# Measurement of red fluorescent dyes for improved multiplexing capabilities and cellular assays on the CLARIOstar® *Plus*

Andrea Krumm
BMG LABTECH GmbH, Allmendgrün 8, 77799 Ortenberg, Germany

- **CLARIOstar *Plus* provides highest sensitivity in measuring red fluorescent dyes**
- **Determination of the limit of detection for AlexaFluor®647: 0.8 pM**
- **Improved HTRF measurements due to a dedicated detector**

## Introduction

Fluorescence-based assays conquered the benches of life science labs due to numerous advantages: fluorophores are often non-toxic, they are affordable and can be measured multiple times. Their intensity is linearly related to their concentration and last but not least the number of assays available is massive. Not only did the use of fluorescence assays per se increase but in particular the use of dyes emitting in the red and infrared range of light has risen.

*What drives the shift to red dyes?*
*Multiplexing.* Research is evolving fast and thus requires obtaining many and exact data in a short time. Thus, multiple assays are run in parallel to allow detection of several biological aspects in one sample. For instance, expression of a fluorescent reporter protein can be referenced to cell viability. However, luminophores and fluorophores measured in the same sample need to be spectrally separated for efficient signal separation. Many traditional assays are based on dyes emitting in the green range (AlexaFluor®488, FITC, GFP). Fluorescent dyes that are combined with such assays are often red-shifted. Similarly, red fluorescent dyes are required for multiplexing with luminescence. As depicted in Fig. 1, luciferases emit light over broad wavelength ranges, often up to 500-600 nm. Separation of luminescent and fluorescent signals is only possible when combining it with red fluorophores emitting over 650 nm.

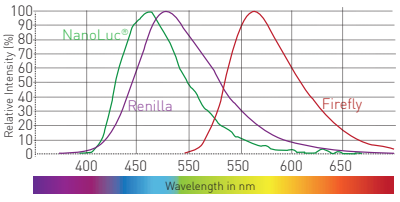


**Fig. 1:** Emission spectra of luciferases. For multiplexing, these can only be combined with red-shifted fluorophores (emission > 650 nm).

*Autofluorescence reduction in cellular and complex samples.*
In complex biological samples there are many unwanted fluorescent components that interfere with the signal of the fluorophore of interest (auto-fluorescence): aromatic amino acid side chains, NAD(P)H, flavin or phenol red occupy the light spectrum from the UV up to the red range. These molecules are mainly found in cellular or complex samples such as serum and plasma.
Using fluorophores emitting further in the red range (>650 nm) prevents acquisition of auto-fluorescence.

Another method circumvents auto-fluorescence in two ways: long lifetime fluorophores detected when auto-fluorescence already faded and their detection in the red. This principle is popular in homogenous assay formats that measure molecule interactions by FRET: homogenous time-resolved Förster resonance energy transfer (HTRF®, Cisbio). A long-lifetime fluorophore is used as donor and transfers energy to a red acceptor fluorophore only if this is found in proximity.
The use of red fluorescent dyes is inevitable when measuring cells or multiple assays at once. Thus, we tested the performance of the CLARIOstar *Plus* with dedicated detectors in measuring the red AlexaFluor®647 and compared HTRF measurements performed with standard and dedicated detectors (PMT).

## Materials & Methods

*AlexaFluor 647 Testing*
Testing of the CLARIOstar *Plus* was performed with AlexaFLuor®647, a bright fluorophore that is excited around 640 nm and emits at 680 nm (Fig. 2).

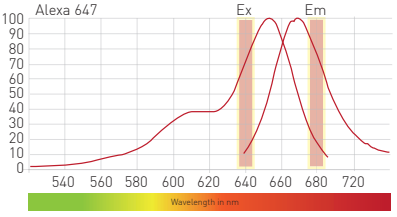


**Fig. 2:** Excitation and emission spectrum of AlexaFluor®647

- black 384 sv microplate (Greiner #784076)
- CLARIOstar *Plus* (BMG LABTECH)
- AlexaFluor 647 (ThermoFisher Scientific)
- PBS (Biochrom #L1825)

The fluorophore was diluted to standards of 100 nM; 20 nM; 4 nM; 0.8 nM and 0.16 nM in PBS. Eight replicates (20 µl) and 85 PBS blanks (20 µl) were pipetted into the plate for determination of the detection limit. Fluorescence was measured on the CLARIOstar *Plus* using the following instrument settings.

