change of 6 mV is needed to change the pH by 0.1. It is very difficult to get reproducibility and accuracy without sufficient flow through a junction. Sluggish, drifting readings are indications that the flow may be impaired.

Fill Solution

Reference filling solution or internal filling solution is the electrolyte that is the contact point between the sample and the reference electrode. The filling solution completes the circuit to measure the voltage change due to the sample. It is comprised of salts that conduct electricity and allow the reference electrode to have a stable voltage for a period of time. The fill solution most often contains potassium chloride, but incompatibility with some

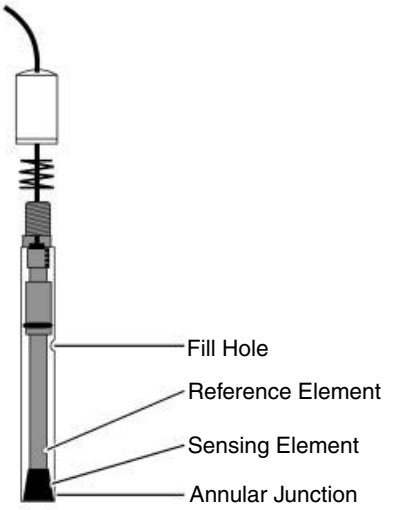

Figure 4.10 Annular pH electrode. Reproduced with permission from Thermo Orion Inc.

samples requires alternate solutions. An example where a different filling solution may be required is with ultra-pure, low ionic strength water. The concentrated KCl would cause the reading to drift as it mixed with the pure water. A lower ionic strength filling solution, such as 2.0 M KCl saturated with Ag^+, would produce faster, more accurate and reproducible readings.

Fill Hole

The filling hole cover on the electrode body must be removed for a positive flow through the junction.

How Does a pH Meter Function?

Theory

pH is an electrochemical measurement of the activity of the hydrogen ion, H^+, in a particular solution. The pH meter measures voltage, in millivolts (mV), from the "battery" created by the electrodes in an aqueous solution (Figure 4.11). The measured voltage is the difference between the electrical potentials of the reference and sensing electrodes. The sensing electrode is usually made of glass which is very sensitive to changes in hydrogen ion activity. Software in the pH meter makes the conversion to solution pH based on previous calibration data stored in its memory. The meter displays the calculated pH of the solution to the operator. Other pertinent information, such as temperature, time and date, and actual millivolts read from the sample are often displayed on more advanced meters.

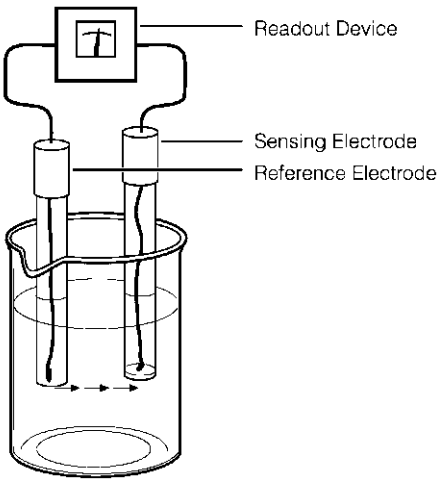

Figure 4.11 Mechanism of pH meter function. Reproduced with permission from Thermo Orion Inc.

Calibration

In order for the meter and electrode system to determine the pH of a sample, it must compare the sample to known values or standards. The standards are specially formulated buffers that have been carefully studied to determine the effect of temperature on their pH. These buffers are used to generate a calibration curve plotting the known pH value versus the measured millivolts to determine the pH of a sample. The millivolt measurement of the sample is plotted on the curve and the pH value is read from the curve.

The pH calibration curve is essentially linear. An average slope is determined at the end of the calibration and reported as percent, with 100% being the theoretical value at that temperature, or as a millivolts per decade of pH where 59.16 is theoretical at 25°C (Figure 4.12). This slope will change with temperature, but it will automatically be corrected by the meter if the correct temperature is entered.

Almost all laboratory pH electrodes are designed so that they read zero millivolts at pH 7. This zero point is called the isopotential point. Calibration curves at different temperatures, and therefore with different slopes, all pass through this point. Buffers are sold with the temperature-corrected pH values on their labels, or they can be found in the research literature.

How Does the Meter Measure the Sample pH?

The meter calculates the sample pH by measuring the mV of the sample, then using the Nernst equation to solve for pH. The Nernst equation is used to describe electrode behavior:

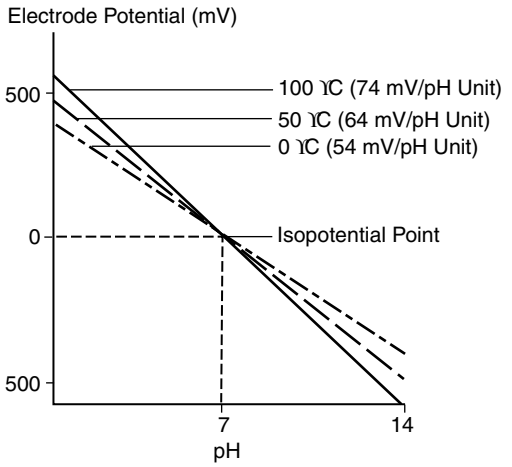

Figure 4.12 Effect of slope on pH determination. Reproduced with permission from Thermo Orion Inc.

$$E_{\text{measured}} = E_0 + S \log a_{\text{H+}}$$

where S is the slope, and E_{measured} is the electrical potential measured at the sensing electrode, and E_0 is the electrical potential measured at the reference electrode, and the log of the hydrogen activity is the pH. Both reference and sensing electrodes are present within a single probe in most pH meters. Due to the presence of the slope term, it is easy to understand the importance of calibration of the meter and electrode system. The meter uses the correct slope for the temperature of the sample, which gives more accurate pH readings. This is especially critical when the pH value varies dramatically from the isopotential point. The further away the pH is from the isopotential point, the greater is the effect a change in temperature produces on the result.

What Is the Purpose of Autobuffer Recognition?

Many meters have an autocalibration feature, autobuffer recognition, that simplifies calibration. This system enables the meter to identify buffers by the buffer's observed millivolt value. For example, a buffer with a millivolt reading of approximately 0 would be identified as pH 7.00, and a reading of around 167 mV would be identified as pH 4.01. If the meter is measuring the temperature, it can automatically adjust for the change in the value of the buffer caused by a change in the temperature.

Common calibration errors occur because of mistakes made in the operation of the autobuffer feature of their meter. Since most meters have pH versus temperature tables or algorithms in their software that will correct for the temperature, the user does not need to adjust the value displayed to account for the buffer pH

change due to temperature as they would in a manual calibration. A second source of problems is not matching a buffer or group of buffers with the correct buffer set in the meter. Many meters allow the user to select the category of buffers they want to autorecognize. These could be the NIST (National Institute of Standards and Technology) buffers (4.01, 6.86, etc.), 4, 7, and 10 buffers, or other sets commonly used in different countries or required by government regulations, such as DIN buffers. Refer to the section "Should you use a non-NIST or non-NIST-traceable buffers to calibrate your pH meter?" for more details. Each set will have the pH versus temperature table or algorithm for that particular formulation, so errors will occur if a different buffer is used. For example, pH 7.00 and the NIST 6.86 buffer will both read close to 0 mV but will have different temperature effects due to their different chemical compositions. Errors in the calibration will give inaccurate sample results.

