HORIBA Scientific

Fluorescence

The Power of Duetta and EzSpec™ Software for Biological Applications

Application Note
Biology/ Biochemistry
FL-2020-12-21

Introduction

Duetta is an analytical instrument capable of recording both the fluorescence and the absorbance of a sample. Absorbance data provides concentration-dependent information on non-fluorescent compounds. Through its user-friendly software, Duetta allows different measurements related to biomolecules like DNA, RNA and proteins including protein concentration, DNA/RNA purity, finding the concentration of an unknown sample based on a standard-based calibration curve, Pass/Fail testing and all standard fluorescence and absorbance related measurements. All these functionalities are possible through dedicated applications in EzSpec™ software for Duetta.

In addition to having UV-visible absorbance spectroscopy capability, Duetta enables measuring fluorescence for a variety of samples in a number of different modes. EzSpec software supports many applications enabling acquisition of fluorescence spectra (emission spectra, excitation spectra and excitation emission spectra). Other applications like capture value for standard curve construction, kinetics and absorbance measurements are also possible in EzSpec software, in the main application. All these acquisition types are very quick thanks to the CCD camera allowing high throughput acquisition and analysis workflows. In addition, the integrated correction of inner-filter effects using absorbance enables avoiding measurement and data analysis errors for highly concentrated samples.

This application note illustrates some of these measurements and highlights the capabilities of this benchtop two-in-one spectrometer for biological laboratory processes.

First, the biochemical applications of fluorescence often utilize intrinsic protein fluorescence. Among biopolymers, proteins are unique in displaying useful intrinsic fluorescence. In proteins, the three aromatic amino acids—phenylalanine, tyrosine, and tryptophan—are all fluorescent. A valuable feature of intrinsic protein fluorescence is the high sensitivity of tryptophan to its local environment. Changes in the emission spectra of tryptophan often occur in response to conformational transitions, subunit association, substrate binding, or denaturation. Tyrosine and tryptophan display high anisotropies that are often sensitive to protein conformation and the extent of motion during the excited-state lifetime[1].

Figure 1: EzSpec Methods

The Protein A280 application measures the absorbance of a protein at 280 nm and enables calculating protein concentration using Beer's law (c=A/ε*b). After determining the measurement related parameters (scan wavelengths, integration time, step increment and band pass), the user must enter the number of tryptophan, tyrosine and cysteine amino acid residues to get a calculated absorptivity at 280 nm for the total protein.

The absorbance measurement requires a blank subtraction that can be done before or after the sample measurement. A previous blank spectrum can also be loaded. Once defined, the method can be saved and loaded for similar future acquisitions.

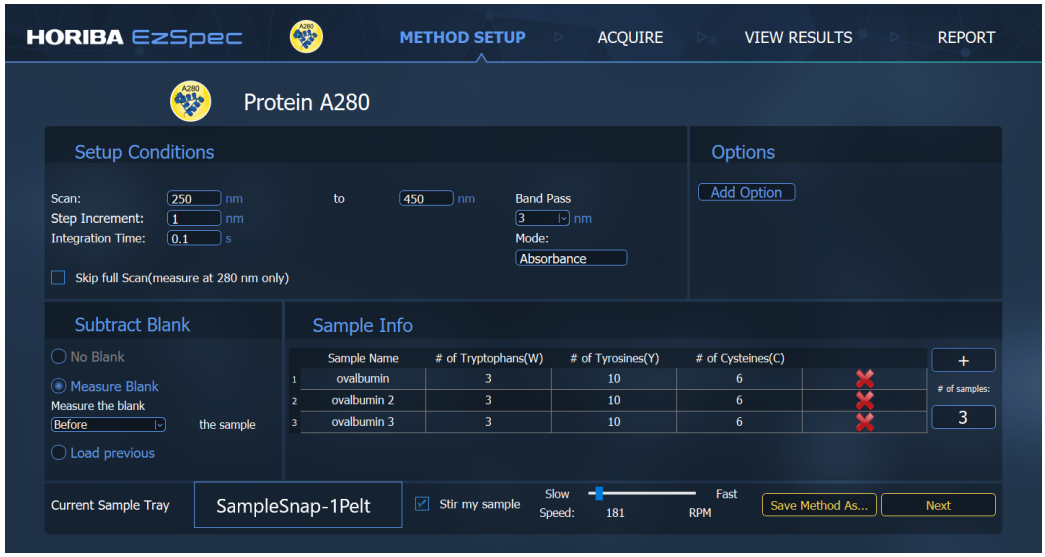


Figure 2: Protein A280 application

The “Next” button will direct the user to the “Acquire” tab where the spectrum of the protein will be displayed.

