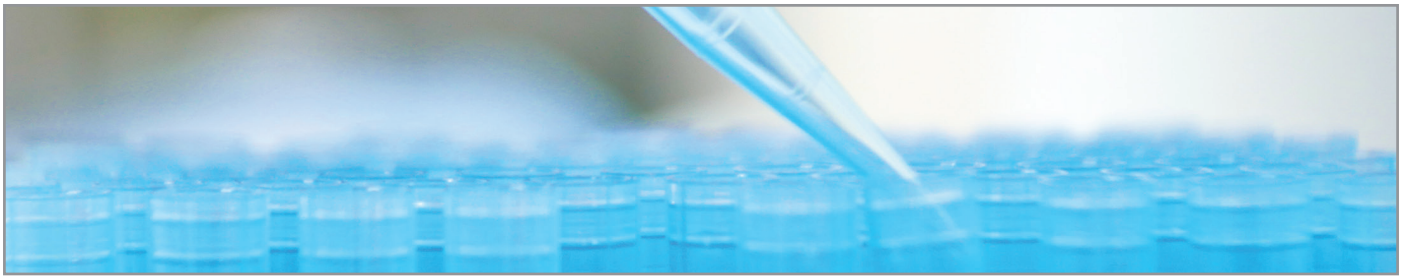


Tips for Preparing Calibration Curve Standards and Avoiding Sources of Error

By Dr. Landon Wiest

Accurate calibration data is the foundation of all quantitation. But, behind every great calibration curve is a tremendous amount of work. Many sources of potential error are waiting at every turn, so it pays to be familiar with how to best ensure precision and accuracy. To avoid the frustration of failed calibration curves, project delays, and wasted time and resources, let's explore some tips to help you prevent some of the pitfalls you can encounter when preparing calibration curve standards. While there are plenty of instrument issues that could cause an unacceptable calibration curve, this article focuses on standard preparation and storage.

Before starting, always make sure you understand the rules and policies (SOPs, regulations, standard guidelines, etc.) of your organization as well as the SDSs and disposal requirements for the chemicals you'll be using. Prioritizing compliance and safety is essential for efficient, effective work, so if you find opportunities for improving existing practices through these tips, talk to your manager first before changing any procedures.

Use Equipment Correctly

While most methods define the compounds and concentrations you need to make, they generally do not provide best practices for actually mixing your standards. The first step toward obtaining a good standard curve is becoming familiar with your equipment and how to use it correctly. It may seem elementary, but knowing how equipment works and using good lab technique should never be taken for granted. Get familiar with the tools at your disposal and practice using them properly until you feel comfortable with the technique. Just because you've used a similar device before does not mean that the new tool will work the same way.

Automatic pipettes are a great example of this: they all do the job, but they don't all work exactly the same way, and experience with one doesn't always apply to another (Figure 1). Here are some small, but important things to consider when preparing calibration curve standards with pipettes.

- *Air vs. Positive Displacement*

  Air displacement pipettes are sensitive to the vapor pressure of the liquid you are measuring. They are typically calibrated using water, so liquids with higher or lower vapor pressures that are volumetrically measured using air displacement pipettes, will have a different volume than the pipette reports. If you need to get accurate and precise volumes of liquids that have vapor pressures different from water, consider using a positive displacement pipette or compensating for the difference by gravimetrically calibrating your air displacement pipette to the proper setting for that particular liquid.

- *Manual vs. Electric Drive*

  Manual drive pipettes require user consistency. Setting the pipette volume and ensuring it doesn't change from aliquot to aliquot is critical for consistent results because even a small turn or bump can be enough to shift the volume setting. You also need to ensure that you're using the same pressure on the plunger at the resistance points when aspirating and dispensing fluids. Electric pipettes are less prone to "drifting" as you handle them, but they dictate the work rhythm, and the user must follow their pace.

Figure 1: Pipettes come in a wide range of styles, including manual vs. electric drive, and air vs. positive displacement. Barrel sizes can feel similar, so always check that you have the correct volume range and are using the proper tip type.

Electronic drive, air displacement pipettes

Manual drive, positive displacement pipettes

- *Tip Types*

  Matching the right tip to the correct pipette is extremely important. Just because a pipette tip fits on a given pipette does not always mean it is the right one. Tips are typically color coded, but it still pays to verify that the tip matches the pipette.

