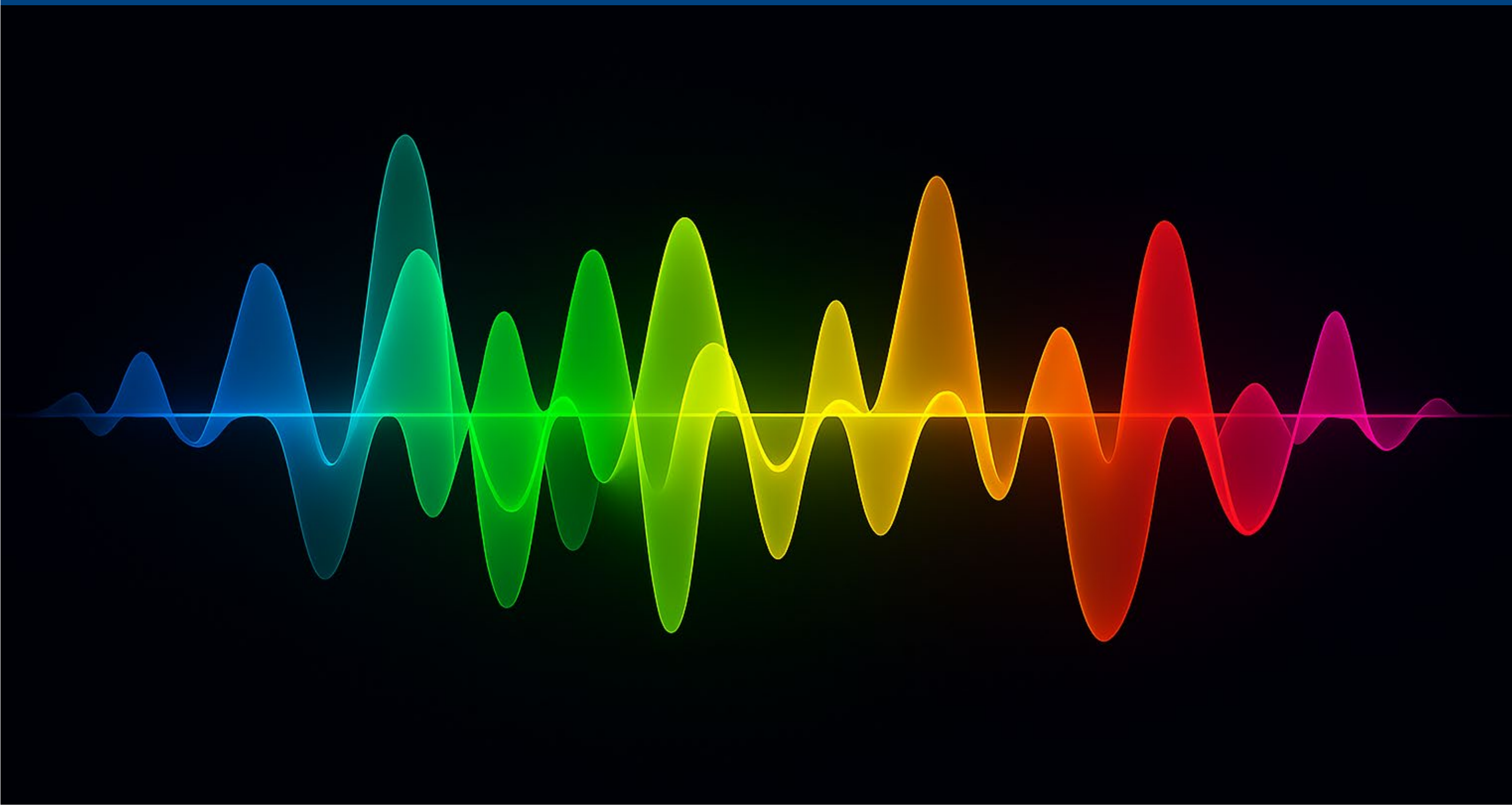
THE MEANING OF GAIN ADJUSTMENT FOR FLUORESCENCE READS



Fluorescence detection emerged in the mid-20th century with advances in photophysics and optics. Today, fluorescence-based readouts are widely used in analytical methods requiring high sensitivity, low detection limits, and precise signal discrimination, including enzymatic assays, immunoassays, and molecular biology applications.