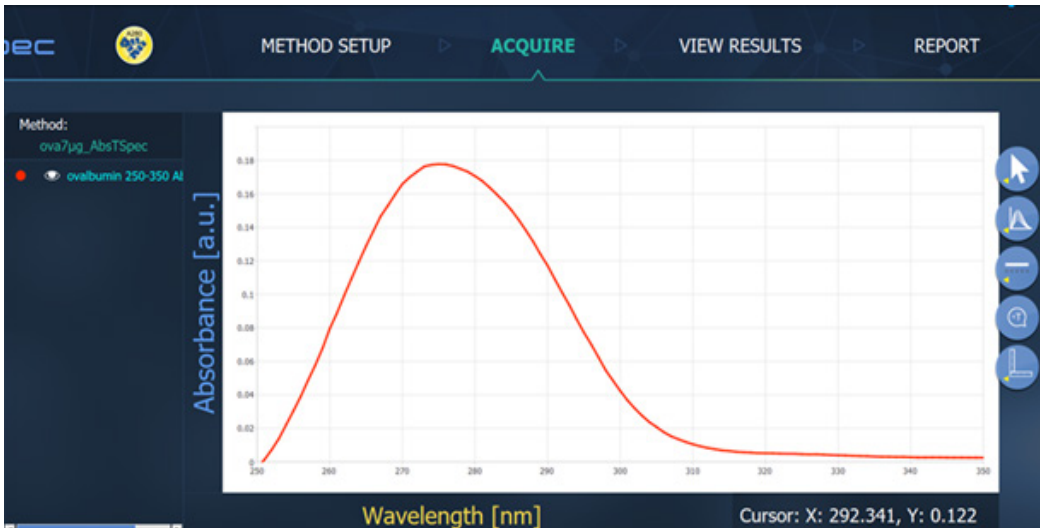
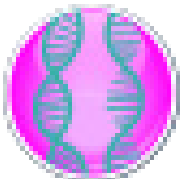


Figure 3: Ovalbumin absorbance spectrum

In the View Results tab, the table displays the absorbance and the calculated molar absorptivity. The concentration is given in “mol/L” and “µM” as shown below with ovalbumin protein.

Figure 4: Results table



DNA/RNA Purity (A260/A280)

DNA concentration calculation is one of the most common needs in molecular biology. Also, for many techniques, like PCR, the purity of DNA is a very important factor.

Duetta enables the concentration calculation of nucleic acids and a purity evaluation based on absorbance. The method is defined using the parameters to scan wavelengths (from 250 nm to 1000 nm), the bandpass, step increment, and integration time. Then, the user inputs the sample information such as dilution factor and the nature of the nucleic acid (i.e. double-stranded, single stranded, or RNA), as shown in Figure 5. As for all the absorbance measurements, a blank is required; it can be measured or loaded from a previous acquisition. Optimizing these parameters is required to get a spectrum, as shown in Figure 6.