- *Calibration*

  Pipettes don't remain calibrated forever. Make sure you get them professionally calibrated regularly, based on your lab's need or requirements, and in compliance with your organization's quality management program. It's always good practice to verify your pipette's calibration gravimetrically with water prior to use.

- *Tip Wetting*

  Positive displacement pipette tips are prone to having air bubbles when they are first filled, so filling and evacuating them a few times before use can help remove bubbles that affect the delivered volume. Also, because of a wetting effect, air displacement pipette tips can have a slightly different volume when first filled as compared to subsequent measurements, so make sure you are consistent in your technique.

- *Pipetting Technique*

  In most cases, pipette tips should be placed just beneath the surface of the liquid. Inserting the pipette tip too deeply into the liquid can result in extra fluid entering the pipette tip prior to aspiration, thereby increasing the measured volume. The pipette should also be held perpendicular to the liquid surface and not at an angle. In an internal study, we compared proper and improper pipetting techniques, and the improper technique resulted in a standard deviation that was nearly nine times higher (Figure 2).

Figure 2: Minimize error by using proper pipetting technique: always hold the tip perpendicular to and just below the liquid surface. Holding it at an angle or deep under the surface can result in inaccurate volumes.

- *Pipette Tip Replacement*

  In most cases, pipette tips should be replaced after use because reusing them can introduce contaminants in subsequent measurements. If you think the tip has potentially been contaminated, discard it.

- *Volume Range*

  It's best to avoid the low end of the pipette's volume range in order to reduce error. Also, choose a pipette whose range more closely matches the volume you are dispensing. For instance, if you have to dispense 10 µL, and you can choose between a 1-10 µL pipette and a 10-100 µL, choose the 1-10 µL pipette because that particular pipette's error is typically lower for a 10 µL aliquot than the larger volume range pipette.

- *Avoid Dispensing Very Small Volumes of Concentrated Solutions*

  If you have a concentrated stock solution that you are diluting to make your calibration levels, and you have to use very small volumes to make the low-level calibration standards, consider making a bridging stock solution that allows you to dilute a larger aliquot to make the lowest calibration level standards.

What other lab equipment do you routinely use when preparing calibration curve standards? Vortex mixers and sonication baths are frequently employed to mix standards. But, to create homogeneity they must be used correctly. For example, when using a vortex mixer, be sure there is enough space in the vial for the solution to mix effectively. The formation of a tiny whirlpool in the liquid indicates there is enough room for thorough mixing. Shaking or sonication can also work, but they take a little longer. When using a sonicator, be aware that thermally labile compounds can degrade because the solution may heat up during sonication. If you have any questions regarding equipment, be sure to consult colleagues, manuals, internet resources, or instrument vendors to answer questions and to learn how to properly use the equipment needed to prepare calibration curve standards.

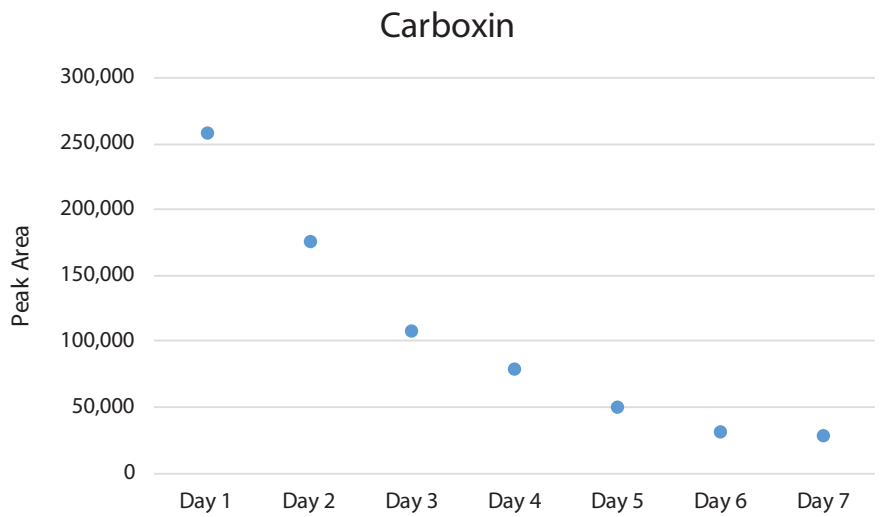
Consider Stability Issues

When preparing working standard solutions, always use high-quality starting materials that are formulated for stability. Visiting **www.restek.com/solutions** is an easy way to find stable stock and custom blends specific to your list of target analytes. Some reference standards manufacturers, such as Restek, obtain ISO certifications to confirm procedures are in place that ensure the labeled concentration is accurate up to the date the product expires (as long as it is stored and handled according to the labeled instructions).