Which Buffers Are Appropriate for Your Calibration Step?

Proper Bracketing

It is essential that the calibration standards cover the entire range of your anticipated sample pH values and include a buffer near the isopotential point. If you will be measuring samples in the pH range of 5 to 6, then buffers 4 and 7 would be appropriate. If you have samples in the 9 to 10 range, buffers 4 and 7 would not generate accurate results as the meter would have to mathematically extrapolate the curve to determine the sample pH, but would not take into account other electrode variables. Calibration buffers with pH of 7 and 10 would be acceptable. Analyzing samples covering a wider range will be more accurately measured with a multiple-point calibration covering a range such as 4, 7, and 10, or even 1.68, 4, 7, 10, and 12.46. Advanced meters do not just draw a best-fit line in these calibrations and use that slope for all measurements. In order to generate the most accurate results, the sample is compared to the line segment that brackets the sample pH. Thus a sample with pH of 11 would be calculated using the slope of the line segment from pH 10 and pH 12.46. This multiple-point calibration strategy maximizes accuracy and saves time by reducing the number of calibrations performed when a wide range of samples is being analyzed.

NIST or Non-NIST-Traceable Buffers

The National Institute of Standards and Technology is a U.S. government agency that sells standards that have been extensively

characterized for certain parameters. NIST sells the salts used to make a range of buffers and has generated tables of the temperature relationship to the pH in chemical references such as the *CRC Handbook of Chemistry* (Weast 1980). These buffer salts are primary reference standards and are very expensive due to the studies that these particular samples have undergone. Most buffers that are commercially available are compared to these buffer salts. Such commercial buffers are labeled NIST-traceable. The European community uses a set of buffers with different chemistries, and these are referred to as the DIN buffers. Most countries recognize other agencies' buffers, but it is best to verify this in cases where the measurements are for international regulatory requirements. As with any chemical measurement where standards are used to quantify a sample, the quality of the standard is critical to accurate sample results. Buffers that are traceable to a defined, accepted agency give more confidence in the sample results than results achieved by untested buffers or buffers of unknown quality. Sample measured against traceable buffers are more easily defended in laboratories where auditing occurs.

What Is Temperature Compensation and How Does One Choose the Best Method for an Analysis?

Temperature compensation is the term for the meter correction for the effect of temperature on the calibration curve. The sample temperature can be received by the meter in various ways. It can be measured by an automatic temperature compensation (ATC) probe, available separately or built directly into the electrode or, in certain new meter circuitries, by the pH electrode itself. Alternately, the operator can manually enter the temperature into the meter. The best method for the user is determined by sample characteristics and cost. An ATC probe or combination pH electrode with internal ATC probe is preferred if sample volume is sufficient. ATC probes are comprised of glass, epoxy, stainless steel, and other materials to provide compatibility with different sample types.

While it is possible for the meter to compensate for the effect of temperature on the calibration, the pH of a solution can itself change with temperature. The solution temperature can shift equilibria within the solution, which can create or scavenge the ions being measured. There is no way to predict or control this temperature effect, so the temperature at which the pH was

determined should be recorded to ensure the data can be reproduced in the future. The internal reference of the electrode also requires time to reach a temperature equilibrium with the sensing bulb (the liquid inner filling solution and a solid metal wire change temperatures at different rates). This temperature effect can be minimized by using electrodes with nonmetallic reference systems.

How Does Resolution Affect pH Measurement?

Resolution is the number of decimal places past the decimal point shown by the meter. Some meters can be set to read, as, 9.0, 9.03, or 9.034. It is best to use only the number of places actually required for the measurement. For example, if a solution must be adjusted from pH 4.3 to 4.5, there is no point in trying to read pH 4.302. The more places that are used, the longer it takes to get a steady reading as the electrode gradually approaches the final value. The resolution of less expensive electrodes is not as high as refillable electrodes. Check the electrode specifications to ensure that the appropriate resolution is being used or the electrode may be slow or never fully reach stability. A stabilized pH reading may still "shift" on the display due to temperature fluctuation. This is a normal occurrence, but the effect will be more pronounced at higher resolution settings.

Why Does the Meter Indicate "Ready" Even as the pH Value Changes?

Because the electrode gradually approaches a final value, there is a trade-off between the time delay before a reading is recorded and getting sufficient precision. Meter design applies different criteria for deciding a sufficient wait period. Changes after the "ready" indicator comes on are usually small. A stabilized, temperature-compensated pH reading may still "shift" on the display due to the temperature fluctuations.

Which pH Electrode Is Most Appropriate for Your Analysis?

Sample Matrix

The sample matrix is the most significant factor to be considered. Proteins, Tris buffers, and other biological samples (agar plates, plasma, cells, fermentation vat samples) cause precipitates with silver ions. The best solution is an electrode without silver, but a free-flowing annular junction can reduce this effect because the greater outward flow of filling solution and ease of cleaning makes it less prone to clogging. Traditionally calomel electrodes

have been used for protein and Tris buffered samples, but these electrodes use a mercury/mercuric chloride reference instead of silver/silver chloride. The health hazards associated with mercury and the expensive special disposal make them less desirable.

Some manufacturers now offer a nonmetallic, redox couple reference system which is not hazardous to biological samples or lab personnel. It contains no ions to cause precipitates with proteins. New electrodes are also available with isolated Ag wire references and AgCl inner filling solutions that do not come in contact with the sample. These electrodes use a polymer gel to keep the sample and silver separated. Electrode manufacturers can guide you to the best electrode for your sample.

Sample Volume and Format

Standard electrodes are typically 12 to 13 mm in diameter, but these might not contact the sample without first transferring a portion of the sample to a suitable container. A longer electrode will be required for samples that cannot be disturbed or transferred. Small sample volumes can be accommodated by semi-micro electrodes with sensing bulb diameters of 4 to 6 mm, while flat surface electrodes can measure the pH of a sample surface, such as agar plates, cheese, or a drop of a limited quantity of sample. Microelectrodes are available, some with needles to pierce septa and measure pH inside a vial. This would benefit sample measurements where exposure to air is not desired. Microelectrodes can also measure pH in microtiter plates.

Temperature

Temperature of the sample is another consideration. Most glass electrodes are stable up to 100°C. Other electrodes are designed to be steam sterilizable or autoclavable. Epoxy bodied electrodes cannot be used at excessively high temperatures; some are stable up to 90°C but many are rated for 80°C.

Combination Electrodes

Combination electrodes are a single electrode that contains both the reference electrode and the sensing electrode within one body. The reference electrode is usually a silver wire with AgCl solution surrounding it, although it can also be a calomel, redox couple, or other reference system. The sensing electrode is usually pH glass but it can be a special transistor in the case of ion specific field effect transistor (ISFET or FET) electrodes. Combination electrodes requires smaller sample volumes than two-

electrode systems, but lack their ability to isolate and vary the reference, which allows for more experimental control.

