Spot-on UV/Vis accuracy with Lunatic & Stunner

Introduction

Verifying the performance of UV/Vis spectrometers is a necessary but time-consuming and labor-intensive step in the development of biologics workflows. Having confidence in the accuracy and precision of an instrument beforehand makes it easier to know the work of verification is worth your time. Lunatic and Stunner have always been perfect for anywhere needing fast, low-volume, and highly accurate quantification of proteins and nucleic acids, but now with certified reference materials and new software they're ready to deliver performance verification quicker and easier.

Lunatic is the next-gen UV/Vis spectrometer that makes quantification easy with a wide dynamic range for proteins or nucleic acids (Figure 1A). It maximizes your throughput with high-speed UV/Vis spectral analysis with just 2 µL of sample and up to 96 samples at a time. The uniquely molded microfluidic circuits prevent sample evaporation and have fixed pathlength microcuvettes that mean your pathlength isn't dependent on moving parts.

Stunner takes protein characterization to the next level by combining the same high-speed UV/Vis spectral analysis from Lunatic with dynamic light scattering (Figure 1B). Using micro-volume Stunner plates, Stunner measures the concentration, particle size, and polydispersity of 96 samples in just 1 hour. With this info, you can control how best to sync up sample quantity and quality.

Different reference materials are necessary for different kinds of qualification. For example, NIST standards are great for verifying the operation of a UV/Vis spectrometer with well-controlled, verified values, but only exist for a limited range of wavelengths and absorbances. Meanwhile, measuring a protein of interest in multiple buffers at several known concentrations is labor-intensive, but results will be highly representative of instrument performance.

A

B

Figure 1: Lunatic: the Concentration Liberator (**A**). Stunner: the Quality Defender (**B**).

Thankfully, Unchained Labs offers the Fundamentals. This kit contains several certified concentrations of stable tryptophan samples that absorb in the UV range, just like proteins. The Fundamentals Check application, a new addition in the Lunatic & Stunner v7.0 software, automatically performs data analysis and makes it easy to evaluate the accuracy, precision, and linearity of Lunatic and Stunner instruments.

This app note describes several methods to verify the accuracy and precision of instruments using UV/Vis absorbance to quantify biologic samples across a broad concentration range.

NIST standards

The National Institute of Standards and Technology (NIST) has standards for the operational validation of UV/Vis spectrometers. NIST Reference Material (RM) 8671 is a humanized IgG1κ monoclonal antibody (NISTmAb) and is recommended for system suitability tests, establishing instrument performance, and assisting in method qualification for therapeutic monoclonal antibodies.[1] NISTmAb is a consistent, readily-available, and independent standard for testing the accuracy and precision of UV/Vis spectrophotometers.

NIST Standard Reference Material (SRM) 2082, also called the NIST Pathlength Absorbance Standards for Microliter Volume Spectrophotometers, can be used to calculate the pathlength of microvolume UV/Vis spectrometers and fixed pathlength cuvettes.[2] Microvolume UV/Vis spectrophotometers tend to report absorbances with a nominal pathlength of 10 mm, so you can determine instrument accuracy by comparing the calculated pathlength to this nominal pathlength. The SRM contains a tryptophan and uracil standard which absorb similarly to proteins and nucleic acids, respectively, making SRM 2082 ideally suited to test Lunatic's and Stunner's UV/Vis capabilities for life sciences applications.

Methods

All measurements were done in octuplicate with 2 µL of sample in High Lunatic plates on 5 Lunatic instruments.

NIST RM 8671, consisting of 10 mg/mL human IgG1κ monoclonal antibody (NISTmAb) in 12.5 mM L-histidine, 12.5 mM L-histidine HCl (pH 6.0), and its formulation buffer were thawed and mixed according to the manufacturer's recommendations. Concentrations were determined using the following modified version of Beer's law:

$$Concentration = \frac{A280 \times 10}{E1\% \times b}$$

A280 is the blank-corrected absorbance at 280 nm with a nominal path length of 10 mm, E1% is the theoretical extinction coefficient of 14.2 mL·mg^{-1}·mm^{-1}, and b is the glycan mass correction factor 0.977.