Endotoxin detection by rFC is a ***fluorescence-based method***:

- the recombinant protein cleaves a peptide releasing a quenched fluorophore;
- the reader excites the fluorophore at a defined excitation wavelength and detects the emitted light at a defined emission wavelength;
- the software converts the measured fluorescence into EU values, i.e. endotoxin activity in the tested sample.

While settings like excitation and emission wavelengths or the number of flashes are relatively straightforward, fluorescence gain remains difficult to fully understand.

Fluorescence Intensity

Fluorescence intensity (FI) is detected using photomultiplier tubes (PMTs) (*Figure 1*).
These devices amplify the incoming light signal from a sample via the photoelectric effect, converting light into an electrical signal. This signal is then quantified by the reader in Relative Fluorescence Units (RFU).

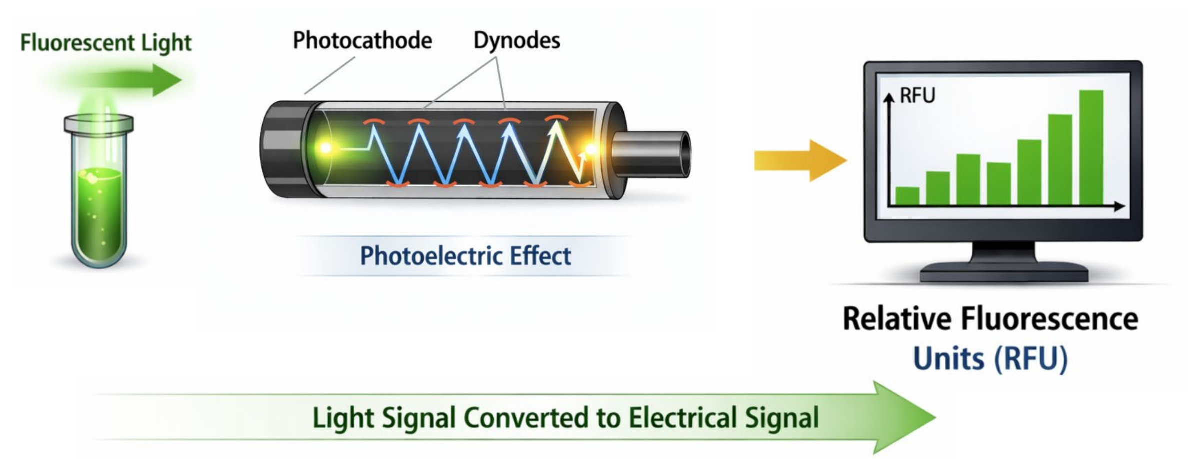


Figure 1: Photomultiplier tube detection of fluorescence: emitted light enters the PMT, is converted into electrons at the photocathode, amplified across dynodes, and measured by the reader as Relative Fluorescence Units (RFU).

Since PMTs and RFU scales are not standardized across manufacturers, absolute fluorescence signals cannot be compared between different instruments. In other words: the scale depends on the instrument and its gain settings. This means that RFU values measured with one gain from one reader do not match RFU values from another reader measured with the same gain.

Absorbance has a fixed scale, but fluorescence does not. This is why fluorescence must be read and interpreted on the same instrument, relative to other measurements or reference signals.

What is fluorescence gain?

Fluorescence gain refers to the voltage applied to the PMT to amplify the incoming fluorescent signal. It acts as an amplification factor that enhances data resolution.

The dynamic range (i.e. the ratio between the brightest and dimmest signals a reader can detect) plays a key role in data quality. Fluorescence gain is the main parameter influencing this range. Adjusting the gain shifts the dynamic range along the assay's concentration curve, without changing its width, only its position.