Refillable or Nonrefillable

Nonrefillable electrodes are less costly and require low maintenance. They may be submersible as there is no hole on the side that must have atmospheric pressure to cause the filling solution to flow. They usually have a fiber or wick junction for the gelled filling solution to leak outside and this flow cannot be stopped. Often these junctions exhibit a "sample memory" which is due to backflow of sample material into the electrode. Several double-junction, low-maintenance electrodes utilize a high-performance polymer for the internal filling solution. These electrodes have open junctions, where the polymer fills a hole in the glass and the silver/silver chloride reference is isolated from the sample by the polymer. These electrodes also can be used with commercial production samples since the silver never contacts the sample, just the gel which has no silver in it. While some polymer systems are prone to hydrolysis, these electrodes offer longer lifetimes, low maintenance and advanced features.

The refillable electrode offers the greatest flexibility and longest lifetime. Most are stored dry so they are better suited for infrequent users, such as classrooms. The filling solution may be altered as required by the samples and when sample contamination occurs. A refillable electrode costs more initially but can be more cost effective due to longer lifetime than a less expensive, nonrefillable electrode.

How Can You Maximize the Accuracy and Reproducibility of a pH Measurement?

New Systems

Prepare and Condition the Electrode

Electrodes need to be conditioned prior to use. The combination electrodes most commonly used in labs have several components that require stabilization for reliable operation. During long-term storage the electrode dries up to some extent, and refillable electrodes stored dry need to be filled with internal filling solution which itself must equilibrate chemically and thermally with the reference material. In addition the glass-sensing electrode needs to be hydrated to measure the pH. The junctions need to be flowing again so that the buffer and sample can create contact with the reference electrode. Refillable electrodes stored wet with their fill hole covers closed need the fill hole opened to create

a positive flow at the junction. Junctions, particularly ceramic designs, become clogged as they dry out due to salt formation from KCl or other sample components, so they require soaking to flow properly. For all of these reasons, the electrode needs time to stabilize before use.

Conditioning is important for a good start to the analysis. The electrode should be soaked for approximately 15 minutes in a commercial storage solution to hydrate and equilibrate the electrode. If storage solution is not available, a mixture of 200 ml of pH 7 buffer and 1 gram of KCl may be used temporarily. Conditioning the electrode around pH 7 is the usual choice for the first calibration point, so it is the best choice for most electrodes. An exception is ion-selective field effect transistor (FET) electrodes which are better conditioned by pH 4 buffer. A calibration curve should then be performed on the reconditioned electrode. If the calibration fails and excessive drift or sluggish behavior is observed, ensure the junction is working and condition the electrode a little longer.

Proceed as per Existing Systems Below.

Existing Systems

Inspect the Reference Filling Solution

Optimally the solution should be filled to just below the fill hole at the start of the day or whenever the level lowers significantly. The electrode should be allowed to equilibrate with its new solution, which may vary slightly in temperature and composition from that already in the electrode.

The meter and electrode system should be calibrated at the beginning of each day of use and then approximately every two hours or when electrode performance is in question. Frequent calibration is recommended because sample components can migrate into the solution if the flow is not adequate or is interrupted for a time. If the pH response is slow, if the solution appears dirty or unusual, or if the samples are known to clog junctions, the filling solution should be changed. When routine maintenance is performed, the filling solution should be drained from the electrode and refilled with fresh solution.

Calibrate the System

The pH meter should be multiple-point calibrated at the beginning of each day of use and then about every two hours for accurate results. The electrode's sensing glass, filling solution, and

junction change slightly during sample analysis and recalibration accounts for this. A one-point calibration is often used for the recalibration, since it will shift the millivolts intercept(E_0) of the line without altering the multiple-point calibration slope from the beginning of the day. The calibration should use buffers that bracket the pH of the samples to be measured, as discussed earlier.

The buffers and samples should be stirred using a magnetic stirring plate with an insulation barrier between the beaker and plate to prevent drift due to the heat generated by the stir plate. The first buffer should be pH 7 as the meter determines the E_0 point. The other pH buffers should be measured in order from lowest to highest, and the electrode rinsed with deionized water between buffers to reduce equilibration time. If the pH meter does not automatically monitor and incorporate temperature effects, these data should be manually entered into the instrument.

Verify the potency of your buffer standards. Buffer solutions expire over time because trace CO_2 from the air will leach into bottles, form carboxylic acid and cause shifting pH values and consuming buffer capacity. This is especially evident in pH 10 buffer where an open beaker will become more acidic over the course of a few hours. Because of varying environmental conditions, the lifetime of a buffer standard cannot be predicted. For this reason single-use buffer pouches have become very popular.

Measure the pH

The electrode must be immersed into the buffer or sample so that the sensor of the electrode and the junction are submersed. The filling solution of the electrode must be at least one inch above the sample surface. The buffers and samples should be stirred using a magnetic stirring plate with a thermal insulation barrier between the beaker and plate. The best reproducibility is achieved when the buffer and samples are measured at the same temperature. The electrode should be rinsed with deionized water, shaken or blotted dry, and rinsed with the next solution to be measured. The electrode should not be wiped dry because static discharge will build up on the electrode and give drifting readings until the discharge has dissipated. Ideally temperature should be recorded with every pH measurement, and a calibration should be run after the last sample to ensure the electrode still meets performance criteria. Quality control samples (i.e., a pH 5 buffer when using a meter calibrated at pH 4 and 7) can be interspersed among the samples.

How Do Lab Measurements Differ from Plant or Field Measurements?

Field measurements lack the controlled environment and sample handling of the laboratory. Field and plant measurements are often measured by placing the electrode directly into the total sample, rather than measuring an aliquot from a larger batch. This can mean less control of sample turbulence or homogeneity which can cause pH drift. The temperature should be recorded and calibration should occur at the site where the samples are measured.

Does Sample Volume Affect the Accuracy of the pH Measurement?

Generally, the sample volume itself doesn't directly affect pH accuracy, but reproducibility will suffer if the electrode sensor and junction are not submersed in the sample. As described below, smaller volumes are more prone to pH alteration from atmospheric CO_2. Temperature and cooling rates can vary with sample volume, so it is best to use the same amount of sample in each measurement. Any sample treatment (i.e., dilution or measuring a supernatant) should be performed for all samples; fewer variables generate more reliable data. Automated sampling systems will operate best with the same volume as the electrode will be lowered into the beaker a fixed amount.

How Do You Measure the pH of Viscous, Semisolid, Low Ionic Strength, or Other Atypical Samples?

Viscous and semisolid samples are best measured with a fast flowing flushable junction electrode or FET technology. Flushable junctions are designed for easy cleaning and will allow a better sample contact with the faster flow. FET sensing electrodes can be cleaned without the polarization issues of glass electrodes. Low ionic strength samples are best measured with refillable electrodes with low ionic strength filling solution.

Calibrating the system with buffers similar in ionic strength to the sample will increase reproducibility when measuring unusual samples. Small volumes of samples with low ionic strength can be affected by exposure to air. The greater ratio of surface area to volume for a smaller sample increases the potential for CO_2 to mix with the sample and cause a pH shift than with a larger sample. This is observed in ultrapure water and is part of the USP injection water testing protocol (USP 645, US Pharmacopeia).

How Can You Maximize the Lifetime of Your pH Meter?

Proper Usage

If the manufacturer's instructions are followed and product ratings adhered to, a quality meter should last many years. Protection of the meter from liquids, wiping up spills and respectful use gives long life. If the meter is to be used in harsh environments, use a meter rated rated for such work. For example, a waterproof system is better suited for work in the field or on ships.

