Application Note

Performing NanoBRET™ with the Thermo Scientific Varioskan LUX multimode reader

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Goal

This note aims at demonstrating the suitability of Varioskan LUX for NanoBRET detection, and it exemplifies the conditions in which this assay can be optimally measured using Varioskan LUX.

Introduction

Bioluminescence Resonance Energy Transfer (BRET) assays, developed during the late 1990's by Promega, have been fundamentally crucial for the understanding of cellular dynamic events, especially protein-protein interactions (PPI). In BRET, an excited energy state of a luminescent donor can transfer energy to an acceptor fluorophore, which will cause the acceptor molecule to emit at a characteristic emission wavelength. These types of assays quickly became useful tools for measuring PPI, and they were considered not only versatile, rapid, and biologically useful, but also suitable for high-throughput screening in live-cell drug discovery of PPI modulators. However, despite their merits, BRET assays have been limited by the low signal strength of BRET donors such as RLuc/RLuc8. Also, these methods often require high donor expression levels, which are not always biologically relevant and can translate into high levels of free donor contributing to the background signal. Lastly, BRET assays are restricted to easy-to-transfect cell lines and they are strongly dependent on the selection of an acceptor molecule that ensures a maximized spectral overlap between the BRET pair.

The introduction of NanoBRET™ by Promega allows overcoming all of these limitations. In NanoBRET, PPI can be studied with a new and significantly more efficient donor-acceptor pair (Figure 1). The new pair consists of an extremely bright blue-shifted luciferase (NanoLuc) as energy donor and a fluorescently labeled red-shifted HaloTag fusion protein as energy acceptor. The new donor (NanoLuc) has a small size (19 kDa), very high emission intensity (460 nm emission peak) and a relatively narrow spectrum, which reduces the chances for donor signal "bleeding" into the acceptor emission channel. In turn, the selection of a red-

emitting fluorescent NanoBRET ligand (618 nm emission) that covalently attaches to HaloTag as acceptor molecule allows for a considerable spectral separation (over 150 nm), thus ensuring very high signal-to-background ratios.

However, despite its superiority over typical BRET assays, NanoBRET performance still depends on the selection of a suitable microplate reader. Promega strongly recommends using an instrument able to measure dual-filtered luminescence with appropriate filters. The selection of sub-optimal filters can jeopardize the assay performance and compromise data quality. According to Promega, the donor signal should
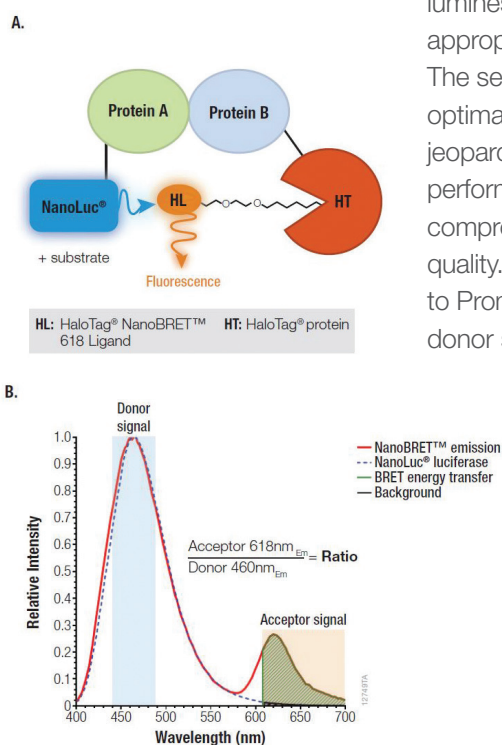

Figure 1. NanoBRET™ assay. In **A**: study of the interaction between proteins A and B is shown, using the NanoBRET pair. Donor is NanoLuc®-Protein A fusion and acceptor is a fluorescently labeled HaloTag®-Protein **B** fusion. In B: the spectral separation and calculation of the NanoBRET ratio is shown. *Reproduced with Permission from Promega*

ideally be measured with a band pass (BP) filter centered around 460 nm and a long pass (LP) filter starting at around 600–610 nm to collect all the acceptor emission signal. Collection of the acceptor signal using an adequate LP filter is particularly crucial to ensure that the assay has a proper sensitivity.

