CLARIOstar® *Plus* simplifies enzymatic reaction monitoring with its novel Enhanced Dynamic Range technology

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- **CLARIOstar® *Plus* detects highly variable signal intensities of a fluorescent β-galactosidase assay**
- **β-galactosidase can be quantified using the CLARIOstar® *Plus* (e.g. for reporter assays)**
- **Enhanced dynamic range eliminates gain considerations and repetitive optimization measurements**

Introduction

How active are enzymes? This is an often addressed question in biochemical laboratories. As enzymes are the catalyzers of most chemical reactions taking place in an organism, they and their regulators are often characterized in detail.
Enzymatic assays typically employ a substrate that is converted to a chromophore, fluorophore or a luciferase substrate in course of the enzymatic reaction. This means in turn that the signal increases with increasing reaction time until all the substrate is converted by the enzyme. As it is hard to foresee at which range of signal intensity an enzymatic assay ends their detection is challenging and is often associated with various rounds of finding the right amplification for fluorescent and luminescent signals. A virtually unlimited dynamic range as found with the enhanced dynamic range (EDR) feature drastically simplifies fluorescent and luminescent enzyme assays and saves time to set up the measurement.

Assay Principle

The enzyme β-galactosidase (β-gal) catalyzes the hydrolysis of β-galactosides by breaking up glycosidic bonds. It is used in biological research to report on cellular senescence which is indicated by senescence-associated β-gal. A second and very popular use of β-gal is as a reporter gene: a DNA construct contains the genetic information for β-gal and a promoter that regulates the expression of the enzyme. Mostly, β-gal serves as control gene. This means it is inserted into the cells of interest during transient transfection along with a reporter gene of interest. In this case β-gal reports on the transfection efficiency. A high transfection efficiency results in high enzyme production; low transfection efficiency results in low enzyme expression. The amount of expressed β-gal can be tested with a non-fluorescent synthetic β-gal substrate: Fluorescein di (β-D-galactopyranoside) (FDG). It is cleaved by the enzyme to galactose and the fluorescent molecule fluorescein (FITC) (Fig. 1). Here, we measured various enzyme concentrations on the CLARIOstar *Plus* with and without use of the novel EDR feature.

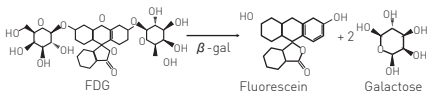

Fig. 1: Principle of the fluorescent β-galactosidase assay. Non-fluorescent Fluorescein di (β-D-galactopyranoside) (FDG) is converted in two steps to the fluorescent Fluorescein and two galactose molecules.

The enhanced dynamic range (EDR) refers to a technology that measures very dim and very intense signals in one plate measurement. This way, a dynamic range of 8 decades can be covered. In contrast, traditional measurements require an amplification of the signal that is typically set by the gain. This limits the range in which signals can be detected (Fig. 2).

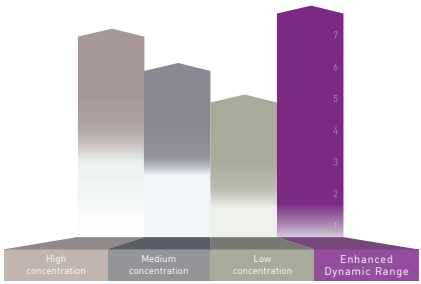

Fig. 2: Enhanced Dynamic Range enables detection of signal intensities spanning over 8 concentration decades (orders of magnitude).

Materials & Methods

- Black 96-well plate with flat bottom (Greiner bio-one # 655076)
- CLARIOstar *Plus*
- β-Galactosidase from *Escherichia coli* (G6008-1KU, Sigma-Aldrich / Merck)
- Fluorescein Di-β-D-Galactopyranoside (F1179, Invitrogen / Thermo Fisher Scientific)

