BMG Labtechnologies

BRET Assays Performed on the FLUOstar OPTIMA

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At a Glance...

- Sequential dual emission detection with very high sensitivity
- On-board (internal) reagent injectors for high throughput and fast kinetics
- A factor 40 difference between positive and negative controls

Introduction

Bioluminescence **R**esonance **E**nergy **T**ransfer (BRET) is a system of choice for monitoring intermolecular interactions *in vivo*. BRET is an advanced, non-destructive, cell-based assay technology that is perfectly suited for proteomics applications, including receptor research and the mapping of signal transduction pathways. The assay is based on non-radiative energy transfer between fusion proteins containing *Renilla* luciferase (Rluc) and e.g. Yellow Fluorescent Protein (YFP).[1,2] The BRET signal is generated by the oxidation of a coelenterazine derivative substrate.

For this application note the BRET^2™ demo kit has been used to prove the feasibility of performing a BRET assay on the FLUOstar OPTIMA microplate reader. The BRET^2 demo kit applies the cell-permeable and non-toxic coelenterazine derivative substrate DeepBlueC™ (DBC) and a mutant of the Green Fluorescent Protein (GFP^2) as acceptor. These compounds show improved spectral resolution and sensitivity over earlier variants.

Materials

All materials were obtained through normal distribution channels from the manufacturer stated.

- *FLUOstar OPTIMA, PN 413-101; BMG, Germany*
- *BRET filter set, PN 009-102; BMG, Germany*
- *Luminescence 384 top optic, optimized for 384 injection from top, PN 11-322; BMG Germany*
- *BRET^2™ demo kit, Cat.# 6310556; PE Life Sciences Inc., Canada*
- *White 384-well OptiPlate™, Cat.# 6007290; PE Life Sciences Inc., USA*

Experimental

A description for the development of BRET^2 protein-protein interaction assays is included with the demo kit. The following section focuses on the microplate reader settings recommended in the assay protocol:

BRET^2 Demo Kit reagents:

- *Non-transfected CHO cell extracts*
- *Negative control (Rluc + GFP^2 not fused together)*
- *Positive control (Rluc-GFP^2 fused together)*
- *DeepBlueC*
- *BRET^2 assay buffer*

FLUOstar OPTIMA settings:

Basic parameter settings for sequential dual luminescence well mode detection are listed below and shown in Fig.1:

- *No. of multichromatics:* *2*
- *1st emission filter:* *410-80 nm*
- *2nd emission filter:* *515-30 nm*
- *Gain for both PMTs:* *3800*
- *Measurement interval time:* *1 s*
- *No. of intervals:* *40*
- *Injection start time:* *10 s*
- *Pump speed:* *260 µL/s*
- *OPTIMA software version:* *1.20-0*

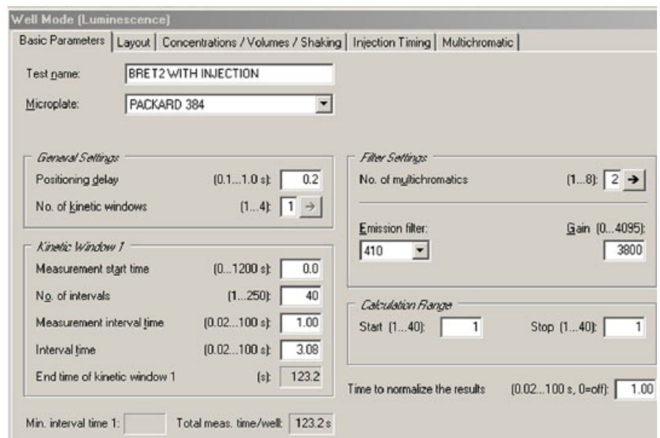

Fig.1: Test protocol with basic parameters

For faster reading time "No. of intervals" and "Injection start time" can be reduced and the emission filters should be placed in adjacent positions to speed filter switching.

Assay Protocol (white 384-well plate):

1. Addition of BRET^2 assay buffer:
   A10-D12: 15 µL of BRET^2 buffer

2. Addition of 10 µL of each cell extracts (Fig.2):
   A10-A12: Non-transfected cells (blank)
   B10-B12: Neg. BRET^2 control (Rluc + GFP^2)
   C10-C12: Pos. BRET^2 control (Rluc-GFP^2)
   D10-D12: BRET^2 assay buffer

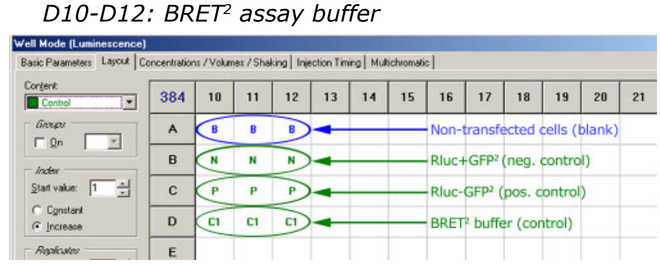

Fig.2: Layout for the BRET^2 demo kit assay

3. Automated injection of DBC and measurement:
Insert the prepared plate in the instrument and fill the injector with DBC solution. Enter for A10-D12 (B, N, P, C1) 25 µL in the "Concentrations/Volumes/Shaking" window for "Volume 1". Set the injection start time to 10 s and start the measurement.

A10-D12: Injection of 25 µL of DBC at 10 µM

On-board reagent injectors allow the measurement of high throughput assays and fast signal kinetics. The data from the measurement was evaluated using the FLUOstar OPTIMA Evaluation Software Package (1.20 or higher).