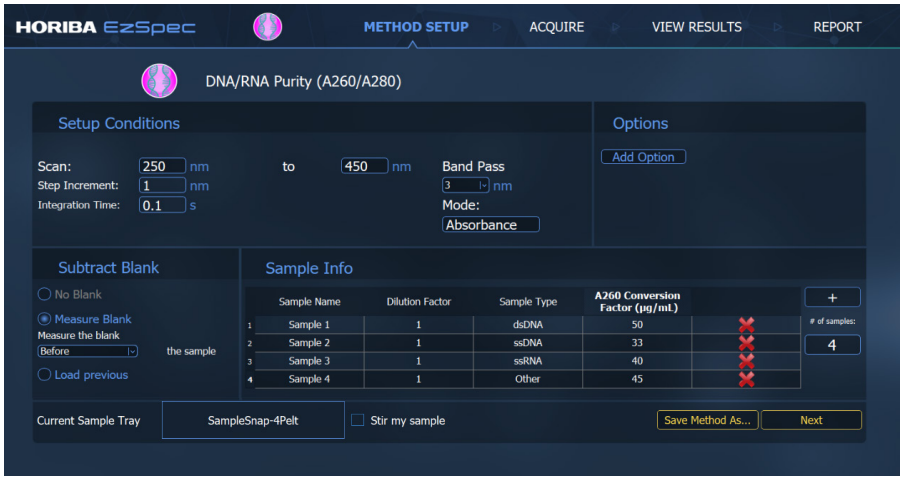


Figure 5: DNA/RNA purity (A260/A280) application

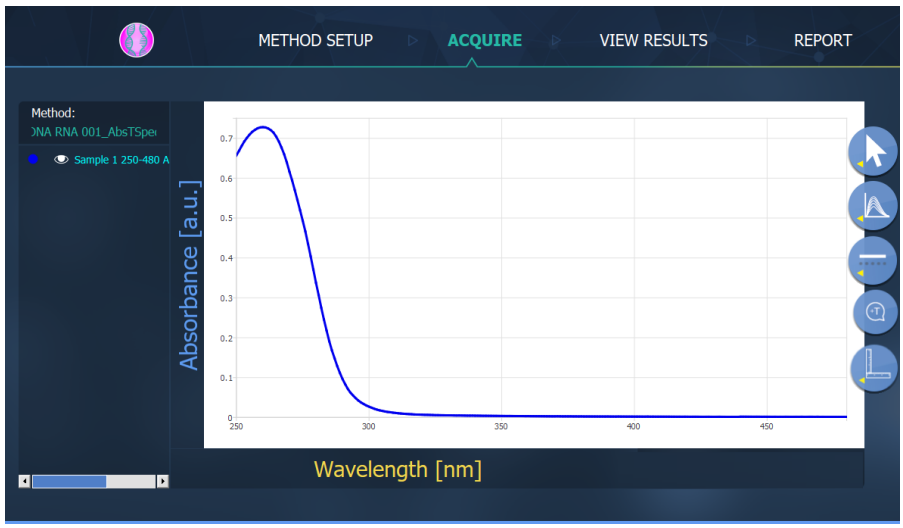


Figure 6: DNA absorbance spectrum

Based on the spectrum, the concentration is calculated, and purity is estimated using a ratio between the recorded absorbance at 260 nm, where DNA and RNA absorb, and 280 nm where proteins absorb. This ratio gives an indication about the contamination of nucleic acid solutions. The background signal at 330 nm is subtracted from each A260 and A280 value before taking the ratio, to account for any high baseline in the absorbance caused by turbidity of the solution. In this example (Figure 7), the studied DNA shows a purity of 1.993, meaning it is a DNA with a very good purity, a value between 1.7 and 2 being considered as pure.

Figure 7: DNA concentration and purity calculation

Build/Use a Concentration Curve

Thanks to this application, an unknown concentration of a sample based on a concentration curve is easy to determine. In the “Capture Value” method within the EzSpec main application, (not detailed in this application note), a concentration curve is built in fluorescence, or both fluorescence and absorbance modes, and loaded (Figure 8) to determine the unknown concentration. A blank measurement is required and can be loaded or measured for each new sample.

