Evaluation of the Slope Spectroscopy Method for Protein Concentration Measurement of Monoclonal Antibodies

Maria Tchamova
Supervisor, Analytical Sciences
Regeneron Pharmaceuticals Inc.

Joe Ferraiolo
Associate Applications Director
Repligen Corporation

Introduction

Protein concentration is an important part of monoclonal antibody production. Traditionally, a UV-Visible spectroscopy method is used to measure protein concentration in this setting, but it has some limitations and challenges. Variable Pathlength Technology delivers significant benefits for protein concentration analysis in a quality control laboratory that manufactures monoclonal antibodies for therapeutic applications.

Importance of Protein Concentration in Monoclonal Antibody Production

Monoclonal antibodies (mAbs) are produced by the immune system to target invaders like viruses, but they can also be manufactured as therapeutic agents to treat disease. Monoclonal antibodies are proteins produced by a single-cell line and designed to bind a specific antigen in the body. This allows users to target and regulate specific cell processes or even to flag cancer cells for destruction.

mAb production is a complex manufacturing process that can be simplified into:

- an upstream cell culture process where a culture of cells is grown under controlled conditions to produce the antibody of interest
- a downstream process, where the antibody is purified through a series of unit operations, typically consisting of resin-packed columns that are designed to remove impurities that have been introduced or generated by the cell culture process
- formulation and dispensing, where the purified antibody is formulated, dispensed, and packaged for administration to the clinic.

Protein concentration is important in the manufacturing process and in the final product. During purification, concentration is used to calculate the optimum protein load for each unit operation and overall yield. Without knowing the protein concentration, users risk overloading the resin, which can lead to loss of product. Or they risk underloading the resin, leading to process inefficiency.

Concentration is also important in the final product. It is critical when assessing the pharmacokinetics of the mAb and selecting an effective dosing regimen for the patient. As a regulatory requirement, users must demonstrate proper protein concentration, protein identity, and purity.

Protein concentration also comes into play during quality control testing of the product. Analytical methods are often concentration-dependent as they require samples and standards to be diluted in an equivalent amount for the analysis.

Inaccurate sample dilutions may lead to invalid or inaccurate test results, which could in turn compromise the integrity and safety of the product. It is important that testing occurs within validated method ranges, ensuring systematic controls are in place to identify out-of-specification or atypical product. If the incorrect concentration is used, it risks operating outside of validated controls.

Certain assays can also be extremely sensitive to concentration. Tests of potency measure the activity of the mAb relative to a known amount of antigen present in a solution. Other methods test for process impurities and report these impurities in the final product relative to the patient dose, ensuring acceptable daily exposure limits are not exceeded.

Challenges With Traditional UV-Vis Method of Measuring Protein Concentration

Traditionally, mAb concentration is measured using UV-Vis spectroscopy. Monoclonal antibodies are proteins composed of chains of amino acids, some of which (e.g., tryptophan) contain aromatic rings which exhibit strong UV light absorption at 280 nm. Because of this, mAbs also absorb UV light most strongly at 280 nm. The number

of aromatic rings in a particular antibody determine how strongly it absorbs light, making this an intrinsic property that is represented by the extinction coefficient of the antibody.

This characteristic allows users to measure antibody concentration in solution using UV spectroscopy and the Beer-Lambert Law, which states:

Absorbance (A) = extinction coefficient (ε) * **pathlength (l)** * concentration (c)

When the extinction coefficient of the analyte at a specific wavelength is known, and pathlength is kept constant, there is a linear relationship between absorbance at that wavelength and concentration of the sample.

As with most instruments, there are limitations. It is difficult to obtain high-resolution measurements without increasing measurement times or producing very expensive and difficult-to-manufacture equipment. As a result, challenges with traditional UV spectrometers present themselves where measurements start to diverge from the Beer-Lambert Law. This is particularly due to stray light and absorbance saturation.

Stray light is light that reaches the detector but is outside the spectral region isolated for measurement. It can originate inside the instrument optics or outside the instrument from light leaks. The more stray light transmitted, the sooner the instrument measurements start to diverge from the linear correlation of absorbance and concentration.