NIST SRM 2082 ampoules of blank buffer solution (10 mM 2-amino-2-hydroxymethyl-propane-1,3- diol (TRIS) buffer, pH 8.0), 1.4 mM tryptophan in TRIS buffer, and 1.0 mM uracil in TRIS buffer, were thawed, thoroughly mixed, and pipetted. Pathlengths were determined for each replicate using the blank-corrected A280 and A260, respectively, a spectral bandwidth of 1.75 nm, a temperature of 25 °C, and equations from the SRM 2082 Certificate. The resulting pathlengths were compared to a nominal pathlength of 10 mm for each measurement.

The percent bias (%bias) of NISTmAb concentration and pathlengths were determined according to the following equation:

$$\%bias = \left|\frac{measured\ value}{reference\ value} - 1\right| \times 100$$

Results

NISTmAb is a representative test molecule for therapeutic protein characterization. According to standards defined by USP, analytical methods for determining drug concentrations should be within 2% of the known concentration, plus or minus the manufacturer uncertainty (MU).[3] The NISTmAb MU is about 0.4% of the reference value of 10 mg/mL, so an analytical quantification method should be within about 2.4% of the reference concentration.

Lunatic is more than a match for the guidelines set for NISTmAb and SRM 2082. All measurements in the 5 tested Lunatics were within 1.25% of the 10 mg/mL reference concentration of NISTmAb (Figure 2), as shown by the whiskers of the box-and-whisker plot. Mean values, shown by the x, and median values, shown by the green line, of the 40 measurements were within 1% of the reference value.

For SRM 2082, all of the pathlengths calculated based on the 8 measured absorbances in 5 Lunatics of the uracil and tryptophan standards were within 2% of the nominal pathlength (Figure 2). Mean and median values of the 40 measurements were with 1.25% of the nominal pathlength.

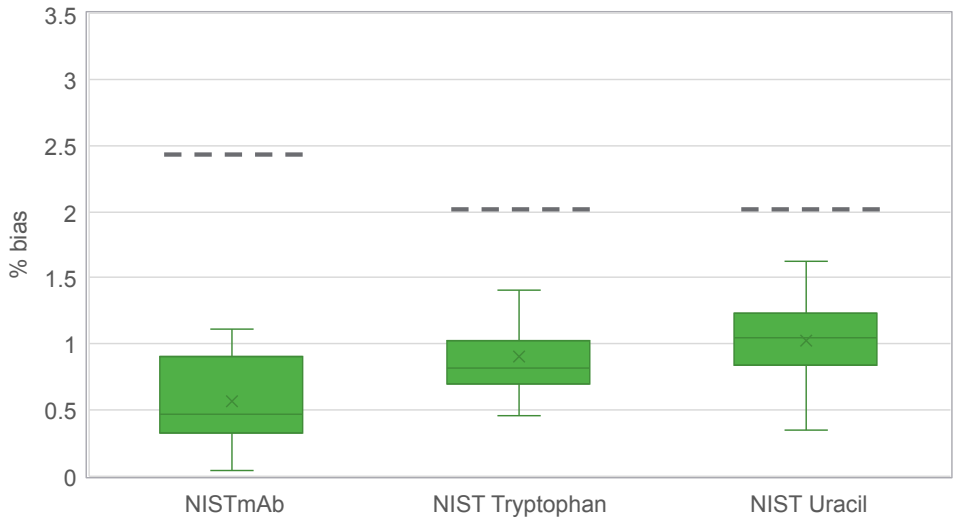


Figure 2: Percent bias of 3 different NIST RMs: monoclonal IgG (NISTmAb), tryptophan, and uracil standards. Samples measured in octuplicate on 5 Lunatic instruments. Dashed lines are 2% plus the percent MU for each substance.

You can prove Lunatic and Stunner's UV/Vis accuracy yourself with independent standards at the wavelengths most relevant to you. SRM 2082 provides certified reference values at 260 and 280 nm, and NISTmAb can qualify methods for quantifying therapeutic antibody concentrations. Lunatic and Stunner are consistently accurate, with results well within 2% of the reference values for all replicates across 5 instruments of the 3 NIST RMs.

The Fundamentals

Verifying UV/Vis performance with NIST standards gives a solid snapshot of instrument operation, but only covers a few absorbance metrics. The Fundamentals makes it easy to prove accuracy, precision, and linearity in the wavelengths most relevant for proteins with 5 concentrations of aqueous L-tryptophan. Since tryptophan residues cause most of a protein's absorbance at 280 nm, the Fundamentals is an appropriate model for most protein applications. Aqueous L-tryptophan is also significantly more stable than proteins at similar absorbances.

