Development of a microplate-based, near-infrared fluorescent detection method for unbound bilirubin

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- **A method to directly detect free bilirubin is sought to better correlate with potential neurotoxicity**
- **The use of red-shifted dyes is preferable due to the potential autofluorescence of biological samples at shorter wavelengths**
- **The utility of the CLARIOstar® for assay development and the PHERAstar® *FSX* as a high throughput platform is highlighted**

Introduction

Bilirubin is a byproduct of hemoglobin turnover which is usually associated with albumin in plasma. However, in the case of hyperbilirubinemia, free bilirubin (Bf) can be present in the plasma at greatly elevated levels. Hyperbilirubinemia is most problematic in children where it affects about 60 % of newborns and 80 % of premature babies. Bf passes through the blood-brain barrier where, if present in large amounts, it elicits neurotoxic effects and leads to seizures and brain damage.[1]

Fluoresprobe Sciences designed fluorescent assays to directly measure Bf, which is beneficial to determine the risk of encephelopathy. They have previously reported the use of fatty acid binding proteins (FABP) that are labeled with fluorescent markers[2]. Increased utility can now be achieved using a Bf sensitive FABP labeled with LICOR 700 DX and a Bf insensitive FABP labeled with LICOR 800 CW. The use of near infrared dyes is ideal for use with undiluted blood.

Improved throughput was achieved by the simultaneous dual emission detection of the PHERAstar *FSX*. Development of a suitable optic module was enabled by the scanning capabilities of a CLARIOstar microplate reader with a red-shifted photomultiplier tube (PMT).

Assay Principle

A ratiometric analysis system was employed that is based on previously characterized mutants of rIFABP. BL22P1B11 is bilirubin sensitive while SNRP1E2 is bilirubin insensitive. These binding proteins were labeled with the LICOR dyes 700DX and 800CW, respectively. The LICOR 700 DX signal is quenched in the presence of Bf while the LICOR 800 CW should be unchanged (Fig 1).

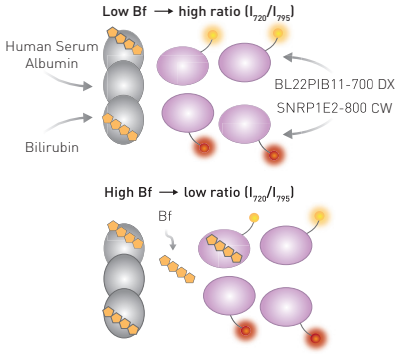

Fig. 1: Principle of the ratiometric assay to measure free bilirubin (Bf).

In the absence of free bilirubin both probes will have a high signal and the ratio below can be calculated.

$$R = \frac{I\,[720]}{I\,[795]}$$

As the levels of Bf increase it will quench the signal of BL22P1B11-700DX and the value of *R* will decrease.

Materials & Methods

- BL22P1B11-700DX and SNRP1E2-800CW (Fluoresprobe Sciences)
- 450 µM Human Serum Albumin (HSA) in 50 mM HEPES buffer and bilirubin (BR)/450 µm HSA (0.5 molar ratio) in 50 mM HEPES buffer prepared by Fluoresprobe Sciences
- PHERAstar *FSX* and CLARIOstar microplate reader (BMG LABTECH)

Spectral scanning

Free rIFABP fluorescently labeled probes were prepared at 1 µM in HEPES buffer and added to a black 96-well plate. Buffer controls were also added and both read on a CLARIOstar equipped with a red-shifted PMT using the following settings:

	BL22P1B11-700DX	
	Excitation Scan	Emission Scan
Excitation wavelength [nm]	547 → 700	623
Excitation bandwidth [nm]	8	16
Emission wavelength [nm]	727	650 → 700
Emission bandwidth [nm]	16	8
Gain	2023	1542

	SNRP1E2-800CW	
	Excitation Scan	Emission Scan
Excitation wavelength [nm]	633 → 745	742
Excitation bandwidth [nm]	8	16
Emission wavelength [nm]	803	769 → 840
Emission bandwidth [nm]	16	8
Gain	718	792

Bf ratiometric readings

BL22P1B11-700DX and SNRP1E2-800DX were combined and added to test wells. To this either 450 µM HSA or 0.5 BR / 450 µM HSA were added. Final concentrations were 1 µM for the fluorescent probes and 50 µM HSA. Control wells with either probe alone were included for calculation of signal to background. The plate was read on the CLARIOstar and PHERAstar *FSX* with the following settings:

