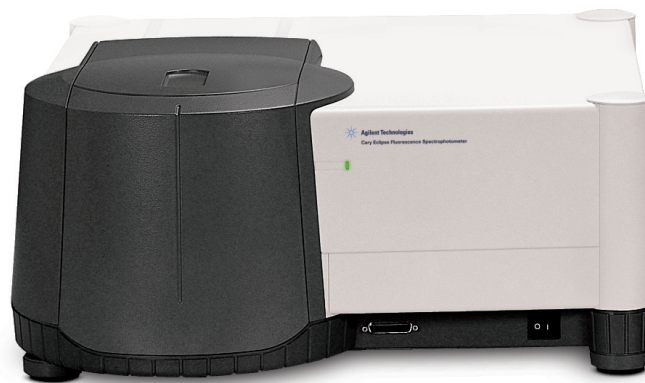


The Linear Dynamic Range and Limits of Detection of Fluorescein using the Agilent Cary Eclipse Fluorescence Spectrophotometer

Technical Overview



Introduction

Fluorescein is one of the most widely used fluorescent labels and is very well characterized. Fluorescein has absorption and emission maxima in the visible region of the spectrum, which is advantageous when it is used as a fluorescent label to monitor biological samples, where background fluorescence from UV excitation and photo degradation can be problematic.

This paper will demonstrate the excellent linear dynamic range of the Agilent Cary Eclipse Fluorescence Spectrophotometer, and also demonstrate the extremely high sensitivity by measuring the limit of detection (LOD) of fluorescein in 0.01 M sodium hydroxide.

The linear dynamic range of a fluorescence spectrophotometer is the region over which the fluorescence emission is directly proportional to the concentration of the sample and is important for quantitative analysis. The linear dynamic range achievable with an instrument is a direct result of the spectrophotometer design¹. Before taking a measurement, it is recommended to determine the linear dynamic range for the fluorophore of interest to ensure that the concentration can be measured in the linear region of the fluorescence spectrophotometer.

The limit of detection of a fluorescence spectrophotometer is a measure of the lowest detectable concentration of a specific sample that can be measured without influence from background signal from the cuvette, the solvent and the instrument. The limit of detection is defined as the concentration that gives rise to an instrument signal of three times the standard deviation of the background signal².

Materials

- Cary Eclipse Fluorescence Spectrophotometer
- Quartz cuvette (10 mm pathlength)
- AR Grade Fluorescein in 0.01 M sodium hydroxide solution (0, 0.1, 1, 10, 50, 100, 500 pM, 1, 10, 50, 100, 540 and 1060 nM, freshly prepared)

Method

Linear dynamic range

Fluorescence intensity measurements were performed using the Cary Eclipse Spectrophotometer with Cary WinFLR software using the instrument parameters listed in Table 1.

Table 1. Operational parameters

Condition	Setting
Excitation wavelength	480 nm
Emission wavelength	520 nm
Excitation slit width	10 nm
Emission slit width	10 nm
Signal averaging time (SAT)	1 s
Excitation filter	Auto
Emission filter	Open
PMT detector voltage	As required

The fluorescence intensity of the blank and the fluorescein solutions were measured using the same instrument conditions, including detector voltage.

If the intensity of a solution was out of range, the PMT detector voltage was adjusted to a lower voltage and the blank and sample measurements repeated. The results of repeated measurements with different detector voltages were used to create a single scale for comparing the intensities observed for the concentration range measured. The linear dynamic range was then determined from the log-log plot of the blank corrected fluorescence concentration compared to the theoretical concentration of fluorescein in 0.01 M sodium hydroxide.

Limits of detection

Time based fluorescence intensity measurements were collected using the instrument parameters listed in Table 2 on the blank, 10, 50 and 100 pM fluorescein solutions at 1 s signal averaging time (SAT) for a total collection time of 60 s.

Table 2. Operational parameters

Condition	Setting
Excitation wavelength	480 nm
Emission wavelength	520 nm
Excitation slit width	10 nm
Emission slit width	10 nm
Signal averaging time (SAT)	1 s
Excitation filter	Auto
Emission filter	Open
PMT detector voltage	1000 V
Total collection time	60 s

The limit of detection was determined using Equation 1.

$$\text{LOD} = [\text{Fluorescein}] \times \frac{3 \text{ Std Dev (Blank)}}{\text{Net signal (Sample - Blank)}} \quad \text{Equation 1}$$

The linear dynamic range and limit of detection data reported in this technical note are only valid under the instrument conditions described. The linear dynamic range and limits of detection will vary depending on the fluorophore, the chosen excitation and emission wavelength, and the instrument setup used.

