Internal Calibration of Microplate Reader Detection System to Improve the Sensitivity, Dynamic Range and the Accuracy of the Spectral Scanning in Fluorometry and Luminometry

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Introduction

When fluorescence signals are measured the results are normally referred as "Relative Fluorescence Units" (RFUs) to emphasis the fact that resulting numeric values are heavily dependent on the settings of the instrument electronics, mainly PMT gain voltage. Results are also somewhat dependent on the environmental aspects, like ambient temperature that affects the electronic noise levels. The practical effect of this relative nature of fluorescence measurements is that result values become quite difficult to be compared, especially if the results are from different measurements done with different instrument settings.

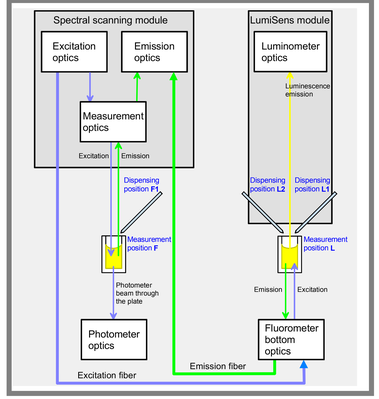
Most of the available instruments partially overcome these difficulties by preventing the use of different electronic settings inside one assay, for example PMT gain voltage is fixed within an assay. This method causes new difficulties that are seen for example as a narrow dynamic measurement range caused by fixed gain voltage. Specially, when high intensity Xenon flash lamps are used for the excitation, the available dynamic range with one gain voltage is commonly reduced to about 3 – 4 orders of magnitude. Fluorometric assay chemistries offer chemical concentration ranges around 5 – 6 orders of magnitude, or even more, so dynamic range of fixed gain voltage is not sufficient.

If Xenon flash lamp is used for the excitation it is necessary to be able to use several PMT gain voltages within one assay to get a good assay performance: both high sensitivity and large dynamic range. When an instrument with fixed PMT gain is used to measure such an assay where the concentration range exceeds the available dynamic range, either high concentration samples are saturated or low concentration samples are not separated from the background, depending on the PMT gain value selected. In both cases certain samples will give incorrect result values.

In addition, the detection system affects also on spectral scanning measurements. The detector system, mainly PMT and monochromator grating, efficiency varies according to the wavelength and that will have an effect on the peak and shape of the spectra.

Thermo Scientific Varioskan Flash® multitechnology reader has in-built calibration system for all measurement technologies covering all mentioned problems in the measurement. This paper shows how detection systems for fluorescence intensity, time resolved fluorescence and luminescence are calibrated to ensure the best possible performance in each individual measurement.

Figure 1. Basic design of Varioskan Flash spectral scanning reader.



Optical Design

The basic optical design of the Varioskan Flash reader is shown in Figure 1. The reader has two optical modules. LumiSens module is used for normal luminescence measurements without any wavelength selection and for luminescence assays where filters are used to separate two or more wavelengths. Another module is a monochromator based spectral scanning module that is used for all the other detection technologies. Both modules are designed so that at least one dispensing head of the instrument's injector units can dispense the well simultaneously with the measurement optics. This makes it possible to dispense the triggering reagent and measure the signal simultaneously and therefore gives possibility to measure very fast flash type chemical reactions.

Both LumiSens module and spectral scanning module have similar calibration system that makes it possible to use several different PMT gain voltages to measure one set of samples. The spectral scanning module includes two reference chips in the plate carrier that produce the calibration signal, one is used for the calibration of the top reading optics and another for the bottom reading optics. The location of the reference chips is shown in Figure 2. These chips produce stable emission signal over the whole spectral range and is used to calibrate four predefined PMT gain voltages.

The basic design for luminometric measurement module is shown in Figure 3. Luminometric measurement module is calibrated using stabilized 560 nm green LED that can produce stable light signal over large intensity range.

Figure 2. Varioskan Flash plate carrier system with PMT gain calibration chips for spectral scanning module.