Experimental procedure
The assay buffer was prepared by weighing 0.8370 g KH_2PO_4, 0.6855 g $Na_2HPO_4 \cdot 2H_2O$, 0.0625 TCEP and 0.0203 g $MgCl_2 \cdot 6H_2O$, dissolving in distilled water and adjusting the volume to 50 ml (fill up to 50 ml).
The β-Galactosidase was dissolved in 1 ml of distilled water (enzyme stock solution) and the substrate fluorescein Di-β-D-Galactopyranoside was dissolved in 10 ml of water (0.5 mg/ml substrate buffer). Standards of 0.5 U/ml; 0.25 U/ml; 0.1 U/ml; 0.05 U/ml; 0.025 U/ml; 0.01 U/ml were prepared by preparing double of the end concentration in distilled water and subsequently diluting it 1:2 in substrate buffer. The substrate concentration was kept constant at 0.25 mg/ml. Total reaction volume was 200 µl and four replicates were measured.
Fluorescence intensity measurements were performed by the CLARIOstar *Plus* multi-mode microplate reader with the settings indicated below.

Rev. 02/2019
Keywords: **enzyme activity, reporter gene**

Instrument Settings

Optic settings	**Fluorescence intensity, Plate mode kinetic**	
	Monochromator	Excitation: 483-14 Dichroic: 502.5 Emission: 530-30
	Gains	EDR or gain as indicated
General settings	Number of flashes	100
	Settling time	0.1 s
Kinetic settings	Number of cycles	28
	Cycle time	600 s
Temperature	monitored	

Results & Discussion

In order to test the suitability of the CLARIOstar *Plus* for measuring β-gal reporter assays, different enzyme concentrations were incubated with the synthetic substrate FDG and its conversion was monitored by measuring FITC fluorescence every 10 minutes.
Traditionally, a signal range needed to be chosen upfront in order to detect such a kinetic measurement. For demonstration purpose, we selected a low gain (800) which translates to low signal amplification and should prevent excessive amplification of the increasing fluorescent signal.
However, detection in this wisely preselected range resulted in overflow measurements for all of the enzyme concentrations as the signal increased further than expected (Fig. 3). The lowest enzyme concentration showed an increase in fluorescence until 1 h 10 min after reaction start (orange line Fig. 3), but flattened out the following timepoints as the signal exceeded the preselected intensity range. A quantification of the enzyme is not possible with the acquired data.

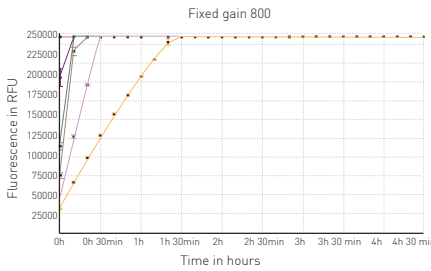

Fig. 3: Measurement of a fluorescent β-gal assay at a falsely chosen gain/signal range.

Measuring the same plate with the EDR feature does not require any preselection. It results in the resolution of each enzyme concentration, from the highest (0.5 U/ml) down to the lowest (0.01 U/ml) (Fig. 4). Thus, the EDR feature prevents the need to prepare and measure a second time. Besides that it reduces the time to run a working enzyme assay as well as concerns when starting the measurement. The data further provided a linear correlation between maximum conversion speed and enzyme concentration (R^2 >0,99) allowing quantification of the enzyme up to 0.1 U/ml (data not shown).

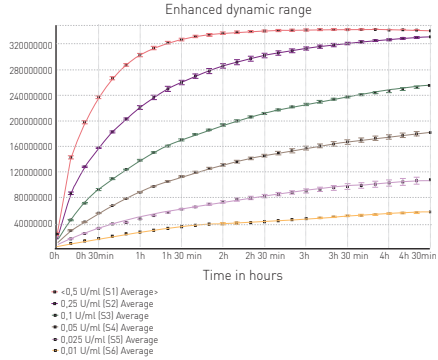

Fig. 4: Measurement of a fluorescent β-gal assay with the EDR feature. Different enzyme concentrations were monitored over 4.5 h.

Conclusion

Here, we demonstrated the detection of a β-gal enzyme assay that is primarily used for reporter assays. With the help of the EDR technology of the CLARIOstar *Plus* β-gal mediated production of fluorescein was conveniently monitored. It allowed the detection of huge signal differences covering a range from 660 RFU up to $3.44*10^8$ RFU. Translated to a reporter assay, this means very high and very low expressions can be measured at the very same plate. The tool simplifies the set-up of enzymatic assays as it does not require time to think about the right gain settings or repeat the assay until optimal settings are found.