The extinction coefficient for tryptophan (E1% = 271.5) is significantly higher than is typical for proteins, like human IgG (E1% = 13.7). This is why the 1, 2, 4, 6, and 8 mg/mL L-tryptophan samples in the Fundamentals kit have similar absorbances to 20, 40, 80, 120, and 160 mg/mL IgG, respectively. For context, Lunatic and Stunner quantify up to 200 mg/mL IgG. The Fundamentals kit tests the accuracy and precision of the upper range of the instrument, while NIST standards test the lower end. Combined, they give a full picture of Lunatic and Stunner performance.

Methods

5 ampoules of 1, 2, 4, 6, and 8 mg/mL L-tryptophan in water with 0.03% NaN_3 from the Fundamentals were stored in the dark at 4° C before use. The standards were vortexed for 20 seconds, sat at room temperature in the dark for 1 hour, vortexed again for 20 seconds, and spun down. Each standard and lab-grade water as a blank were pipetted into a High Lunatic plate, according to the template in the Fundamentals Check app (Figure 3). Target concentrations and MUs were entered into the software and the samples were measured. This was repeated for 5 Lunatics.

Results

Overlaying the 16 replicate tryptophan absorbance spectra of the Fundamentals kit standards from a representative Lunatic shows the impressive reproducibility of the instrument (Figure 4A). Each spectrum is shown as a semi-transparent line, so their regions of overlap appear darker. A linear regression of the measured vs. target concentrations shows the high degree of accuracy and precision with a slope of almost 1 and an R^2 of 0.9999 (Figure 4B).

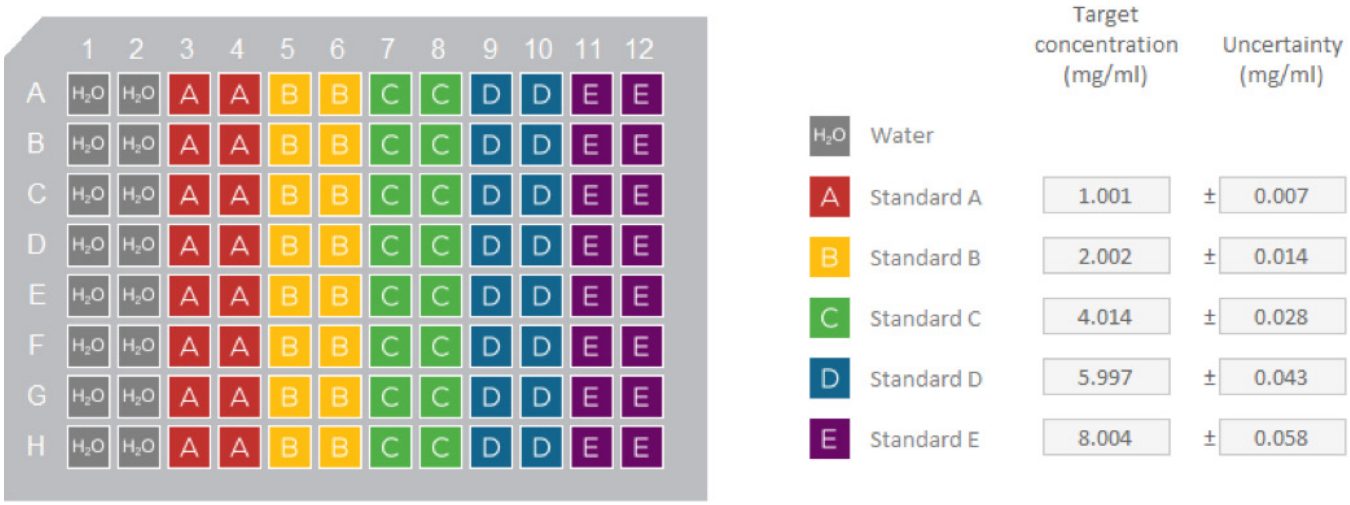


Figure 3: Lunatic & Stunner v7.0 software guides you through setting up the plate for a Fundamentals Check and saves the input target concentrations and uncertainty of the CRM with the results.