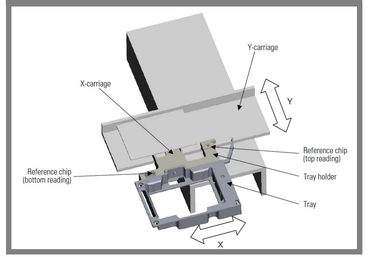
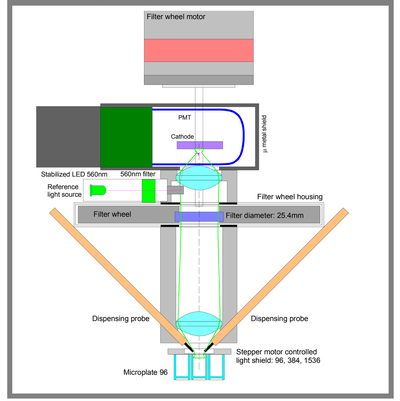


Figure 3. Detailed optical design of Varioskan Flash LumiSens module.



Calibration process

The calibration process is divided into three phases: Initial factory calibration, start-up calibration and runtime calibration.

Factory calibration process is the main system for the instrument calibration and it is fully integrated process that is completely based on these reference samples inside the unit. During this process, the instrument will measure all possible information about the instrument electronics, mechanics and optics, for example PMT sensitivity curves, lamp intensity and spectral characteristics, monochromator relative sensitivity spectra etc. This factory calibration takes about an hour and after that all this information describing how instrument performs is stored inside the instrument memory. Factory calibration needs to be done only after the instrument has been assembled in the factory, or if the internal software is changed or major service has been done.

Start-up calibration is performed every time when instrument is switched on to eliminate possible effect of environmental effects or aging of the electronics. This process is a shortened version from factory calibration where instrument checks that all calibration values are still the same that they were during the factory calibration, all calibration data is still completely valid.

Runtime calibration is performed before and during each measurement run. It includes the calibration of PMT gain voltages for those wavelengths that will be used in the measurement with spectral scanning module when LumiSens module for luminometry is always calibrated with 560 nm signal. The calibration has the following steps:

Before the measurement

PMT gain voltage calibration with the reference signals. The reference chip (when spectral scanning module will be used in assay) or the 560 nm LED (when LumiSens module will be used) sends couple of calibration signals with certain intensity difference between the signals, covering the whole dynamic range. All those wavelengths that have been selected to be used in the assay are calibrated using the reference signals. These reference signals are measured with predefined (during factory calibration) PMT gain voltages. Based on this calibration data, instrument will form a conversion table where relative differences between the PMT gain voltages are normalized and defines light intensity levels where each PMT gain voltage should be used. This conversion table is then used to normalize measurement results that have been measured with different gain voltages. The effect of this PMT gain calibration is shown in Figure 4.

During the measurement

Xenon lamp intensity calibration. When Xenon lamp flashes, each flash is an individual phenomenon, every flash has little different intensity, flash time etc. Therefore, a small portion of the flash is directed directly to the reference detector that will analyze flash properties (intensity, length etc.). This information is used to eliminate the excitation intensity variations. The effect of Xenon lamp intensity calibration is shown in Figure 5.

Figure 4. Effect of the PMT gain calibration.
A) Fluorescein calibration series was measured with different PMT gain voltages without PMT gain calibration. Each PMT gain selection gives its typical about three decades linear dynamic range and same PMT signal response is obtained with several fluorescein concentrations.
B) When several PMT gains are calibrated against each others continuous linear dynamic range over seven order of magnitude can be obtained. Each gain is responsible for measuring certain area of the dynamic range.

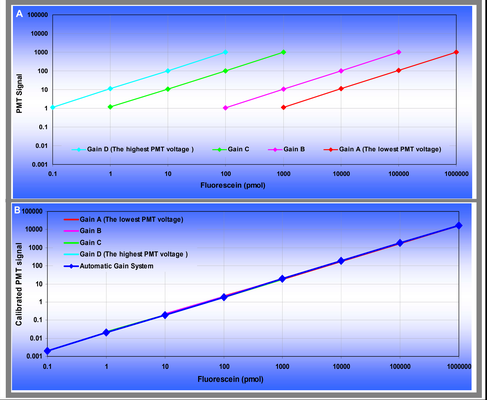
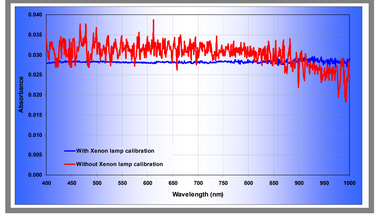


Figure 5. Effect of Xenon lamp intensity calibration.