Results and Discussion

The absorption and emission spectra of fluorescein in 0.01 M sodium hydroxide are shown in Figure 1. The measured and blank corrected intensity of the fluorescein solutions was used to calculate the fluorescein concentration for the samples measured.

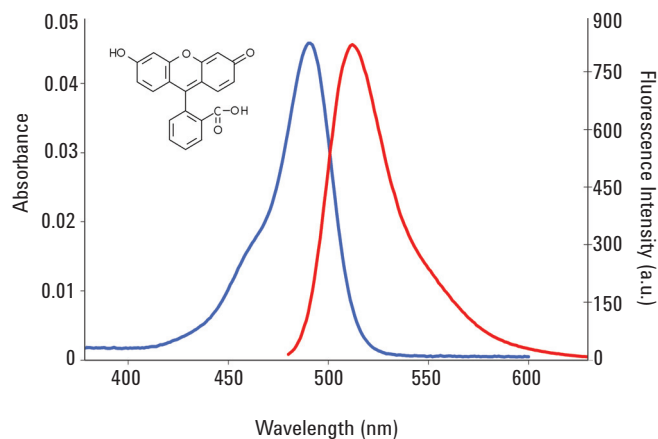


Figure 1: Excitation (blue) and emission (red) spectra of fluorescein in 0.01 M sodium hydroxide collected using 1 s SAT, 1 nm data interval, and 10 nm excitation and emission slit widths.

The fluorescein concentration was then plotted against the theoretical concentration of fluorescein solution to generate a calibration plot (Figure 2). Using the calibration plot, the linear dynamic range for the Cary Eclipse can be determined.

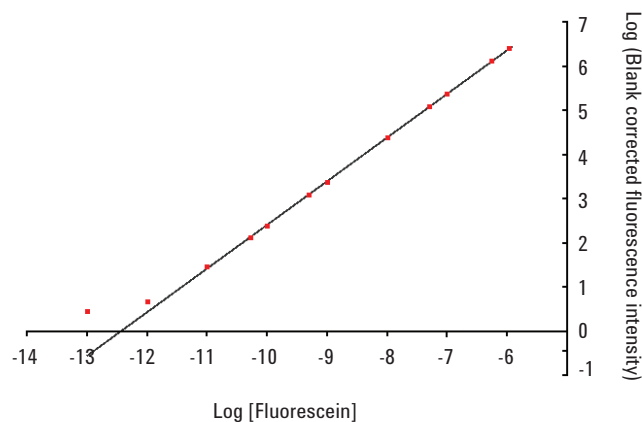


Figure 2: Calibration plot showing the log of the measured fluorescein concentration [Log (Blank corrected fluorescence intensity)] against the theoretical fluorescein concentration (Log [Fluorescein]).

The linearity of the instrument was confirmed by performing a linear regression on the collected data ($r^2 = 0.99993$), which shows the range of fluorescein concentrations over which the Cary Eclipse Fluorescence Spectrophotometer remains linear. Figure 2 shows linearity over five orders of magnitude, from 10 pM to 1.06 μ M fluorescein in 0.01 M sodium hydroxide.

Limit of detection

Five Cary Eclipse Fluorescence Spectrophotometers were used to determine the limit of detection of fluorescein in 0.01 M sodium hydroxide. The average limit of detection was determined to be 0.48 pM. Alternatively, the limit of detection can be determined from the linear dynamic range plot (Figure 2). From the linear regression, an equation for the line of best fit was determined for each instrument. The limit of detection was calculated by substituting 3 times the standard deviation of the blank into this equation. Using this method, the mean limit of detection collected on the five instruments was calculated to be 0.45 pM, which is in excellent agreement with calculation based on measurements on multiple instruments.

Summary

Fluorescein in 0.01 M sodium hydroxide was used to demonstrate the wide linear dynamic range of the Cary Eclipse Fluorescence Spectrophotometer. Fluorescein in 0.01 M sodium hydroxide is linear over five orders of magnitude. The limit of detection was determined to be 0.48 pM and confirmed using the linear regression method.

With excellent sensitivity and a wide linear range, the Cary Eclipse Fluorescence Spectrophotometer is an ideal instrument for the analysis of fluorescein and other probes used for analysis of biological samples.

References

1. ASTM Designation: E 578 – 07: Standard Test Method for Linearity of Fluorescence Measuring Systems.
2. ASTM Designation: E 579 – 04: Standard Test Method for Limit of Detection of Fluorescence of Quinine Sulphate.

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