The average concentration, mean recovery, and relative standard deviation (RSD) vs. the target concentration were calculated by Lunatic & Stunner analysis for each of the standards (Table 1). The average concentration was within 1% of the target concentration of every standard. All RSDs were less than 1%, even at the highest concentration, showcasing Lunatic's high degree of precision.

The Fundamentals kit allows you to easily verify Lunatic and Stunner's linearity, precision, and accuracy with certified standards available from Unchained Labs. These standards were carefully selected for stability, ease-of-use, and to cover as much of Lunatic and Stunner's absorbance range as possible, in the region of the absorbance spectrum most important to protein quantification.

Protein dilution series

The most direct measure of a UV/Vis instrument's accuracy, precision, and linearity is a protein dilution series at known concentrations. However, using protein standards can be time-intensive and require significant amounts of sample. Thankfully Lunatic and Stunner can prove excellent inter- and intra-instrument

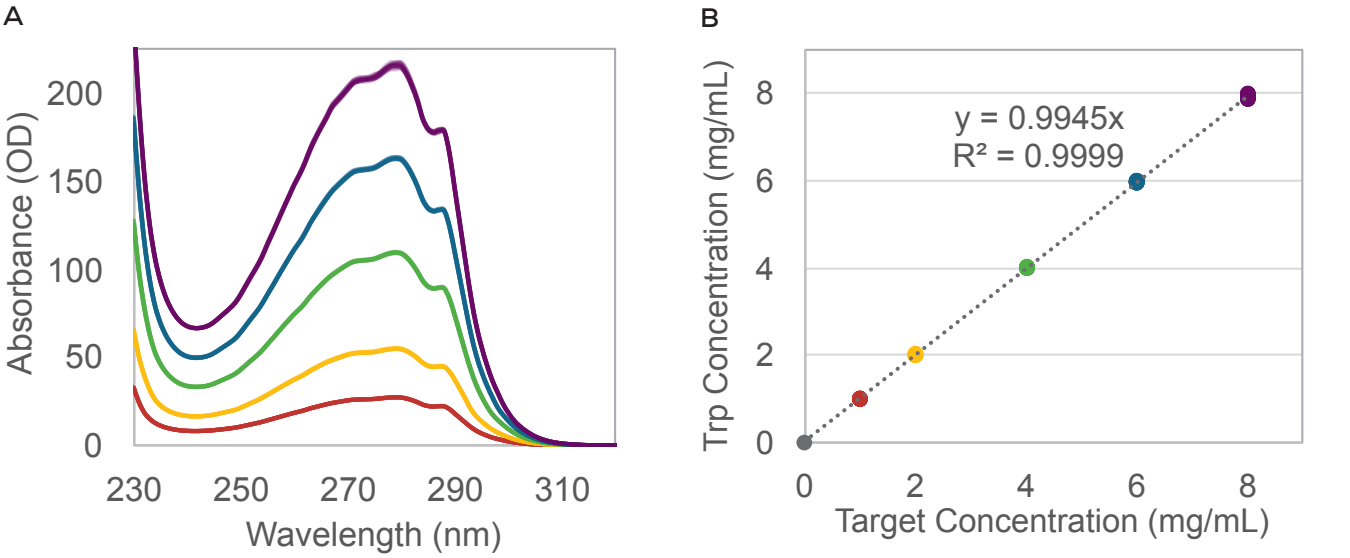


Figure 4: Overlaid spectra of the 16 replicates at each L-tryptophan concentration of a Fundamentals check (**A**). Averages and linear regression of the standard vs. target concentrations of the same Fundamentals check. All 16 replicates are shown but in most cases are too similar to be resolved as separate points (**B**).

Sample	Target conc. (mg/mL)	Avg. conc. (mg/mL)	Mean recovery	RSD
Standard A	1.001	1.001	100.0%	0.18%
Standard B	2.002	2.014	100.6%	0.39%
Standard C	4.014	4.013	99.98%	0.22%
Standard D	5.997	5.98	99.71%	0.28%
Standard E	8.004	7.932	99.10%	0.52%

Table 1: Target and average concentrations, mean recovery, and RSD from a Fundamentals check.

reproducibility using only 2 µL of sample and by reading 96 samples in about 5 minutes, addressing the major challenges to using protein standards.