In this note, we aim at assessing the performance of Varioskan LUX when running NanoBRET assays as well as offering a broad methodological guide for selecting the optimal NanoBRET reading settings in this reader.

Materials and Methods

Instruments & accessories

Varioskan LUX multimode reader with luminescence capability (i.e. Thermo Scientific, VL0L00D1)

Emission filter 460 nm HBW80 D25mm (donor emission) (Thermo Scientific, F46080)

Emission filter 610 nm Long pass D25mm (acceptor emission) (Thermo Scientific, F610LP)

Emission filter 600 nm Long pass D25 mm (acceptor emission) (can be obtained from various suppliers, i.e. Edmund Precision Longpass 600nm 25nm, R5000181866-15061, 62985)

Reagents and materials

96-well microplates (Thermo Scientific™ Microlite 1+, white plates, cat. number 7471)

384-well microplates (PerkinElmer OptiPlate-384 HS, gray plates, cat. number 6005310)

1X PBS/0.1% BSA

NanoBRET™ Control Protein Panel: contains 5 samples that represent different amounts of fractional occupancy: 0%; 0.1%; 1%; 10% and 100% (can be obtained, upon request from Promega)

NanoBRET™ Nano-Glo® Substrate, part of NanoBRET™ Detection System (N1571 or N1661)

Experiment set-up

NanoBRET assay

Prepare a 2X solution of NanoBRET Nano-Glo Substrate by making a 250-fold dilution in 1X PBS/0.1% BSA. For each of the vials representing various amounts of fractional occupancy, dispense 50 µL into each well of a white 96-microplate, and add an equal amount of the 2X NanoBRET Nano-Glo Substrate (50 µL). This last reagent is recommended to be dispensed with Varioskan LUX. Within 10 minutes of substrate addition, measure donor emission and acceptor emission using Varioskan LUX, with the selected filters.

Varioskan LUX settings for NanoBRET assay

Adequate filters need to be installed in the instrument before starting. For that purpose, follow the instructions for the installation of new filters into the Varioskan LUX L module, available in the instrument manual. Of note, when installing the F600LP or F610LP filters, a bandwidth needs to be defined in Thermo Scientific SkanIt™ software, even though these filters don't have a bandwidth. Use a number between 60-999 nm.

For programming the SkanIt session:

- Add a Kinetic Loop and select the total time (or number of readings) and kinetic interval
  For this assay, 20 readings are made with 1 min of kinetic interval. Note: The kinetic decay of both the acceptor and donor emission signals is very significant after the first 10 minutes of the reaction (illustrative example shown in Figure 2). However, the decay of donor and acceptor signal keeps the same rate, thus the calculated NanoBRET ratios are not affected at longer measurement times.
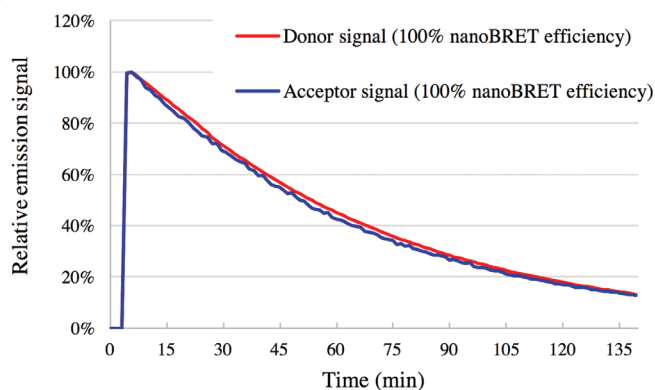

Figure 2. Representative kinetic decay data obtained for the acceptor and emission signals. Donor signal was measured using a 460/80BP filter and acceptor signal was measured with 610/LP filter in this example. Similar acceptor and donor kinetic decays are also detected at lower fractional occupancy values.

- Add a Dispense step into the kinetic loop

In this case, 50 µL of the NanoBRET Nano-Glo Substrate are dispensed at reading 1, using Medium-High speed

- Add a Luminescence measurement step using Filter Optics into the kinetic loop

Select the correct positions of the filter pairs that will be used for measuring NanoBRET; set up a measurement time of 1000 ms, and use Automatic Dynamic Range. Note: Changing the gain settings to increase the acceptor emission signal is not typically helpful in improving NanoBRET results (changing the gain will amplify both the signal and the background noise). However, in the case that the acceptor emission results being saturated, the gain can

be decreased to get non-saturated readings

- Add a Luminescence Spectrum step (optional)

Measure the acceptor and emission spectrum in wells with 0% fractional occupancy (no nanoBRET signal) and 100% fractional occupancy (maximal nanoBRET signal). Use a wavelength range of 400-700 nm (1 nm step) and a measurement time of 100 ms.