Proper Cleaning

Precipitates at the Electrode Junction

The precipitate that forms at the electrode junction is crystallized potassium chloride from the inner filling solution. This "KCl creep" is created as the inner filling solution containing KCl comes through the junction when there is no sample or liquid on the external side of the junction. The water of the filling solution evaporates and crystals form. The creeping KCl should be rinsed away with deionized water and the filling solution height checked prior to putting the electrode back into service.

Clogged Electrode Junctions

There are several junction types, and they require different cleaning techniques. Consult the instruction manual for your particular electrode. In general, soaking or sonicating in a commercial cleaning solution for your sample type or a dilute hydrochloric acid solution can often remove sample buildup. Protein accumulation often requires a cleaning solution with pepsin for faster removal. After cleaning, the filling solution chamber of the electrode should be flushed with copious amounts of deionized water, then rinsed with filling solution several times. The filling solution rinses ensure that the electrode is put back into service with the proper concentration instead of a diluted filling solution. If your junction requires frequent cleaning, a different reference system or filling solution should be investigated. A junction cannot always be sufficiently cleaned; the electrode must be replaced.

Proper Storage

Precisely follow the manufacturer's recommendations for electrode storage. Some electrodes, such as gel-filled electrodes, should be stored in pH storage solution. They might be ruined if stored dry. The standard refillable electrodes are often stored with

filling solution, and the fill hole cover closed. The sensing element is capped and kept moist with a few drops of pH storage solution. The filling solution can be emptied and then refilled when the electrode is returned to use. Some sleeve junction electrodes can be stored dry. Crystals may appear from evaporated residual filling solution, but they can be rinsed away with deionized water prior to returning the electrode to service.

Refillable electrodes offer a longer life as they are better designed for storage and do not have a fixed filling solution volume to dictate lifetime. Gel electrodes have a finite amount of continually leaking gel and when the gel is depleted, the electrode must be replaced. Refillable FET electrodes can be stored dry and refilled prior to use. The lifetime of any electrode is dependent upon level of care and maintenance, sample/application, type of filling solution and amount of filling solution if it is non-refillable.

TROUBLESHOOTING

Is the Instrument the Problem?

Meter

The meter alone, without the electrode, can be tested to verify performance. A quality pH meter can be tested easily by using a shorting cap over the electrode input to shunt or close off the BNC connector. This will allow the meter's internal diagnostics or self-test to check circuits and ensure that the electronics are functioning properly. There will be an error displayed on the meter if any tests have failed. Consult the instruction manual for details.

Slope

The best indicators of the electrode condition are the slope of the calibration curve and response time required to obtain a stable pH reading. A clean, well-performing electrode will produce a slope close to 100% or 59.16 mV/decade, which is the theoretical slope for pH determined by the Nernst equation. As any electrode ages, the percent slope decreases. This natural occurrence can be slowed by proper use and care of the electrode. The recommended operating range varies slightly by manufacturer but is usually 92% to 100% of the theoretical ideal above. The electrode should be replaced when the slope falls below the manufacturer's recommended operating range.

Response Time

The response time, or the time it takes until the reading stabilizes, will become longer as the sample components coat the sensing glass bulb. This can often be remedied with cleaning and/or replacing the filling solution. There is a point when the electrode may have damage that won't be recovered by cleaning. If the calibration data fall within the manufacturer's specifications, the sample may be causing the problem.

Is the Sample the Problem?

If the sample reading seems inappropriate, measure the pH of a buffer standard. A correct measurement of the standard points to a sample problem. If the electrode is sluggish or does not stabilize when measuring the standard, clean and recalibrate the electrode. Reanalyze the buffer standard with the cleaned electrode in the buffer to verify system operation. If this measurement is accurate, measure the sample again. If the sample still does not give a stable reading, further investigation into the measurement techniques and sample itself is recommended.

Sometimes the "expected" value is not obtained for a measurement but the correct value is. The problem is simply an incorrect perceived value. Competing ions, sodium ions at pH of 12 or above, or a sample that coats the electrode can affect pH measurements. pH sensing glass is optimized for hydrogen ions, but sodium ions are also detected to a lesser extent. This sodium error increases at high pH levels. A nomogram found in the electrode instruction manual can be used to correct the pH reading in samples with high sodium content. Other compounds or ions could be "complexed" out of solution or bound up or change its form so that it doesn't affect the sample any more.

Often the sample can be better analyzed using a different electrode design. Inexpensive gel electrodes with wick junctions—where the sample can migrate back into the gelled reference fill solution—are not as effective as refillable electrodes in complex matrices. Samples that may contaminate the filling solution are best analyzed with flushable electrodes. Samples need a sufficient amount of water to give a pH reading; a diluted sample may be measured more reproducibly. Samples and buffer should always be measured at the same temperature if possible. The sample may change its composition with temperature variation. pH is a

relative measurement, so it might be necessary to optimize your sample preparation method.

Service Engineer, Technical Support, or Sales Rep: Who Can Best Help You and at the Least Expense?

The electrochemical measurement of hydrogen ion activity is simple, yet complex. Due to the many factors and interactions, many users increase errors in their measurements inadvertently. The best way to optimize your results is to educate yourself about your measurement system. Follow the instructions that the equipment manufacturer provides.

There are many versions of the standard glass pH electrode. Be sure that you are using the best electrode for your sample type. The sample only has contact with the electrode. So, if the electrode is not working properly, you cannot expect accurate results. The electrode preparation and conditioning steps are critical and vary by electrode type. Knowledge of your sample guides you to the proper measurement system and calibration procedures.

If you do need technical support for your analysis, it is best to call the company that manufactured your electrode. Due to the minimal cost of a pH meter as compared to other laboratory instruments and the replaceable electrode, service engineers are not a cost-effective option.

SPECTROPHOTOMETERS (Michael G. Davies and Andrew T. Dadd)

This overview addresses some of the basic aspects of UV-visible spectrophotometry and summarizes some of the standard operating procedures. It provides the reader with the fundamental background to select and operate a UV/Vis instrument addressing both specific and general requirements. This section also presents a number of methodologies that are currently available to successfully perform quantitative and qualitative analysis of macromolecules (e.g., proteins and nucleic acids) and small molecules including nucleotides, amino acids, or any UV/Vis-absorbing compounds

What Are the Criteria for Selecting a Spectrophotometer?

Most entry level instruments perform the most common applications involving the analysis of proteins and nucleic acids. The following information is provided to help you refine your choice of instrumentation.

What Sample Volumes Will You Most Frequently Analyze?

Advances in manufacturing of cuvettes has allowed greater flexibility both in terms of volumes and concentrations for the assessment of UV/Vis spectra or single/multiple wavelengths. Cell volumes as low as 10 μl may be employed in some instruments, whereas special holders that position the cuvette in the light path might be required for others. Further details on cell types is provided later in this chapter. It is worth noting that continuous-flow as well as temperature-controlled cell holders are available for specific applications.

External Computer (PC)

PC control is especially beneficial for logging and archiving data via disk, LIMS (Laboratory Information Management System) and networks, and for producing customized reports. Spectrophotometers managed by an external PC will almost always provide more functions to analyze and manipulate data. Most free-standing instruments perform scanning, kinetics, quantitative analysis, and other functions, but the ability to store and manipulate data is usually limited. Some manufacturers sell software for use with an external PC that expands data manipulation and functionality. A combination of lower-cost instrument and supplemental software sometimes provides the most function for the least money. This may be offset by the extra bench space requirements and the cost of a PC.