High concentration protein samples often have very high viscosities, which can interfere with some microfluidic applications. The newest updates to Lunatic and Stunner Client v7.0 increase Lunatic's and Stunner's maximum viscosity from 40 cP to 100 cP by slightly modifying the pump procedure. A quick load option is also now available for loading 16 gelating samples at a time.

Methods

All measurements were done in octuplicate with 2 µL of sample in High Lunatic plates on 5 Lunatic instruments.

Human serum albumin (HSA) was prepared to a stock concentration of 365.5 mg/mL in 0.85% NaCl, then gravimetrically diluted to 365, 114, 34.6, 10.7, 3.42, 1.04, and 0.323 mg/mL in the same buffer. The 2 highest concentration samples and buffer as a blank were measured using quick load and the Protein (Turbidity) app. The remaining samples with buffer as a blank were measured with the standard settings. Concentrations were determined with an E1% of 5.37.

Polyclonal human IgG was prepared in phosphate-buffered saline (PBS), pH 7.4, and acetate buffer (25 mM acetate, 100mM NaCl, 0.025% Tween-80, pH 5.0) to a stock concentration of 254 and 201 mg/mL, respectively. Both IgG stocks were then gravimetrically diluted to 200, 170, 140, 110, 80, 50, 20, 12.5, 7.5, 2.5, and 0.1 mg/mL in their respective buffers. Concentrations were determined with an E1% of 13.7 and buffer blanks.

Results

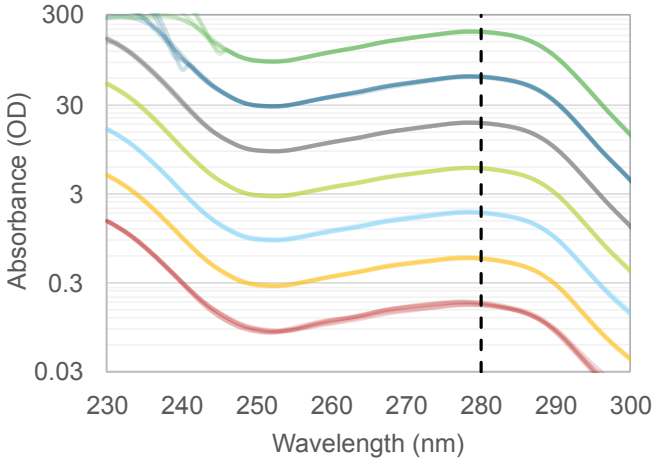
Overlaying the semi-transparent averaged spectra from each instrument at the 7 HSA concentrations shows darker portions where the instruments gave the same results (Figure 5A). This demonstrates Lunatic's high degree of inter-instrument precision across the UV/Vis spectrum, as does the inter-instrument %CVs or standard deviations (Figure 5B). This method can be used to determine the degree of inter-instrument agreement for labs with multiple Lunatics.

For the 11 sample dilution series of IgG in PBS, the high R^2 of the measured vs. target concentration illustrates accuracy, precision, and linearity for Lunatic's concentration measurements across its entire concentration range (Figure 6). And even when switching IgG to an acetate buffer, Lunatic still delivers an R^2 of nearly 1 across a range of 0.1–200 mg/mL, as shown by the target concentration vs. A280 (Figure 7).

Conclusion

UV/Vis quantification on Lunatic and Stunner delivers superb accuracy and precision that you can prove for yourself across a broad range of protein and nucleic acid concentrations. NIST standards are high-quality, verified RMs to assess the function of UV/Vis spectrometers. For a broader absorbance range, the Fundamentals kit makes for fast qualification of UV/Vis absorbance on Lunatic and Stunner

A



B

Conc. (mg/mL)	%CV or SD
365	0.400%
114	0.901%
34.6	0.230%
10.7	0.335%
3.42	1.44%
1.04	0.831%
0.323	0.010 mg/mL

Figure 5: Overlaid average spectra from 8 replicates of gravimetrically-diluted HSA in 0.85% NaCl measured on 5 Lunatics with a dashed line at 280 nm (**A**) and the average concentrations and intra-instrument %CV or SD (**B**). Y -axis is in log scale.