PMT gain voltage selection for each sample. The optimal gain voltage is selected individually for each sample well using one flash test measurement. Based on the emission signal level from this trial flash measurement, the Varioskan Flash selects the optimal gain voltage and performs the actual measurement. When next sample is measured, similar trial flash measurement is performed and a new gain is selected for the new sample. This trial flash test measurement is performed before each measurement in all measurement types, end-point, kinetic and spectral scanning. The schematic diagram of this process is shown as an example in Figure 6.

Figure 6. PMT gain selection process during the measurement.
10 ms trial flash is used prior to each measurement to select the correct PMT gain. The PMT gain that was used for the previous measurement is used for the first trial flash. If needed, PMT gain in changed and new trial flash is performed until a correct gain is found.

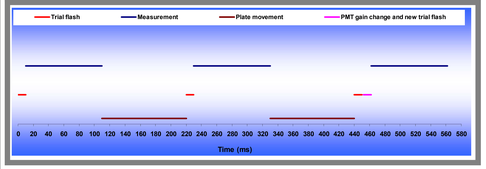
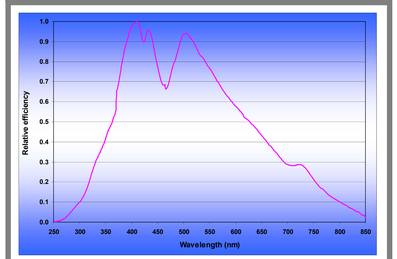


Figure 7. Wavelength dependent efficiency of the monochromator based detection system of Thermo Scientific Varioskan Flash.
As seen, the instrument has the highest detection sensitivity at 409 nm and the sensitivity is remarkably lower at for example low UV and near IR areas.



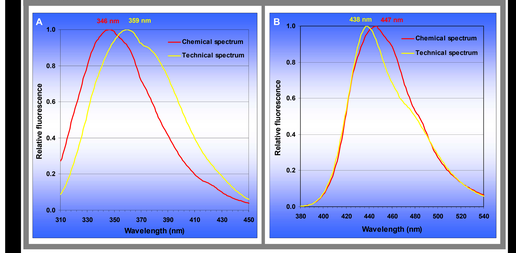
Spectral Correction

Efficiency of any detection system is affected by the electronic components included, specially the PMT and in monochromator systems the monochromator grating. The relative efficiency varies according to the wavelength and has an effect specially on the spectra. If these instrument dependent effects are not eliminated from the spectra then the spectra do not precisely represent the true chemical spectra and are called technical spectra. The difference between technical and true chemical spectra is dependent the case, when spectra is measured in the range where instrument's relative efficiency changed rapidly strong difference between technical and chemical spectra is seen.

Figure 7. shows the wavelength-dependent efficiency of the detection system of the Varioskan Flash reader. This reader has a unique spectral correction feature which can be used to correct the shift of the technical spectra caused by the effects of the instrument's detection optics.

When these variations in the reading efficiency of the instrument are reflected to the measured spectra totally incorrect spectra can be seen. The technical (uncorrected) and true chemical (corrected) spectra of tryptophan and 4-methylumbelliferone are shown in Figure 8. The comparison of the spectra clearly shows the effect of the spectral correction on the peak emission wavelengths and the difference between peak wavelengths of technical and chemical spectrum can be up to even 15-20 nm with certain labels, for example tryptophan.

Figure 8. Examples of the differences in technical and chemical fluorescence spectra of two common fluorometric labels.
A) When tryptophan label is used, a strong spectral shift to the higher wavelength is seen in technical spectra due to electronics higher relative sensitivity to wavelengths over 350 nm.
B) With Methyl-umbelliferone an opposite effect is seen: Instrument electronics is more sensitive around 400 – 430 nm than around 440 – 470 nm.



Conclusions

- With this fully automatic gain selection it is possible to use optimal gain voltage to measure each sample individually.
- When high emission signal sample is measured, a low gain voltage is used to ensure sufficient measurement range without the detector saturation.
- Similarly, when low emission signal sample is measured, the high gain voltage is used to guarantee good signal to noise ratio and therefore good assay sensitivity.
- Automatic gain calibration and selection makes it possible to obtain simultaneously both high assay sensitivity and dynamic range sufficient for any fluorometric application.
- Spectral correction feature is needed to measure instrument independent chemical spectra and can be valuable in assay optimization when small changes in wavelengths can improve the assay performance considerably.

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