Single Beam or Double Beam versus Diode Array

There are three modes of optical configuration available in UV-visible spectrophotometry.

Single-beam instruments with microprocessor control have good stability and simple optical and mechanical configurations. A light source is monochromated (a single wavelength is selected) usually by a diffraction grating (or a prism in older instruments) and then passed through the sample cuvette. Comparison between reference and sample is achieved by feeding the postdetector signal to a microprocessor that stores the reference data for subtraction from the sample signal prior to display of the result.

The light beam within a double-beam spectrophotometer is split or chopped and passed through both the sample and reference solutions to obtain a direct reading of the difference between them. This is useful in applications where the reference itself is changing and constant baseline subtraction can be employed for compensation, as can occur in enzyme analyses of biological

systems. In a double-beam system a portion of the originating light energy is passed through the sample, and optically matched cuvettes need to be used for proper results.

A third optical configuration is the diode array. Here light is monochromated after passing through the sample. Transmitted light is then focused and measured by an assembly of individual detector elements arranged to collect a complete range of wavelengths. No sample compartment lid is necessary. Wavelength selection is dictated by the choice of detector elements (approximately 500 at 1 nm/diode), providing a more limited spectral range than single- or double-beam instruments.

Wavelength Range

Nucleic acids and proteins require the UV range 230 to 320 nm almost exclusively. Other compounds can be analyzed by monitoring specific wavelengths and scanning within the visible range. Until recently instruments were categorized into visible only (>320 nm) or UV and visible (190–1100 nm) primarily as a reflection of lamp technology. With improvements in lamp design and detector technology, another class of instrument can monitor absorbances between 200 and 800 nm with a single lamp. These compact instruments are designed mostly to measure the purity and concentration of nucleic acids and proteins, and some also possess basic scanning capabilities. For in-depth identification and verification studies or for a core facility, an instrument capable of scanning between 190 and 1100 nm is recommended.

Wavelength, Photometric Accuracy, and Stray Light

Wavelength accuracy describes the variation between the wavelength of the light you set for the instrument and the actual wavelength of the light produced. The variation in most instruments ranges from 0.7 to 2 nm. Should an instrument suffer from wavelength inaccuracy, the largest variation would be observed at wavelengths on either side of the absorbance maximum for a molecule, where there is a large rate of change of absorbance with respect to wavelength (Figure 4.13) and when working with dilute solutions. Note in Figure 4.13 the significant decrease in absorbance at wavelengths near 280 nm and above. Any wavelength variation by the instrument will produce very skewed data in these changeable regions of DNA's absorbtion spectra. This phenomenon also explains why A_{280} results in a very dilute sample have to be interpreted with caution.

Photometric accuracy describes the linearity of response over

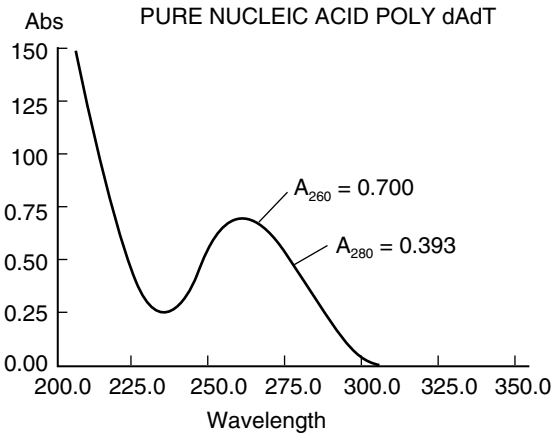

Figure 4.13 UV-visible absorption scan of DNA. Reproduced with permission from Biochrom Ltd.

the absorbance range. Normally this is expressed up to two absorbance units at specific wavelengths as measured against a range of calibrated standard filters from organizations such as the NIST. Typically it is within 0.5%. As most photodetectors are generally accurate to within 1%, the main factors compromising accuracy are errors in light transmission, most commonly stray light.

Stray light is radiation emerging from the monochromator other than the selected wavelength. This extra light causes the measured absorbance to read lower than the true absorbance, creating negative deviations from the Beer-Lambert law (Biochrom Ltd., 1997), ultimately ruining the reliability of subsequent concentration measurements. Stray light has a relatively large effect when sample absorbance is high, as in high concentrations of DNA measured at 260 nm. Dilution of concentrated samples or use of a smaller path length cell removes this effect.

Spectral Bandwidth Resolution

Bandwidth resolution describes the spectrophotometer's ability to distinguish narrow absorbance peaks. The natural bandwidth of a molecule is defined by the width of the absorbance curve at half the maximum absorbance height of a compound, and ranges from 5 to 50 nm for most biomolecules. The bandwidth of DNA is 51 nm, when measured from the spectrum in Figure 4.13. It can be shown that if the ratio of spectral to natural bandwidth is greater than 1:10, the absorbance measured by the spectrophotometer will deviate significantly from the true absorbance. A spectrophotometer with a fixed bandwidth of 5 nm or less is ideal for biopolymers, since there is no fine spectral detail, but for samples with

sharp peaks such as some organic solvents, transition elements, and vapors like benzene and styrene, higher resolution is required.

Good Laboratory Practice

There has been an increase in laboratory requirements to conform with Good Laboratory Practice (GLP) techniques according to FDA regulations (1979). The FDA requires that results be traceable to an instrument and the instrument proved to be working correctly. Instrument performance criteria for spectrophotometers have been defined by the European Pharmacopoeia (1984) as being spectral bandwidth, stray light, absorbance accuracy, and wavelength accuracy. Standard tests are laid down and are checked against the appropriate filters and solutions to confirm instrument performance.

Beyond the Self-Tests Automatically Performed by Spectrophotometers, What Is the Best Indicator That an Instrument Is Operating Properly?

The Functional Approach

Measure a series of standard samples via your application(s) in your instrument and, if possible, a second spectrophotometer. Calibrated absorbance filters can be obtained from NIST and from commercial sources (Corion Corporation, Franklin, MA; the National Physical Laboratory, Teddington. London; and Starna, Hainault, U.K.). Quantitated nucleic acid solutions are commercially available (Gensura Corporation, San Diego, CA) but do not provide reproducible data over long-term use. Nucleic acid and protein standards prepared from solid material as required is recommended provided that the concentrations are carefully determined. Do not rely on the quantity of material indicated on the product label as an accurate representation of the amounts therein.

The Certified Approach

National and international standards organisations will likely require some or all of the following tests.

Bandwidth/Resolution

For external checks against a universally adopted method the Pharmacopoeia test is used. (*European Pharmacopeia*, 1984). The ratio of the absorbances at 269 and 266 nm in a 0.02% v/v solution of toluene *R* (*R* = reagent grade) in hexane *R* is determined as in the *European Pharmacopoeia* (2000).

Stray Light

Stray light is determined using a blocking filter that transmits light above a certain wavelength and blocks all light below that wavelength. Any measured transmittance is then due to stray light. The *European Pharmacopoeia* (2000) specifies that the absorbance of a 1.2% w/v potassium chloride *R* should be greater than 2.0 at 200 nm, when compared with water *R* as the reference liquid.