However, once any reference material is opened and mixed with other standards or solvents, chemical reactions can occur over time that cause degradation, making your prepared calibration standards inconsistent from day-to-day, or even hour to hour. The example shown in Figure 3 illustrates how the pesticide carboxin, which is stable in the purchased stock formulation, can degrade over time when part of a prepared multiresidue pesticide calibration standard.

There may also be temperature or light sensitivities that must be taken into consideration, particularly for storage. You must establish how long the new mixtures are stable so as to be certain that they will produce accurate calibration curves when used. Conduct stability studies in advance so you know exactly how you need to handle and store working standards and how long they will produce precise and accurate quantitative results. Keep in mind that different concentrations can degrade at different rates.

Figure 3: Degradation of carboxin in a prepared multiresidue pesticide calibration standard (1 ppb starting concentration).



Carboxin is present in a 30-compound pesticide standard (cat.# 31976) that is part of a 10 ampul LC multiresidue pesticide kit containing 204 analytes (cat.# 31971) where each ampul contains a solution specifically formulated and grouped for maximum long-term stability. A 1 ppb calibration standard containing all kit components was prepared in aqueous celery extract and acetonitrile (90:10) to illustrate the importance of testing individual components in complex calibration mixtures over time.

In addition to stability, analyte solubility can be an issue. If you have compounds that are not very soluble in the dilution solvent, you could encounter some serious calibration problems. Always research analyte chemistry to confirm that the compounds and their various calibration levels will be both soluble and stable in the prepared solutions.

Establish a Fail-Safe Workflow

Creating a written workflow ahead of time for preparing calibration curve standards will make work in the lab much more efficient. Using a spreadsheet with pre-calculated volumes and concentrations to document the workflow steps is a good practice that ensures you will not miss steps or add an incorrect amount. It's a great way to preplan the amounts of chemicals and solvents you'll need and envision the routine before doing it.

Adding a color-coded spreadsheet to your workflow is an easy way to provide an additional level of security. Use colors to link the reference materials, concentrated stock solutions, and final standards of different calibration levels. It's easy to grab the wrong vial or ampul when everything looks the same. If you match the colors of the spreadsheet cells to the labels or caps you use in the lab, you minimize the chances for error. Once you've made your workflow, print it out, bring it to the lab, and keep it in front of you while you're working (Figure 4).

Figure 4: Using a pre-calculated, color-coded workflow for preparing calibration standard solutions and moving vials in a rack to track step completion is a simple way to prevent costly errors.

When you are ready to start work in the lab, first check all of the amounts of chemicals, solvents, and supplies to make sure you have enough of everything. Then, begin following the workflow you created. While performing each step, incorporate some kind of system that lets you keep track of the work you've completed. For instance, you may be making 10 or more calibration levels, each with different reference materials, stocks, diluents, and/or internal standards. In this situation, it's very helpful to work with a vial rack so that once a step is complete for a given vial you can move it to another row to indicate where you are in the procedure. Colleagues ask questions, you get phone calls…there are numerous possible interruptions and distractions that can make it hard to know where you've stopped. If you use color coding and stick to a routine that helps you track your progress, it will help tremendously in avoiding adding the wrong standard to the wrong calibration level, or even skipping a step by accident.

In summary, when preparing calibration curve standards, minimizing potential sources of error is essential for obtaining accurate quantitation. Paying attention to the strategies discussed here—and asking questions when you need to—is an excellent way to prevent problems and ensure good results. Time spent learning about equipment and developing proper technique, understanding stability and solubility limitations, and developing a fail-safe workflow is a good investment that will improve certainty in your analytical results. To learn more about reference standards, visit **www.restek.com/standards.**

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