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	CLARIOstar	
	Chromatic 1	Chromatic 2
Excitation	660-30	
Dichroic	691.5	
Emission	720-24	794-32
Gain	718	792

	PHERAstar *FSX*
Excitation	660
Emission A	795
Gain A	2349
Emission B	720
Gain B	1315

Results & Discussion

Our goal was to enable the use of PHERAstar *FSX* plate readers to detect this ratiometric Bf assay. First, the CLARIOstar was used to determine the spectral properties of the fluorophore. The results of our assessment are shown in figure 2. The Ex / Em scans of BL22PIB110-700DX clearly show maxima at 689 nm and 695 for Ex and Em, respectively. Furthermore, the Em maximum for SNRP1E2-800 CW can be seen at 800 nm. Although the Ex maximum for SNRP1E2-800CW is not observed in these scans the results are sufficient for our purpose as they allow us to compare the Ex spectra for BL22PIB110-700DX and SNSRP1E2-800CW.

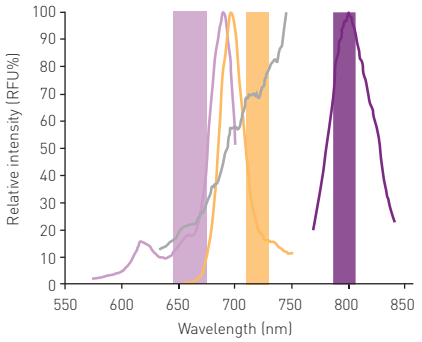

Fig. 2: Ex/Em Scans of BL22PIB11-700 DX and SNRP1E2-800 CW Relative intensity expressed as percent of maximum is shown for BL22PIB11-700DX Ex scan (■) and Em scan (■) as well as SNRP1E2-800 Ex scan (■) and Em scan (■). Overlays indicate the selections used to create a PHERAstar optic module with Ex (■), Em A (■) and Em B (■).

Appropriate filters for the PHERAstar *FSX* optic module were selected based on the spectra shown in Fig. 2. An excitation wavelength suitable for both BL22PIB110-700DX and SNSRP1E2-800CW was chosen and near-peak emission wavelengths for each probe were selected.

The resulting optic module was then tested on samples that contained no bilirubin or sufficient excess bilirubin so that Bf should be observed (Table 1). As expected a strong fluorescent signal was observed at 720 nm for the BL22PIB110-700DX probe under zero bilirubin conditions which was reduced in the presence of bilirubin. In contrast, the signal for the SNSRP1E2-800CW was less affected by the presence of bilirubin probe. Thus the ratio of the signals also decreased when comparing zero bilirubin samples to those with Bf.

Table 1 Ratiometric Bf Assay

		PHERAstar *FSX*	CLARIOstar
50mM Human Serum Albumin	720 (avg.)	143,530 RFU	247,024
	795 (avg.)	71,681	132,113
	Ratio	2	1.87
Human Serum Albumin and Bilirubi	720 (avg.)	110,356	185,800
	795 (avg.)	61804	118,061
	Ratio	1.77	1.57

For comparison the results from the CLARIOstar are shown. The LVF monochromator™ was set up to mimic the filters employed in the PHERAstar *FSX* optic module. The results in Table 1 show that comparable performance can be achieved between the CLARIOstar and PHERAstar *FSX* microplate readers.

Conclusion

This application note highlights the utility of the CLARIOstar as an assay and module development platform for the PHERAstar *FSX*. Using red-shifted PMTs on the CLARIOstar enabled scanning of NIR dyes and a suitable optic was developed. The performance of the module was tested and found suitable for the desired detection of Bf in samples. Importantly, the CLARIOstar also performed well in the same assay which helps to explain the ease of transition to the PHERAstar *FSX* from the CLARIOstar.

References

1. Wan ASL, Mat Daud S, Teh SH, Choo YM and Kutty FM (2016). Management of neonatal jaundice in primary care, *Malays Fam Physician*, **11**: 16-19
2. Huber, AH, Zhu, B, Kwan, T, Kampf, JP, Hegyi, T and Kleinfeld, AM (2012). Fluorescence Sensor for the Quantification of Unbound Bilirubin Concentrations, *Clin Chem*, **58**: 869-876