Wavelength Accuracy

This is determined using a standard that has sharp peaks at known positions. According to the *European Pharmacopoeia* (2000), the absorbance maxima of holmium perchlorate solution, the line of a hydrogen or deuterium discharge lamp, or the lines of a mercury vapor arc can be used to verify the wavelength scale.

Wavelength Reproducibility

Wavelength reproducibility is determined by repeatedly scanning a sharp peak at a known position, using the same standard as for wavelength accuracy.

Absorbance Accuracy (Photometric Linearity)

The absorbance of neutral density glass filters, traceable to NIST, NPL (National Physical Laboratory) *www.nist.gov*, *www.physics.nist.gov* or other internationally recognized standards, is measured for a range of absorbances at a stated wavelength. Neutral density filters provide nearly constant absorbances within certain wavelengths of the visible region, but measurements in the UV require metal on quartz filters or a liquid standard such as potassium dichromate *R* in dilute sulphuric acid *R* (*European Pharmacopoeia*, 2000). Metal on quartz filters can exhibit reflection problems, and dirt can contaminate the metal coating. The liquid must be prepared fresh for each use; sealed cells of potassium dichromate prepared under an argon atmosphere are commercially available.

Photometric Reproducibility

Photometric reproducibility is determined by repeatedly measuring a neutral density filter.

Noise, Stability, and Baseline Flatness

Noise is determined by repeatedly measuring the spectrum of air (no cuvette in the light path) at zero absorbance. This is achieved by setting reference on air. It is specified as the calculated RMS (root mean square) value at a single wavelength. The

stability is the difference between the maximum and minimum absorbance readings at a specified wavelength (at constant temperature). The RMS (square root of $[a_1^2 + a_2^2 + a_3^2 + \ldots]$, where a represents the absorbance value at each wavelength) is calculated over the whole instrument wavelength range for the spectrum of air to provide the baseline flatness measurement.

Which Cuvette Best Fits Your Needs?

Small Volumes

Cuvettes with minimal sample volumes of 250 μl or greater usually do not require dedicated cuvette holders and are compatible with most instruments. Cuvettes with minimal sample volumes between 100 and 250 μl might require a manufacturer-specific, single-cell holder, and cuvettes requiring sample volumes below 100 μl almost always require specialized single-cell holders that are rarely interchangeable between manufacturers. These ultra-low-volume cuvettes have very small sample windows (2×2 mm) that require a specialized holder to align the window with the light beam. Some manufacturers recommend the use of masked cells to reduce overall light scatter.

If the light path length of your cuvette is less than 10 mm, check if the instrument automatically incorporates this when converting absorbance data into a concentration. The Beer-Lambert equation assumes a 10 mm path length. A double-stranded DNA sample that produces an absorbance at 260 nm of 0.5 in a cuvette of 10 mm path length produces a concentration of 25 μg/ml. The same sample measured in a cuvette with a 5 mm path length produces an absorbance of 0.25, and concentration of 12.5 μg/ml if the spectrophotometer does not take into account the cuvette's decreased path length. Capillaries of 0.5 mm path length can analyze very concentrated samples without dilution, but the quantitative reproducibility can suffer because of this extremely short light path.

Disposable Cuvettes

Plastic cuvettes are not recommended for quantitative UV measurements because of their reduced transmittance below 380 nm, which may seriously compromise accuracy and sensitivity of some quantitative methods. Polystyrene cuvettes may be replaced by a methacrylate-based version that supposedly allow higher transmittance values over the common plastic cuvettes. Cuvettes composed of novel polymers with superior absorbance properties are in development. However, caution should be exercised to ensure solvent compatibility using any material.

What Are the Options for Cleaning Cuvettes?

Dirty cuvettes can generate erroneous data, as they can trap air bubbles or sample carryover. Cuvettes made from optical glass or quartz should be cleaned with glassware detergent or dilute acid (e.g., HCl up to concentrations of 0.1 M) but not alkalis, which can etch the glass surface. When detergent is insufficient, first inspect your cuvette. If it is comprised of a solid block of glass or quartz and you see no seams within the cuvette, you can soak it in concentrated nitric or sulfochromic acids (but not HF) for limited periods of time. Then the cuvettes must be rinsed with copious amounts of water with the aid of special cell washers ensuring continuous water flow through the cell interior. Exposure to harsh acid must be of limited duration due to the possibility of long-term damage to the cuvette surface. Alternatively, polar solvents can also be employed to remove difficult residues. One cuvette manufacturer claims to provide a cleaning solution that is suitable for all situations (Hellmanex, Hellma, Southend, U.K.). Seams are indicative of glued joints and are more commonly present in low sample volume cuvettes. The interior sample chambers of seamed cuvettes can be treated with acid but not the seams. Cuvettes made from other materials or mixtures with glass should be treated with procedures compatible their chemical resistance.

How Can You Maximize the Reproducibility and Accuracy of Your Data?

Know Your Needs

Must your data be absolutely or relatively quantitative? If your situation requires absolute quantitation, your absorbance readings should ideally fall on the linear portions of a standard calibration curve. Dilute your sample if it's absorbance lies above the linear portion, or select a cuvette with shorter path length. If your absorbance values reside below the linear portion and you can't concentrate your samples, include additional calibration standards (to the original standard curve) that are similar to your concentration range. The objective is to generate curve-fitting compensation for values outside linear response.

Know Your Sample

What are the possible contaminants? Are you using phenol or chloroform to prepare DNA? Could the crushed glass from your purification kit be leaking out with your final product? If you can predict the contaminants, methods exist to remove them, as described in Chapter 7, "DNA Purification" and Chapter 8, "RNA

Purification." Many spectrophotometers also can compensate for contaminants by subtraction of reference or 3-point net measurements. If you can't predict the contaminant, scan your sample across the entire UV-visible spectrum, and compare these data to a scan of a purified sample control. The type of interference is indicated by the wavelengths of absorbance maxima that are characteristic of particular molecular groups and such information is available in Silverstein et al. (1967). Possible contaminants may be signified by comparison of outstanding absorbance peaks against an atlas of reference spectral data (e.g., commercially available from Sadtler, Philadelphia, PA). However, reference data sometimes do not give an accurate match, and it is more accurate and relevant to exploit the attributes of a fast scanning spectrophotometer and generate spectra of materials involved in the sample preparation procedure. This can give a direct comparison on the same instrument. Combined with the use of a PC for archiving, it is a convenient way to build up specific sample profiles for searching and overlays.

Cell suspension measurements at 600 nm (A_{600}) provide a convenient means of monitoring growth of bacterial cultures. Provided that absorbance is not above 1.5 units, A_{600} correlates quite well with cell numbers (Sambrook et al., 1989). The geometry of an instrument's optical system affects the magnitude of these absorbance measurements because of light scattering, so A_{600} values can vary between different instruments.

Opaque, solid, or slurried samples may block or scatter the light, preventing accurate detector response. A special optical configuration is required to deal with these samples to measure reflectance as an indicator of absorbance. This requires a specifically designed source and sample handling device, and costs can surpass the spectrophotometer itself.