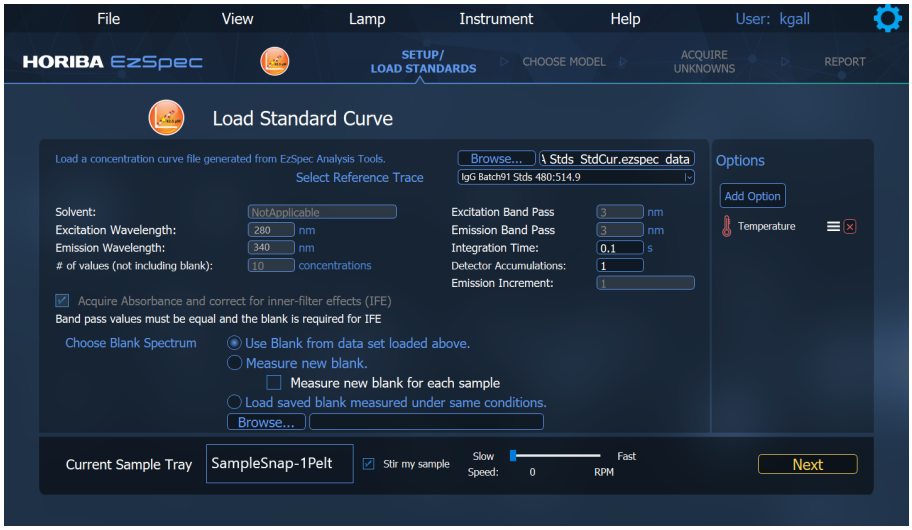


Figure 8: ”Load Standard Curve” setup window

By clicking on the “Next” button, the user can choose the convenient order of polynomial fit (1, 2, 3 or 4) to build the concentration curve as shown in Figure 9.

The unknowns are acquired, and the model can be used to determine the concentration of the unknown sample(s) that will be plotted directly on the curve (red dots).

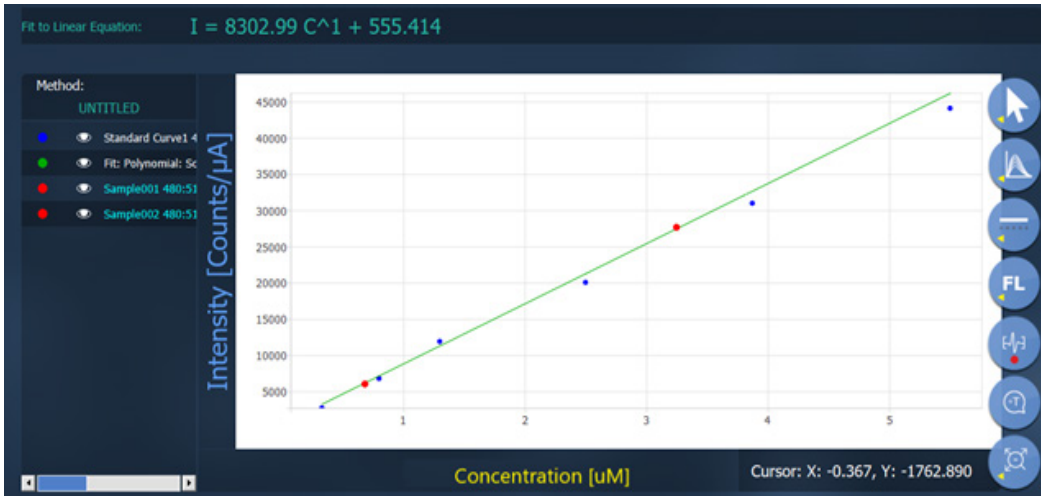


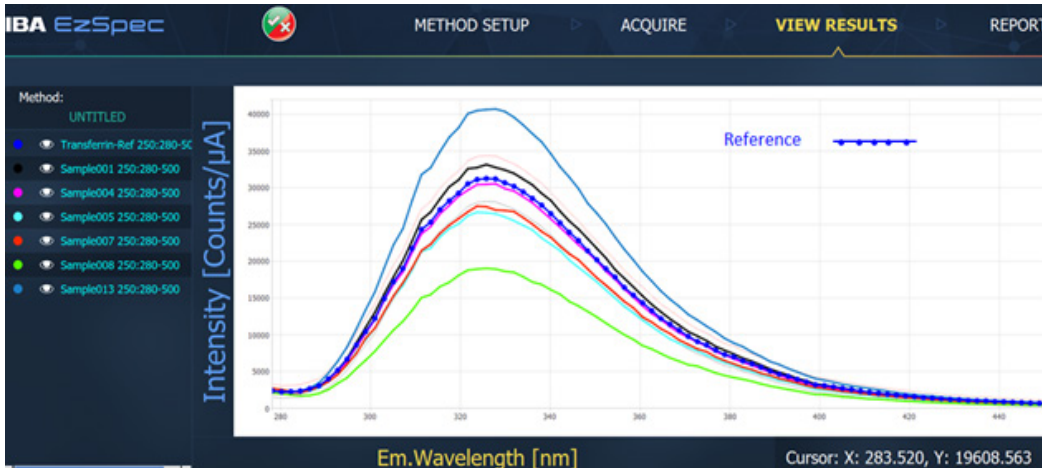
Figure 9: Concentration determination of unknown sample