Data processing

For data processing, following steps are recommended:

- Calculate NanoBRET ratio values for each sample by dividing the acceptor emission values by the donor emission values (Acceptor Em / Donor Em)

In this assay, acceptor emission signals are measured at 600 or 610 nm, while the donor emission signal is measured at 460 nm

- Calculate the corrected NanoBRET ratio values by subtracting the NanoBRET ratios of the no-ligand control from the NanoBRET ratios of the samples

In current example, the no-ligand control corresponds to the sample with 0% of fractional occupancy (lacking the fluorescence HaloTag ligand)

- Perform a linear regression analysis of the corrected NanoBRET ratios's dependencies on the fractional occupancies and calculate the slope of the curve

A linear curve should be obtained if the NanoBRET signal is properly detected by the instrument

- Calculate the limits of quantitation (LOQ) and the limits of detection (LOD) using following equations.

Note: The no-ligand control (below) corresponds to the 0% fractional occupancy sample used in this panel (indicated in Promega kit instructions)

$$LOQ = 10 * \frac{SD\ (no-ligand\ control)}{slope}$$

$$LOD = 3 * \frac{SD\ (no-ligand\ control)}{slope}$$

- Calculate the screening window coefficient values (Z') to assess the assay performance at each fractional occupancy using the means and standard deviations of the NanoBRET ratios:

$$Z' = 1 - \frac{3\ SD\ (no-ligand\ control) +\ 3SD\ (ligand\ control)}{|Mean\ (ligand\ control) - Mean\ (no-\ ligand\ control)|}$$

Results and Discussion

The NanoBRET assay performed here utilizes the Promega Control Protein Panel. Because the covalently bound HaloTag ligands cannot be exchanged once attached, different ratios of the "occupied" and "unoccupied" fusion proteins are combined in this panel to create fractional occupancy. This is done by mixing fixed ratios of purified NanoLuc-HaloTag fusion proteins that has been pre-incubated with either the fluorescent HaloTag acceptor ligand or a non-fluorescent ligand. This strategy creates stable fractional occupancies mimicking different ligand-bound acceptor protein levels. To achieve 0% occupancy, 100% of the HaloTag fusion protein is bound with a non-fluorophore ligand, while 100% fractional occupancy is achieved by using 100% of the HaloTag fusion occupied with the fluorescent ligand. In all the samples, the total amount of NLuc protein fusion remains constant.

The tested dynamic range covered 3 orders of fractional occupancy magnitudes (0.1-100%). When using two different emission filters for collecting the acceptor signal and performing the assay in 96-well microplates, the NanoBRET signal detected by Varioskan LUX remains linear (Figure 3).

The LOQ and LOD values, parameters that serve as measures of instrument performance, are also very similar when collecting the acceptor signal with a BP filter of 600 or 610 nm (Table 1). An LOQ around 0.2% fractional occupancy in both cases indicates that a sample that contains as little as 0.2% donor-acceptor (BRET) pairs relative to the total donor population can be statistically distinguished from a sample that contains only donor molecules, using Varioskan LUX. NanoBRET is especially good as screening tool. From the results obtained here it can be also concluded that a screening assay optimized to be performed with 1% maximal nanoBRET efficiency produces excellent quality parameters (Z' > 0.8) regardless of the acceptor emission BP filter (600 or 610 nm) that is used for measuring with Varioskan LUX.

The assay also performs well when run in 384-well microplates and the LOQ measured in this case is only slightly increased to 0.35% (Figure 4). This makes the pairing of NanoBRET and Varioskan LUX particularly useful for assaying acceptor-donor pairs in large chemical inhibitor screens.