Know Your Instrument's Limitations

Instruments costing the equivalent of tens of thousands of dollars might generate reproducible data between absorbance values of 0.001 and 0.01, but the scanning instruments found in most laboratories will not. Ultra-dilute samples are better analyzed using a long path length cell or a fixed wavelength monitor of high specification. A low sample volume cuvette might reduce or eliminate the need to dilute your sample.

How low an absorbance can your instrument reproducibly measure? Perform a standard curve to answer this question. Note

that absorbance can be reproducible, but if the absorbance measurement does not fall on the linear part of the calibration curve, it might not correlate well with concentration.

What Can Contribute to Inaccurate A_{260} and A_{280} Data?

Instrument Issues

Aging, weakened UV lamps can generate inaccurate data, as can new deuterium lamps that were not properly warmed up (20–40 minutes for older instruments). Start-up is not an issue for most instruments produced within the last 10 years, which usually only require 10 minutes and may be accompanied by automatic internal calibration (required for GLP purposes). Lamp function is discussed in more detail below.

Sample Concentration

Measuring dilute samples that are near the sensitivity limits of the spectrophotometer is especially problematic for A_{280} readings. The sharp changes on either side of 280 nm (Figure 4.13) amplify any absorbance inaccuracy.

Contaminants

Contaminating salt, organic solvent, and protein can falsely increase the absorbance measured at 260 nm. Contaminants can be verified and sometimes quantitated by measuring absorbance at specific wavelengths. The additive effect on the spectrum is detected by alteration in the relevant absorbance ratio ($A\lambda_1/A\lambda_2$) as shown in Figure 4.14.

Absorbance at 230 nm

Tris, EDTA, and other buffer salts can be detected by their absorbance of light at 230 nm, a region where nucleotides and ribonucleotides generally have absorbance minima. At 230 nm this also is near the absorbance maximum of peptide bonds, indicating the presence of proteins. Therefore readings at 230 nm or preferably a scan incorporating wavelengths around 230 nm can readily show up impurities in nucleic acid preparations. High-absorbance values at 230 nm indicate nucleic acid preparations of suspect purity. In preparation of RNA using guanidine thiocyanate, the isolated RNA should exhibit an A_{260}/A_{230} ratio greater than 2.0. A ratio lower than this is generally indicative of contamination with guanidine thiocyanate carried over during the precipitation steps.

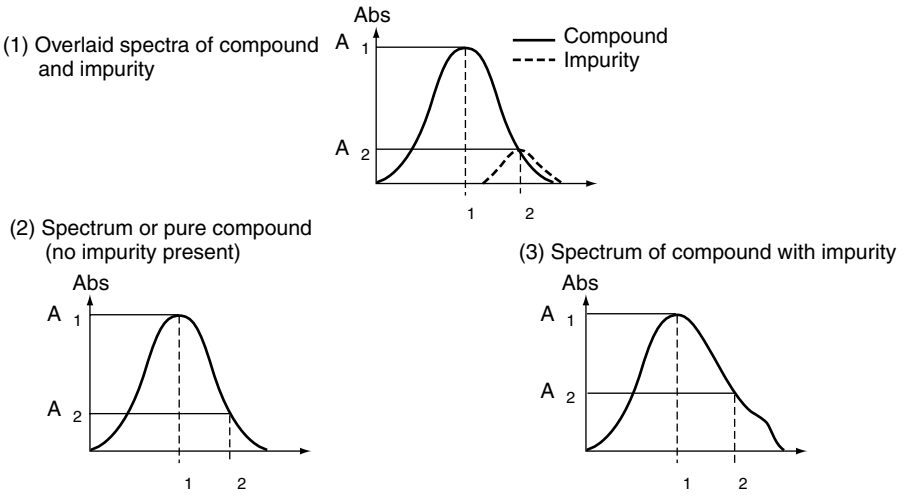

Figure 4.14 Detecting contaminants by absorbance ratio. Reprinted by permission of Biochrom Ltd.

Absorbance at 320 nm

Nucleic acids and proteins normally have virtually no absorbance at 320 nm, although absorbances between 300 and 350 nm may be indicative of aggregation, particularly in the case of proteins. Subtracting the absorbance at 320 nm from the absorbance detected at 260 nm can eliminate absorbance due to contaminants such as chloroform, ethanol, acetates, citrates, and particulates that cause turbidity. Background absorbance at 320 nm is more likely to skew the A_{260} readings of very dilute nucleic acid solutions or samples read in ultra-low-volume (<10 μl) cuvettes.

Does Absorbance Always Correlate with Concentration?

The Beer-Lambert law (Biochrom Ltd., 1997) gives a direct proportional relationship between the concentration of a substance, such as nucleic acids and proteins, and its absorbance. So a graph of absorbance plotted against concentration will be a straight line passing through the origin. Under straight line conditions, the concentration in an unknown sample can be calculated from its absorbance value and the absorbance of a known concentration of the nucleic acid or protein (or an appropriate conversion calibration factor).

When this Beer-Lambert relationship between absorbance and concentration is not linear, DNA and protein cannot be measured accurately using one factor (i.e., molar extinction coefficient) or

concentration for calibration. For the greatest accuracy the absorbance readings have to be calibrated with known concentrations similar to those in the samples. The calibration standard range should cover the sample concentrations, which are measured to allow curve-fitting compensation for values outside linear response. Deviations from linearity result from three main experimental effects: changes in light absorption, instrumentation effects, and chemical changes.

Changes in light absorption can be produced by refractive index effects in the solution being measured. Although essentially constant at low concentrations, refractive index can vary with concentration of buffer salts, if above 0.001 M. This does not rule out quantitation as measurements can be calibrated with bracketing standard solutions or from a calibration curve.

Instrumentation effects arise if the light passing through the sample is not truly monochromatic, which was mentioned earlier in the section on spectral bandwidth. The Beer-Lambert law depends on monochromatic light, but in practice at a given spectrophotometer wavelength, a range of wavelengths, each with a different absorbance pass through the sample. Consequently the amount of light measured is affected and is not directly proportional to concentration, which results in a negative deviation from linearity at lower light levels due to higher concentrations. This effect only becomes apparent if absorbance peaks are narrow in relation to spectral bandwidth; it is not a problem with specifications set as discussed in that earlier section.

Chemical deviations arise when shifts occur in the wavelength maximum because of solution conditions. Some nucleotides are affected when there are pH changes of the buffer solvent, giving shifts of up to 5 nm. The magnitude of absorbance at 260 nm changes for DNA as it shifts from double-stranded to single-stranded, giving an increase in absorbance (hyperchromicity). In practice, frozen DNA solutions should be well thawed, annealed at high temperatures (80–90°C) and cooled slowly before measurements.

Why Does Popular Convention Recommend Working Between an Absorbance Range of 0.1 to 0.8 at 260 nm When Quantitating Nucleic Acids and When Quantitating Proteins at 280 nm?

Most properly functioning spectrophotometers generate a linear response (absorbance vs. concentration) between absorbance values of 0.1 and 0.8; hence this range is considered

safe to quantitate a sample. If you choose to work outside this range, it is essential that you generate a calibration curve containing a sufficient number of standards to prove a statistically reliable correlation between absorbance and concentration. Such a calibration study must be performed with the cuvette to be used in your research. Cuvette design, quality, and path length can influence the data within such a calibration experiment. Calculations of protein and peptide concentration also require linearity of response and the same principles apply to their measurements.