In addition, measurements hit an absorbance limit or saturation. Due to these instrument limitations, samples often require dilution to ensure that measurements remain within the linear range of the instrument. This is particularly difficult for high concentration samples as it leads to larger dilution factors. The need to perform multiple dilutions exposes higher risk for error due to analyst and equipment variability.

Having to dilute samples typically leads to a laborious process, especially in the quality control space. Quality control (QC) laboratories operate under stringent conditions to ensure that product tested and released into the clinic meets all regulatory expectations and ensures a safe administration to the patient.

A typical QC workflow involves the following steps:

1. A daily instrument check.
2. Collection of a background measurement to account for any stray light limitations.
3. Collection of the blank measurement. Typically, the blank is the diluent that is used to prepare the samples. This is done to ensure that minimal diluent interference is observed or to correct for the interference that is observed.
4. Measurement of an assay control, which can be either a purchased or an in-house prepared standard that is qualified and expected to produce measurements within a specified range.
5. Sample dilution in triplicate to mitigate variability from analyst technique. Depending on the target concentrations, sample preparations may also require serial dilutions to avoid pipetting small volumes.
6. Each sample preparation is measured on the UV spectrometer.
7. Once all data is collected, scatter correction and concentration are calculated manually for each measurement, as well as for the average of all preparations.
8. Data review. Because manual calculations pose more risk for error, additional data-review steps are often required, consuming more time and resources in the QC laboratory.
9. Optimal concentration ranges are implemented for each product based on the extinction coefficient of the products. This makes it difficult to apply and implement a platform approach of total protein measurement within the QC lab.

Evaluation of Variable Pathlength Technology in GMP Quality Control Laboratories

In light of the limitations discussed with the traditional method, this paper explores the benefits and challenges experienced with a new slope spectroscopy method using variable pathlength technology found in the CTech™ SoloVPE® System.

(1) Principles of Variable Pathlength Technology (VPT)

Similar to traditional UV methods, the variable pathlength spectroscopy (sometimes referred to as slope spectroscopy) method also employs the Beer-Lambert Law. However, instead of keeping pathlength constant and varying the concentration, the VPT can vary the pathlength while keeping the concentration constant. In other words, if the extinction coefficient of the analyte at a specific wavelength is known, and concentration kept constant, there is a

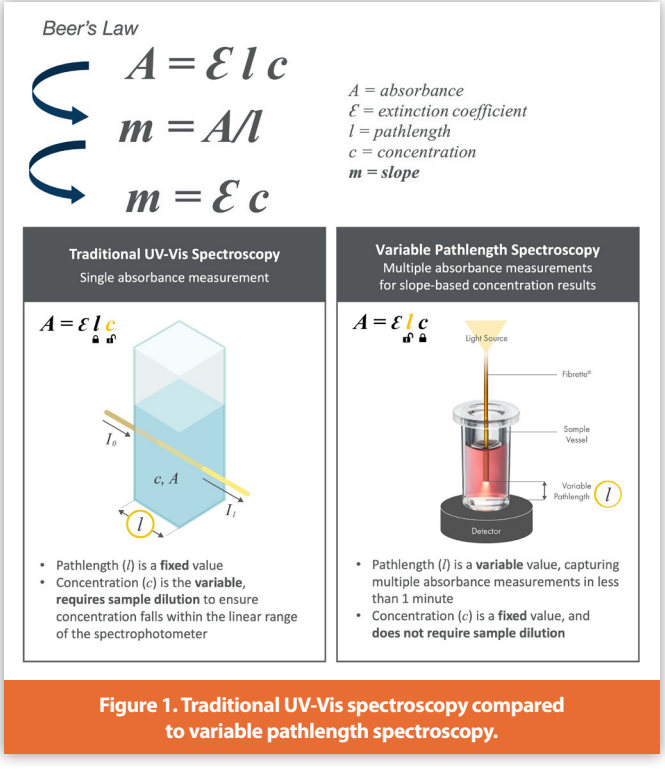

Figure 1. Traditional UV-Vis spectroscopy compared to variable pathlength spectroscopy.

linear correlation between the absorbance at that wavelength and the pathlength of the instrument (Figure 1).