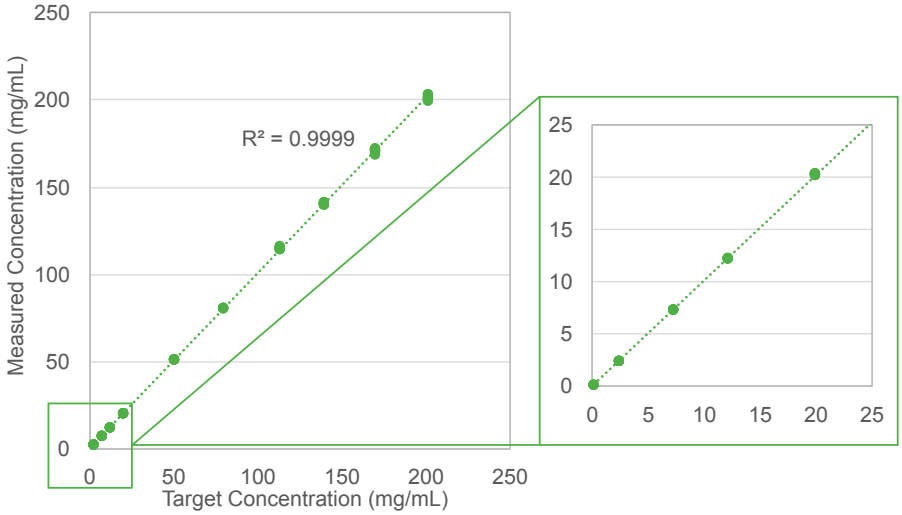


Figure 6: Measured concentration vs. target concentration of 11 samples of gravimetrically diluted IgG in PBS measured in octuplicate from 0.1–200 mg/mL. Zoomed-in figure shows concentrations from 0.1–20 mg/mL. Replicates are too similar to be resolved as separate points in most cases.

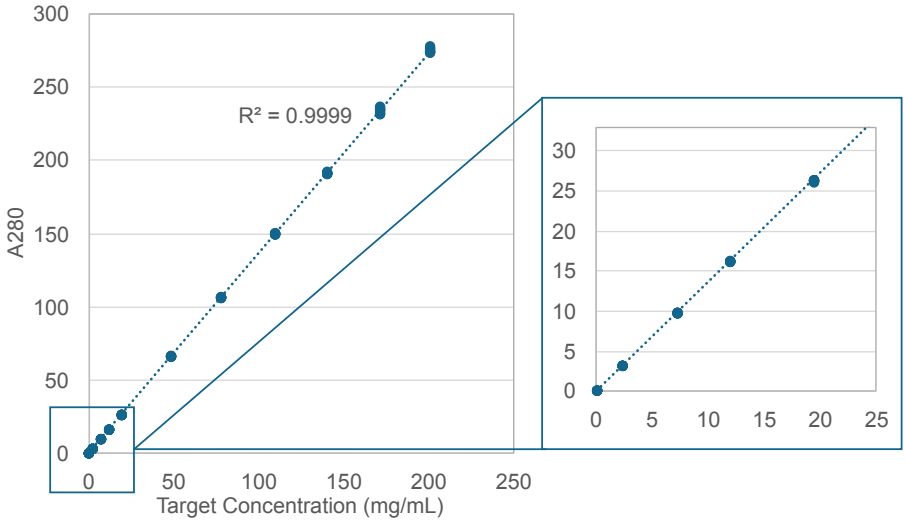


Figure 7: Absorbance vs. target concentration of 11 samples of gravimetrically diluted IgG in acetate buffer measured in octuplicate from 0.1–200 mg/mL. Zoomed-in figure shows concentrations from 0.1–20 mg/mL. Replicates are too similar to be resolved as separate points in most cases.

with stable samples of certified concentrations and easy-to-use reporting software. You'll be blown away by Lunatic and Stunner's accuracy, precision, linearity, and simplicity.

References

1. RM 8671, NISTmAb, Humanized IgG1κ Monoclonal Antibody, National Institute of Standards and Technology; U.S. Department of Commerce: Gaithersburg, MD (15 March 2020).
2. SRM 2082, Pathlength Absorbance Standards for Microliter Volume Spectrophotometers, National Institute of Standards and Technology; U.S. Department of Commerce: Gaithersburg, MD (15 March 2020).
3. United States Pharmacopeia 42 Chapter ‹857› Ultraviolet-Visible Spectroscopy.

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