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A

$$y = 36.568x^{1.0234}$$
$$R^2 = 0.99976$$

B

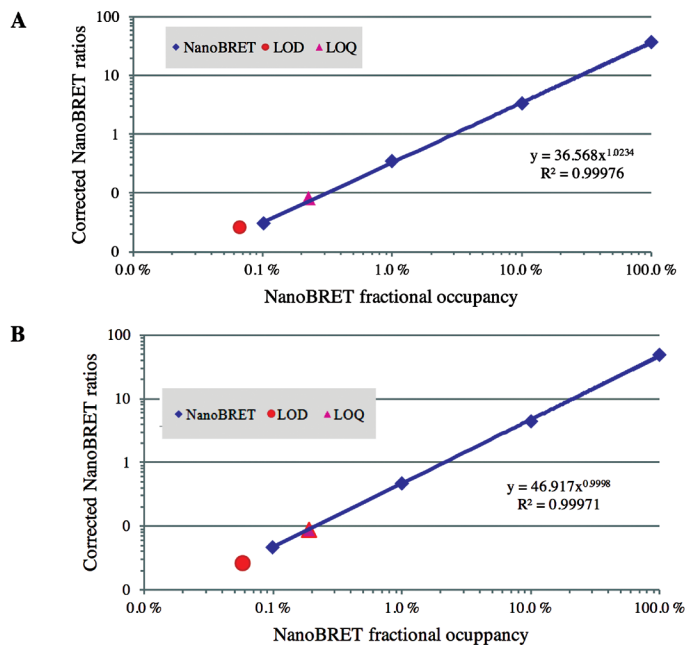$$y = 46.917x^{0.9998}$$
$$R^2 = 0.99971$$

Figure 3. Data obtained for the NanoBRET™ Control Protein Panel performed in 96-well microplates and measured with Varioskan LUX using two different acceptor emission filters (A: 610/LP and B: 600/LP). NanoBRET ratios were calculated after measuring donor and acceptor emission signals at different percentages of fractional occupancies. Measurement of the donor signal was done in both cases using a 460/80BP filter.
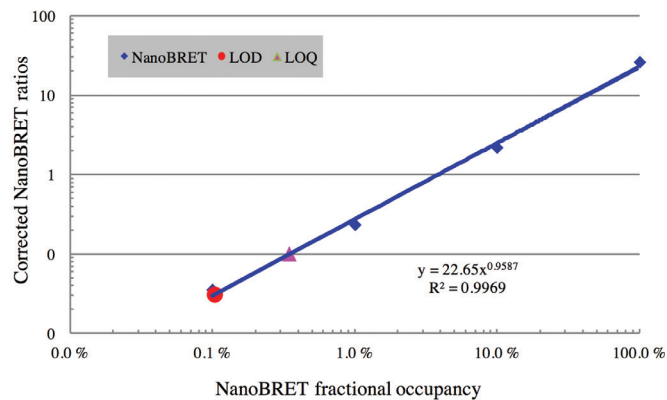
$$y = 22.65x^{0.9587}$$
$$R^2 = 0.9969$$

Figure 4. Data obtained for the NanoBRET™ Control Protein Panel performed in 384-well microplates and measured with Varioskan LUX. NanoBRET ratios were calculated after measuring donor and acceptor emission signals at different percentages of fractional occupancies. Measurement of the donor signal was done using a 460/80BP filter, while for the acceptor signal the selected filter in this case was the 610/LP.

Conclusions

Choosing filter optics for multiple luminometric detection in Varioskan LUX, allows the performance of NanoBRET, a technology developed by Promega that is particularly suited for the dynamic studies of PPI in live cells. We recommend measuring donor emission with a band pass (BP) filter centered at around 460 nm (emission 450nm/BP 80nm) and a long pass (LP) filter starting at either 600 or 610 nm (emission 600nm/LP or 610nm/LP) for acceptor emission.

Table 1. Statistical performance of the NanoBRET™ Control Protein Panel performed in 96-well microplates and measured with Varioskan LUX using two different acceptor emission filters

Donor emission: 460 nm HBW80 D25mm; Acceptor emission: 610 nm Long pass D25mm			
Fractional occupancy (%)			
	0.1	1	10	100
Z'	0.06	0.84	0.99	0.99
LOD	**0.07%**			
LOQ	**0.22%**			
Donor emission: 460 nm HBW80 D25mm; Acceptor emission: 600 nm Long pass D25mm			
Fractional occupancy (%)			
	0.1	1	10	100
Z'	0.04	0.86	0.97	0.99
LOD	**0.05%**			
LOQ	**0.19%**			

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