Some of the key features of variable pathlength spectroscopy:

Pathlength selection is controlled and optimized by the instrument. The instrument generates 5 to 10 absorbance measurements at different pathlengths within the linear range.

In addition, the proprietary software calculates the sample concentration using the generated slope, the user-supplied extinction coefficient, and the Beer-Lambert Law.

These key features drastically simplify the workflow of the QC protein concentration method, allowing the QC workflow to be simplified to four major steps:

1. Perform daily instrument check to ensure the instrument is set up and operating as intended.
2. Collect assay control measurement as a secondary layer of control to ensure stringent QC regulations are met.
3. Collect sample measurement. A single sample measurement can be implemented.
4. Report concentration.

(2) Benefits of Variable Pathlength Spectroscopy

Looking at the significantly shortened workflow, many of the benefits of SoloVPE System over traditional UV-Vis methods are apparent.

While both require a daily instrument check, the VPT instrument's internal daily check automatically populates a pass/fail result compared to required calibrated standards in traditional UV methods.

Both methods require an assay control to ensure that QC regulations are met. However, with VPT device, there is an option to purchase a confirmed standard already supplied by the vendor that comes with a certified value. For traditional methods, typically in-house controls must be prepared and qualified in-house.

The VPT system does not require any sample dilution, whereas traditional UV typically requires dilutions in triplicate.

The VPT instrument does not require a blank measurement, as the samples are not diluted and the diluent isn't tested, whereas traditional UV requires this blank measurement to ensure minimal background interference.

The VPT device automatically reports the sample concentration, whereas traditional UV requires manual calculations.

And finally, the sample used for the VPT measurements is often recoverable, allowing its use for other QC assays. Traditional UV requires sample manipulation, which makes it difficult to recover the sample for reuse.

In summary, by removing sample dilution and automating result reporting, roughly 45 minutes is saved per sample, if sample dilutions, measuring every preparation, manually calculating results and having a second verifier to review calculations are factored in.

The invalid rate is also lowered, due to eliminating any variability from analysts, as well as pipettes. Saving time and lowering the invalid rate enables a much higher throughput method within the QC laboratory.

(3) Applications of the Variable Pathlength Spectroscopy

Variable pathlength technology has multiple applications across monoclonal antibody production. Regeneron Pharmaceuticals data has shown the technology is suitable for use in areas such as: the research and development sector during product discovery and formulation development; process areas where operators can take immediate, on-the-floor measurements to continue manufacturing operations; quality control laboratories, which focus on product quality; and even some external contract manufacturing organizations, by easily transferring the variable pathlength method for other uses.

In quality control, protein concentration analysis is used for in-process release and stability testing, as well as for mid-assay concentration measurements. The slope spectroscopy method was validated for a range of concentrations, from 0.1 mg/ml to 279 mg/ml. This method was also validated for a range of extinction coefficients and an array of product excipients.

Various analytical techniques require confirmation of sample concentrations during the assay, such as after a filtration step or after a reconstitution step. The VPT instrument makes these steps more efficient as operators do not have to stop mid-assay to dilute samples and manually perform calculations. Furthermore, the variable pathlength spectroscopy method can be used for cleaning studies, ensuring the product has been cleared of the process.

(4) Method characteristics evaluated during validation

Overall, the VPT system eliminates the challenges we experience using the traditional UV-Vis method. But some method characteristics must still be evaluated in detail, especially when implementing and validating the technology in the QC space for the first time.

Example characteristics that Regeneron evaluated include:

- Matrix specificity—important to ensure that sample matrix components do not interfere with the protein detection
- Scatter correction—important to determine if scatter correction is appropriate for each product that is analyzed
- Linearity and range—important to ensure the method is suitable for the full range of sample concentrations
- Accuracy—ensure accurate measurements for samples with known protein concentration
- Repeatability and precision—ensures method consistency by introducing high method variability (range of different analytes and instruments)

Table 1 shows some of the precision data generated at Regeneron for its mAb products. Four different analysts and three different instruments were used across a total of eight analyses. Each analysis consisted of triplicate measurements, producing a total of 24 measurements for this one sample. All 24 measurements produced consistently accurate results with low variability.

Table 1.