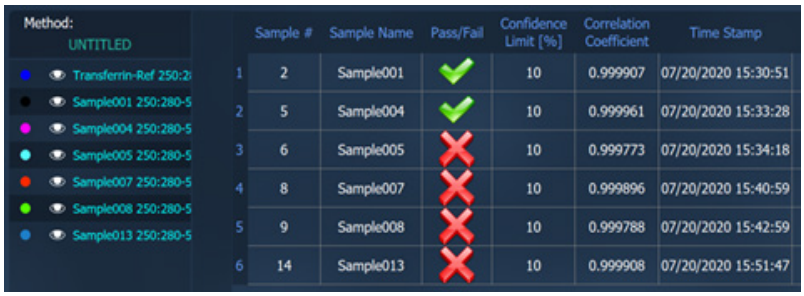
Pass/Fail

Dedicated to qualitative control applications, and useful for pharmaceutical formulation consistency or contamination detection, this software enables testing if the trace from an acquired sample matches a reference trace within a defined confidence limit. If it does, the sample is listed as Pass. If not, then the sample is listed as Fail. The correlation coefficient for each sample tested is also shown in the results. Figure 10 shows the method setup window where the user inputs both the reference and a percent value to set the weighted confidence limit. A blank spectrum is required and can be extracted from a new measurement or loaded from previous data files. The sample spectrum can be compared to the reference according to the shape of the curve after spectra normalization (Normalize Spectra), or according to both shape and intensity (Match Intensities).

Figure 10: Pass/Fail App Method setup window

The tested samples will display in the “Acquire” tab in a table. In parallel, a graph will display in “View results” tab (Figure 11). In this case, the spectrum of the measured sample is considered as “pass” because it falls within the set upper and lower confidence limits.





	Sample #	Sample Name	Pass/Fail	Confidence Limit [%]	Correlation Coefficient	Time Stamp
1	2	Sample001	✓	10	0.999907	07/20/2020 15:30:51
2	5	Sample004	✓	10	0.999961	07/20/2020 15:33:28
3	6	Sample005	✗	10	0.999773	07/20/2020 15:34:18
4	8	Sample007	✗	10	0.999896	07/20/2020 15:40:59
5	9	Sample008	✗	10	0.999788	07/20/2020 15:42:59
6	14	Sample013	✗	10	0.999908	07/20/2020 15:51:47

Figure 11: Pass/Fail App results examples

Conclusion

Knowing the accurate concentrations of DNA, RNA and protein solutions is a very important step in any laboratory workflow that involves protein/DNA/RNA extraction, purification, labeling and analysis. It enables enhancing amplification of DNA, for example in, PCR (polymerase chain reaction) and ensures reproducibility by monitoring protein yields and losses during protein isolation procedure. Quantifying protein/DNA/RNA using absorbance is a rapid, label-free, and non-destructive method, for which an incubation step is not required.

Duetta with EzSpec software offers various tools for the most common biological applications. The user-friendly interface makes lab routine tasks easy to accomplish for any user profile. In addition, the indicators (e.g. Pass/Fail App) help to automatically interpret the results. By combining both absorbance and fluorescence in a compact format, a powerful software, and unique features (correction for absorbance), the Duetta brings added value to any laboratory.

References

[1] Principles of Fluorescence Spectroscopy, second edition, Joseph R. Lakowicz Springer US 1999 pp 529-575 10.1007/978-1-4757-3061-6

[2] HORIBA Application Note (2019). Automatic Correction of Fluorescence Spectra for Primary and Secondary Inner-filter Effects (IFE) with Duetta™

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