Typically, high gain values offer strong amplification and are ideal for detecting weak signals. Conversely, bright signals require lower gain values to avoid excessive amplification. High gain increases sensitivity, allowing differentiation between low signals and background. However, it can lead to signal overflow if the sample is too bright. Low gain prevents detector saturation with strong signals but reduces sensitivity, making it harder to distinguish weak signals from background noise.

Gain settings scale the fluorescence signal to keep it within the readable range of the detector. Measuring bright samples with high gain may saturate the detector, resulting in unusable data. On the other hand, using low gain for weak signals may cause them to be lost in background noise.

To avoid these issues and provide the best possible dynamic range between the highest and the lowest measurement values of your assay, fluorescence gain is typically adjusted on a specific sample

In Ph Eur 2.6.32 and USP<86>, the need to perform a gain adjustment is defined. But it could be avoided if the reader allows it to do it.

What is the effect on the sample quantification of a wrong gain adjustment?

A wrong gain adjustment does not have any impact on quantification or patient safety. The worst-case scenario is an invalid result caused by signal overflow at the highest concentration of the standard curve or poor discrimination at the lowest points.

Table 1 shows an example showing the variation in net dRFU values with different gain adjustment settings:

Table 1: Impact of Gain Adjustment Value on Fluorescence Signal (Net dRFU) across the standard curve. Higher gain amplifies signal intensity without changing the proportional relationship between concentration and response.

	Gain Adjustment Value	**56**	**66**	**76**
Sensitivity		NET dRFU		
	5 EU/ml	15.488	50.555	139.607
		15.156	49.470	136.610
	0.5 EU/ml	1.640	5.354	14.785
		1.584	5.170	14.277
	0.05 EU/ml	153	498	1.375
		153	499	1.378
	0.005 EU/ml	13	43	119
		13	41	113

Increasing the gain adjustment results in higher net dRFU values. If these values remain within the acceptable RFU range that your reader can handle, this will not represent an issue.

Figure 2 shows the impact on the shape of the standard curve and the potential quantification of an unknown sample.

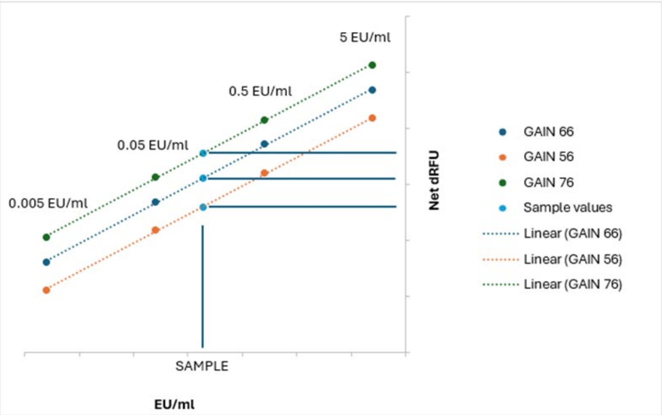


Figure 2: Effect of Gain Adjustment on Fluorescence Response. Each line represents the standard curve at different gain settings (56, 66, 76), showing how signal intensity (Net dRFU) scales with endotoxin concentration (EU/ml) without altering proportionality.

Just like the points on the standard curve, the RFU value associated with the sample will change with gain adjustment, either increasing or decreasing.

This variation occurs because gain acts as an amplification factor, scaling the signal intensity without altering the underlying relationship between concentration and fluorescence. However, as shown in the graph, this does **not** affect the sample's position on the standard curve or its calculated concentration.

In other words, *while the absolute RFU values shift, the proportionality within the curve remains intact, ensuring that quantification is consistent and reliable as long as measurements stay within the instrument's dynamic range*.

The endotoxin value comes from the standard curve, not from the gain setting. Like camera ISO does not change the scene: it only makes the image brighter or darker on screen. A high ISO makes the same scene look brighter. A low ISO makes the same scene look darker. The objects, shapes, and positions stay the same.