Target Conc. (mg/mL)	Analyst	Instrument	Run	Prep	Measured Conc. (mg/mL)	R2	Percent Recovery (%)	Average Measured Conc. (mg/mL)	SD (mg/mL)	%RSD
76.8	A	I	1	1	77.4	0.9998	101	77.6	1.1	1.4%
				2	77.5	0.9998	101			
				3	78.1	0.9997	102			
			2	1	76.5	0.9998	100			
				2	77.0	0.9998	100			
				3	76.6	0.9998	100			
	B	I	3	1	76.5	0.9997	100			
				2	78.5	0.9997	102			
				3	78.4	0.9998	102			
			4	1	77.1	0.9998	100			
				2	79.1	0.9997	103			
				3	78.4	0.9997	102			
	C	II	5	1	77.3	0.9999	101			
				2	76.9	0.9998	100			
				3	77.0	0.9999	100			
		III	6	1	79.6	0.9999	104			
				2	78.3	0.9999	102			
				3	81.0	0.9998	105			
	D	II	7	1	77.3	0.9998	101			
				2	76.7	0.9998	100			
				3	76.5	0.9998	100			
		III	8	1	77.8	0.9999	101			
				2	76.8	0.9999	100			
					76.9	0.9999	100			

Quality control precision data collected during validation.

Precision was also evaluated across four analysts and three instruments for lower concentration samples as shown in Table 2. Again, all 24 measurements produced accurate results compared to the target and low variability.

(5) Challenges with high concentration samples

As shown in Table 1 and 2, the precision data observed for low to mid concentration samples was well-controlled and very easy to apply into the QC laboratory.

However, some challenges emerged when introducing the VPT System for higher concentration samples, above the range of 120 mg/ml.

As shown in Figure 2, the UV absorbance values above a certain pathlength begin to saturate and diverge from the linear range of the Beer-Lambert law. This leads to large variability in sample measurements, especially across multiple analysts and multiple instruments, and sometimes produced artificially low concentrations for a sample.

There is still a linear range at lower pathlength that can be used for these concentration measurements. Correcting this issue was achieved by limiting the instrument analysis to only five or six points, where the instrument is able to choose the smallest pathlength when measuring high concentration samples, and the largest pathlength

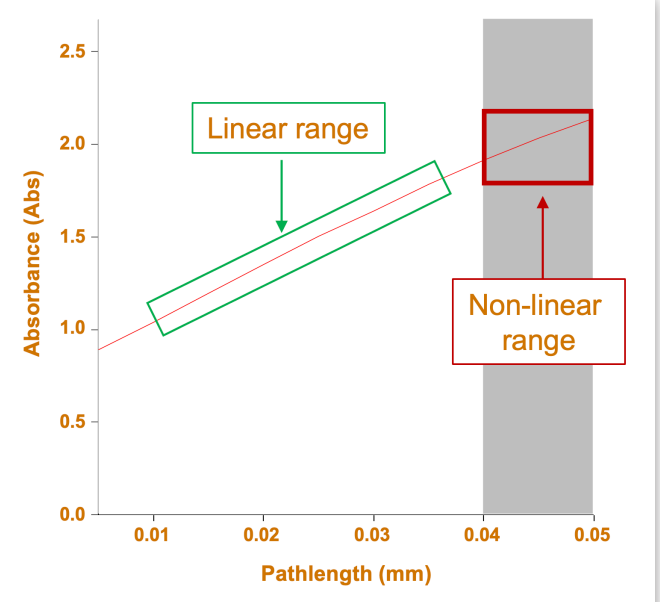

Figure 2. The VPT instrument generates 5 to 10 absorbance measurements at different pathlengths. For the high concentration samples, this 10-point analysis sometimes exceeds the linear range of the instrument.

Table 2. Low-mid concentration precision data.