Deuterium lamps can generate linear responses up to three units of absorbance; the linear response of xenon lamps decreases at approximately two units of absorbance.

Is the Ratio $A_{260}:A_{280}$ a Reliable Method to Evaluate Protein Contamination within Nucleic Acid Preparations?

The original purpose of the ratio $A_{260}:A_{280}$ was to detect nucleic acid contamination in protein preparations (Warburg and Christian, 1942), and not the inverse. This ratio can accurately describe nucleic acid purity, but it can also be fooled. The stronger extinction coefficients of DNA can mask the presence of protein (Glasel, 1995), and many chemicals utilized in DNA purification absorb at 260 nm (Huberman, 1995). Manchester (1995) and Wilfinger, Mackey, and Chomczynski (1997) show the very significant effects of salt and pH on absorbance of DNA and RNA preparations at 260 and 280 nm.

If you doubt the validity of your $A_{260}:A_{280}$ data, check for contaminants by monitoring absorbance between 200 and 240 nm, a region where nucleic acids absorb weakly if at all, as described above. As discussed in Chapter 1, "Planning for Success in the Lab", a contaminant is problematic only if it interferes with your application. If contaminant removal is necessary but impractical, Schy and Plewa (1989) provide a method to assess the concentration and quality of impure DNA preparations by monitoring both diaminobenzoic acid fluorescence and UV absorbance.

What Can You Do to Minimize Service Calls?

Respect the manufacturers suggested operating temperatures and humidity levels, and avoid dust. Spills should be avoided and cleaned up immediately. This is because some materials not only attack instrument components but can also leave UV-absorbing residues and vapors.

How Can You Achieve the Maximum Lifetime from Your Lamps?

Deuterium

Older designs of deuterium lamps require that the lamp be powered up and kept on prior to sample measurement. The best indicator of vitality in these older designs is the hours of UV lamp use. As lamps approach the manufacturer recommended lifetimes, the light energy fades, producing erratic, irreproducible absorbance measurements. Deuterium lamps also lose effectiveness when stored unused and should not be kept longer than one year before use.

Should you automatically discard a deuterium lamp when it reaches the predicted lifetime? The answer is no. Deuterium lamps can generate accurate, reproducible data beyond their predicted lifetimes. Simply monitor the accuracy of an older lamp with control samples. Recently designed pulsed technology deuterium lamps turn on only when a sample is read (demand switching), resulting in lifetimes of five years or more.

Frequently switching the power on and off will prematurely weaken most deuterium lamps, but not the demand-switched lamps described above, which can last through thousands of switching cycles.

Tungsten

Tungsten lamps tend to give longer lifetimes—at least six months if left on continuously and several years when used during normal working hours. During long use, instruments tend to drift because of warming-up, while background noise decreases. It is better to leave instruments on during the working day and re-reference if lower noise measurements are required. Switching frequently may shorten total lamp lifetime unless the control circuits have been designed to minimize lamp wear on switching.

Xenon

Xenon lamps flash on only when a sample is read, resulting in lifetimes of 1000 to 2000 hours or more of actual use. Lifetime is not affected by frequent switching on and off.

The Deuterium Lamp on Your UV-Visible Instrument Burned Out. Can You Perform Measurements in the Visible Range?

With current internal calibration software, instruments can still self-calibrate and operate through the visible range without the

deuterium lamp. Tungsten sources cover the range from 320 to 1100 nm, giving overlap at the lower end of the range into the UV. Likewise an instrument with a nonfunctional tungsten lamp will accurately generate UV absorbance data.

What Are the Strategies to Determine the Extinction Coefficient of a Compound?

The Beer-Lambert law defines absorbance A as equal to the product of molar absorptivity (extinction coefficient E) cell path length L and concentration C. The extinction coefficient defines the absorbance value for a one molar solution of a compound, and is characteristic of that compound.

$$A = ECL$$

An extinction coefficient can be empirically calculated from the absorbance measurement on a known concentration of a compound, as discussed in Chapter 10. Some extinction coefficients for nucleotides are shown in Table 10.2 of Chapter 10. Data for individual products can usually be found in manufacturers' information leaflets.

Issues of absorbance critical to the quantitation of nucleotides, oligonucleotides, and polynucleotides are discussed in greater detail in Chapter 10.

What Is the Extinction Coefficient of an Oligonucleotide?

A common approach applies a conversion factor of 33 or 37 μg per A_{260} for oligonucleotides and single-stranded DNA, respectively, and this appears sufficient for most applications. For a detailed discussion about the options to quantitate oligonucleotides and the limitations therein, refer to Chapter 10.

Is There a Single Conversion Factor to Convert Protein Absorbance Data into Concentration?

The heterogeneity of amino acid composition and the impact of specific amino acids on absorbance prevents the assignment of a single conversion factor for all proteins. The protein absorbance at 280 nm depends on contributions from tyrosine, phenylalanine, and tryptophan. If these amino acids are absent, this wavelength is not relevant and proteins then have to be detected by the peptide bond in the region of 210 nm. The Christian-Warburg citation provides a strategy to convert protein absorbance to concentration, but this requires modification based on composition (Manchester, 1996; Harlow and Lane, 1988).

Several methods are available in the literature, from which a relatively accurate extinction coefficient may be derived (e.g., Mach, Middaugh, and Lewis, 1992). Provided that the amino acid composition is known, an equation can be used to determine *E* that takes into account the number of tyrosines and tryptophans, as well as the number of disulfide bonds (if known); the latter less critical. It is sometimes imperative to conduct the measurements under denaturing conditions (e.g., 6 M Guanidine-HCl) for accurate evaluation of the extinction of a protein, particularly when the majority of the aromatic residues are buried within the protein core. This may be revealed by comparing the normal or second derivative spectra in the presence and absence of the denaturing agent.

What Are the Strengths and Limitations of the Various Protein Quantitation Assays?

There are four main reagent-based assays for protein analysis:

1. Bradford (Coomassie Blue) has the broadest range of reactivity and is the most sensitive. The drawback is its variable responses with different proteins due to the varying efficiency of binding between the protein and dyestuff. The optimum wavelength for absorbance measurement is 595 nm. Sensitivity can be improved by about 15% for longer reaction times up to 30 minutes for microassays, and responses can be integrated over a longer period. Detergents give high background responses that require blank analyses for compensation.

2. BCA, measured at 562 nm, is about half the sensitivity of the Bradford method but has a more stable endpoint than the Lowry method. It also has a more uniform response to different proteins. There is little interference from detergents. It is not compatible with reducing agents.

3. Lowry, measured at 750 nm, is almost as sensitive as the Bradford assay, but it has more interference from amine buffer salts than other methods.

4. Biuret, measured at 546 nm, is in principle similar to the Lowry, but involving a single incubation of 20 minutes. Under alkaline conditions substances containing two or more peptide bonds form a purple complex with copper sulphate in the presence of sodium potassium tartrate and potassium iodide in the reagent. There are very few interfering agents apart from ammonium salts and fewer deviations than with the Lowry or ultraviolet absorption methods. However, it consumes much more material. In general, it is a good protein assay, though not as fast or sensitive as the Bradford assay.

Smith (1987) lists compounds that interfere with each assay and illustrates problems associated with the use of BSA as a standard (see also Harlow and Lane, 1988; Peterson, 1979).

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