Rev. 02/2019
Keywords: **AlexaFluor® 647, HTRF**

*Instrument settings*

| | | |
|---|---|---|
| Optic settings | Fluorescence intensity, top optic, end point | |
| | Detector | Dedicated red-shifted PMT |
| | Filters | Ex: 640-10<br>Dichroic: LP664<br>Em: 680-10 |
| | Gain | EDR |
| General settings | Number of flashes | 100 |
| | Settling time | 0.1 s |

*Detector comparison for HTRF measurements*

For comparison of standard and dedicated detector in HTRF measurements, a Cisbio kit based on Eu Cryptate donor (emission 620 nm) and XL665 (emission 665 nm) acceptor was used.

- white 96 well half area microplate (provided by Cisbio)
- CLARIOstar *Plus* (BMG LABTECH)

*Instrument settings*

| | | |
|---|---|---|
| Optic settings | Time resolved fluorescence, dual chromatic | |
| | Detector | Dedicated red-shifted PMT and standard PMT |
| | Filters | Ex. Ex TR<br>Dichroic: LP TR<br>Em 1: 665-10,<br>Em2: 620-10 |
| | Integration times | Start: 60 µs, Time: 400 µs |
| General settings | Number of flashes | 200 |
| | Settling time | 0.1 s |

## Results & Discussion

A standard curve of AlexaFluor647 was measured to determine its detection limit on the CLARIOstar *Plus* (Fig.3). The standard curve from 100 nM down to 0.16 nM resulted in a highly linear correlation as indicated by an $R^2$ of > 0,9999. Calculation of the detection limit according to IUPAC standard resulted in a low limit of 0.8 pM (Table 1).

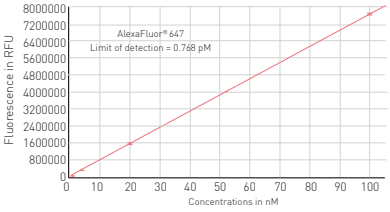


**Fig. 3:** Standard curve of AlexaFluor647.

Table 1. Limit of detection calculation of AlexaFluor647 measured on the CLARIOstar *Plus*

| | |
|---|---|
| Standard deviation of the blank (and %CV) | 19.6 (2.2%) |
| Slope of linear regression | 76676 |
| Limit of detection - LOD (3* $SD_{Bl}$/Slope) | 0.8 pM |

The high sensitivity is not limited to red dyes. The LOD of 0.15 pM for FITC (data not shown) shows that the dedicated PMT is a suitable solution for multiplexed detection of red and green fluorophores.

Testing the dedicated red-shifted detection of the CLARIOstar *Plus* for HTRF measurements revealed improvements in assay window, measurement stability and consequently assay quality. The deltaF is a measure of the assay window which relates the increase in FRET ratio of positive controls to the negative control. This measure was highly improved using the dedicated detector as compared to the standard detector (Fig. 4) high positive control +17%, low positive control +15 %). As additionally the %CV, a measure of signal stability, of the blank was reduced using the dedicated detector the complete assay quality was improved. The Z' value is indicative of assay quality and includes signal stability as well as distance between positive and negative controls. HTRF assays with low signals greatly benefit from the novel detector as Z' is improved from 0.27 to 0.66 (143 %). HTRF assays with high signals display very high quality with a Z' > 0.9, irrespective of the detector (Fig. 4).

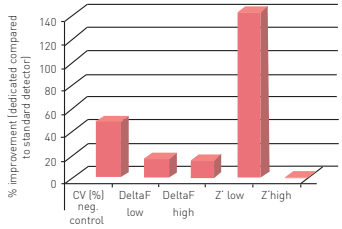


**Fig. 4:** Improvement of HTRF measurements with a dedicated detector as compared to a standard detector. Assays with low and high energy transfer were tested on five instruments. DeltaF for assay window and Z' for assay quality were analyzed.

## Conclusion

Due to increased use of cell-based assays and multiplexing, fluorophores emitting in the red are on the rise. Here, we demonstrate the suitability of the CLARIOstar *Plus* to detect such dyes. It displays high sensitivity with a low LOD for AlexaFluor647 (0.8 pM) and significantly improved HTRF measurements. This makes the CLARIOstar *Plus* a reliable device when it comes to complex samples and multiplex measurements.