Analyst	Instrument	Run	Target Conc. (mg/mL)	Prep	Measured Conc. (mg/mL)	R^2	Percent Recovery (%)	Average Measured Conc. (mg/mL)	SD (mg/mL)	% RSD	Target Conc. (mg/mL)	Prep	Measured Conc. (mg/mL)	R^2	Percent Recovery (%)	Average Measured Conc. (mg/mL)	SD (mg/mL)	% RSD
A	I	1	1.04	1	1.08	0.9999	104	1.04	0.02	2.3	49.7	1	49.7	0.9998	100	49.8	0.6	1.1
				2	1.08	1.0000	104					2	49.9	0.9998	100			
				3	1.08	0.9999	105					3	49.8	0.9997	100			
		2		1	1.02	0.9999	98					1	49.5	0.9998	100			
				2	1.02	0.9999	98					2	49.3	0.9997	99			
				3	1.02	0.9999	99					3	49.3	0.9997	99			
B	I	3		1	1.01	0.9999	97					1	49.4	0.9998	99			
				2	1.01	0.9999	97					2	50.0	0.9998	101			
				3	1.01	0.9999	98					3	50.7	0.9996	102			
		4		1	1.03	0.9999	99					1	49.8	0.9998	100			
				2	1.02	1.0000	99					2	51.3	0.9998	103			
				3	1.02	0.9999	98					3	51.0	0.9996	103			
C	II	5		1	1.02	0.9999	98					1	50.0	0.9999	101			
				2	1.06	1.0000	102					2	49.5	0.9999	100			
				3	1.05	0.9999	101					3	49.5	0.9999	100			
	III	6		1	1.06	0.9999	102					1	49.3	0.9999	99			
				2	1.06	0.9999	102					2	49.2	0.9999	99			
				3	1.03	0.9999	99					3	49.5	0.9999	100			
D	II	7		1	1.03	1.0000	100					1	49.7	0.9998	100			
				2	1.04	0.9999	100					2	50.2	0.9998	101			
				3	1.06	0.9999	102					3	49.6	0.9998	100			
	III	8		1	1.05	0.9999	101					1	50.0	0.9999	101			
				2	1.05	0.9999	101					2	48.8	0.9999	98			
				3	1.04	0.9999	100					3	49.5	0.9999	100			

Table 3. The top chart shows six measurements of Product A with a target concentration of 210 mg/ml using10 pathlength measurements. The bottom chart shows six measurements of Product B with a target concentration of greater than 180 mg/ml. In this case, only five pathlength measurements on the instrument were used.

Product A (target: 210 mg/mL)	Measurement	Concentration (mg/mL)	R^2	Max Pathlength	Max Absorbance	Slope	Average Concentration (mg/mL)	%RSD	SD (mg/mL)
10-point No Dilution	1	179.8	0.9990	0.050	2.0	28	189.0	2.5	4.8
	2	189.7	0.9993	0.050	2.0	30			
	3	189.0	0.9990	0.050	2.0	30			
	4	192.3	0.9991	0.050	2.1	30			
	5	190.1	0.9995	0.050	2.1	30			
	6	193.1	0.9992	0.050	2.2	31			
Product A (target: 210 mg/mL)	**Measurement**	**Concentration (mg/mL)**	**R^2**	**Max Pathlength**	**Max Absorbance**	**Slope**	**Average Concentration (mg/mL)**	**%RSD**	**SD (mg/mL)**
5-point No Dilution	1	194.0	0.9998	0.030	1.0	28	193.4	0.9	1.8
	2	192.3	0.9996	0.025	1.1	28			
	3	192.9	0.9996	0.035	1.1	28			
	4	192.3	0.9998	0.030	1.0	28			
	5	192.3	0.9997	0.025	1.0	28			
	6	196.8	0.9996	0.025	1.0	29			

when measuring low concentration samples. This ensures that all measurements remain within the linear range.

When considering Product A, this is where the method begins to diverge from the Beer-Lambert Law. Variability between the six sample measurements, taken by the same analytes on the same instrument, is very high at 2.5% RSD and 4.8 mg/ml standard deviation (Table 3).

Looking at Product B, it can be observed that the instrument limits the maximum pathlength it uses so that maximum absorbance is kept within the range of one absorbance unit, compared to the 10-point method above. This measurement is able to produce much higher R-squared values (0.9% RSD) and much lower variability (1.8 mg/ml standard deviation).

The 10-point measurement for Product C resulted in the highest RSD, delta concentrations, and highest standard deviation as shown in Table 4. In contrast, the two 5-point methods produced a much tighter range across all measurements, with much lower overall variability (1.2% RSD) and standard deviation (2.7 mg/ml). Scatter correction did not impact the final results for Product C, as both measurements resulted in the same average reported concentration. This indicates that scatter correction is a characteristic to be assessed for each product.

The results of these measurements show that VPT is able to mitigate the challenges seen with high concentration products (Table 4).

Table 4. With Product C, we measured with a target concentration of 220 mg/ml using three different techniques. Data was collected across a range of 10 different analytes in order to add variability. Top row:10-point measurement with no sample dilution. Middle row: 5-point measurement with no sample dilution and no scatter correction. Bottom row: 5-point measurement with no sample diluation and applied scatter correction at 320nm.

Product C (target: 220 mg/mL)	Range Across 10 Analysts (mg/mL)	Δ	Average (mg/mL)	SD (mg/mL)	% RSD
10-point No Dilution	206.9 to 233.5	26.6 mg/mL	218.8	5.4	2.5%
5-point No Dilution, No Scatter Correction	214.5 to 228.0	13.5 mg/mL	222.5	2.7	1.2%
5-point No Dilution, With Scatter Correction	218.0 to 225.8	7.8 mg/mL	222.5	2.7	1.2%

	Variable Pathlength Spectroscopy Multiple absorbance measurements for slope-based method	Traditional UV-Vis Spectroscopy Single absorbance measurement for linear range-based method
Instrument Check	System internal daily quick check	Daily instrument check requires calibrated standard
Reference Standard	Purchased ConfiRM Standard used as assay control	In-house prepared and qualified assay control
Dilution	No sample dilution	Large sample dilution in triplicate
Correction	No blank measurement or background correction required	Requires blank measurement and background correction
Analysis	Automatically reports sample concentration using system software	Manual calculation of sample concentration
Recoverable Sample	Recoverable sample – useful intermediate check for QC assays	Sample is diluted and may not be recovered for use in QC assays

Summary

Protein concentration is a critical attribute and a regulatory requirement for the manufacturing of monoclonal antibodies. Concentration is used both in the upstream and downstream manufacturing processes to determine safe and effective patient dosing, and in analytical methods for product release and stability. When implementing the VPT system, especially in the quality control space, it's important to consider the range of protein concentrations that will be evaluated and tested for each product. This ensures that optimal instrument parameters are applied from the beginning.

The patented variable pathlength technology unlocks the power of data-dense slope spectroscopy, which characterizes samples by collecting multiple absorbance data points at several pathlengths to create a section curve (absorbance vs. pathlength plot). This can be analyzed in real-time to verify linearity in compliance with the Beer-Lambert law, as the linear region of the section curve is directly proportional to the concentration of the sample based on the sample's extinction coefficient.

The variable pathlength method saves significant time compared to traditional UV methods, especially in the quality control space. By eliminating sample dilutions, on-bench time and human error are reduced. By automating the sample reporting, documentation times and errors are lessened, as well as the need to have a second verifier. As a platform technology, the system reduces sample prep time, uses low sample volumes, eliminates background correction, and produces rapid results. Furthermore, the variable pathlength spectroscopy method is easily transferred to other labs and sites across the business without training analysts to properly dilute samples and calculate concentrations. These benefits result in a high-throughput assay. For a QC lab testing and releasing an average of 155 samples per week, the use of the slope spectroscopy method would save about nine hours per day, or 39 days each year, compared to a traditional UV-Vis method.

Supporting References

1. Shih E, Salerno M. The Power of Slope Spectroscopy. C Technologies, Inc.: Bridgewater, NJ, 2008; Accesed 9/1/2021. https://ctech.repligen.com/wp-content/uploads/2021/03/The-Power-of-Slope-Spectroscopy-1.pdf
2. Publication Number DOC0057(EN): Solo VPE System Specifications. C Technologies Inc., Bridgewater, NJ, 2018 <https://support.ctechnologiesinc.com/docs/kbi/DOC0057.pdf>
3. Huffman S, Soni K, Ferraiolo J. UV-Vis Based Determination of Protein Concentration: Validating and Implementing Slope Measurements Using Variable Pathlength Technology. BioProcess Int. 12(8) 2014: 66-73.