Results and Discussion

When the donor and acceptor are in close proximity the energy resulting from the catalytic degradation of the DBC is transferred from Rluc to GFP^2 which will then emit fluorescence at its characteristic wavelength.

The kinetic curves (raw data) of the negative control are shown in Fig.3 for both channels where the low values of the 515 nm channel indicate that no resonance energy transfer occured.

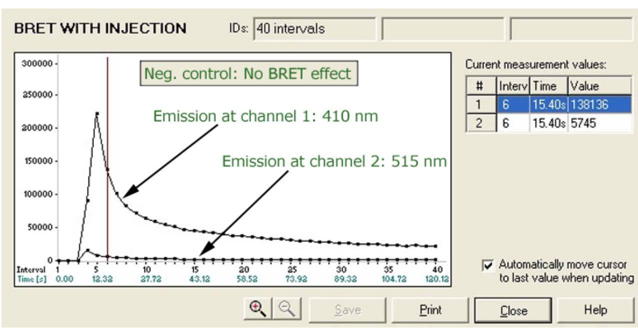

Fig.3: No BRET effect: Emission only at 410 nm

The positive control in Fig. 4 shows the raw data of both kinetic curves, where the values at 410 nm are reduced and the channel at 515 nm shows elevated values due to the BRET effect.

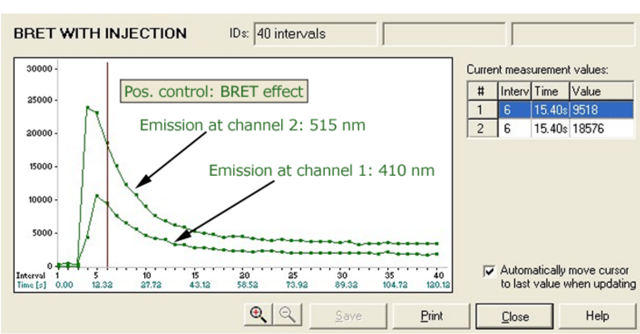

Fig.4: BRET effect: Additional emission at 515 nm

The BRET signal is a ratiometric measurement and indicates the occurrence of protein-protein interaction *in vivo*. This type of detection eliminates data variability caused by fluctuations in light output which can be found with variations in assay volume, cell types, number of cells per well and/or signal decay across the plate. In Fig.5 the blank corrected $BRET^2$ ratio for both, negative and positive control, are shown and were determined as:

$$BRET^2\ ratio = \frac{\text{(Emission at 515 nm - emission at 515 nm of non-transfected cells)}}{\text{(Emission at 410 nm - emission at 410 nm of non-transfected cells)}}$$

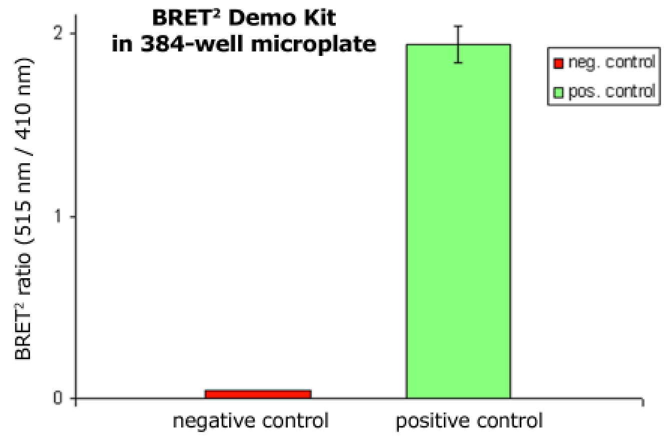

Fig.5: Ratio of negative and positive control

In the FLUOstar OPTIMA evaluation software the calculation corresponds to the quotient of „Raw data – blank" for channel 2 (515 nm) and channel 1 (410 nm) of an arbitrarily selected range of the emission curves. The signal for negative and positive control here reveals a value of around 0.05 and 1.9 respectively, which leads to a factor of around 40 and a clear discrimination between these controls. The high factor between these controls is caused by the artificial fusion construct of the positive control (Rluc-GFP^2) resulting in an extremely high BRET. Real assays samples will presumably result in lower ratios.
Nevertheless BRET assays show no photo-bleaching or photo-isomerization of the donor protein, or auto-fluorescence from cells or microplates which can be caused by incident excitation light and therefore has potential advantages over similar FRET assays. Furthermore the large spectral resolution between donor and emission peaks in $BRET^2$ (115 nm) greatly improves the signal to noise ratio over traditionally used BRET and FRET technologies that typically have only an ≈50 nm spectral resolution.[3]
In conclusion, ratiometrically quantifiable $BRET^2$ assays on the FLUOstar OPTIMA have been successfully demonstrated. The FLUOstar OPTIMA's internal reagent injectors for 384-well plate format allow higher plate throughput and measure faster kinetics than the vast majority of microplate readers available on the market.

References

1. Xu Y, Piston DW, Johnson CH. A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. Proc Natl Acad Sci USA 1999; **96**:151-6.

2. Angers S, Salahpour A, Joly E, Hilairet S, Chelsky D, Dennis M, Bouvier M. Detection of β_2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc Natl Acad Sci USA 2000; **97**:3684-9.

3. Mahajan NP, Linder K, Berry G, Gordon GW, Heim R, Herman B. Bcl-2 and Bax interactions in mitochondria probed with green fluorescent protein and fluorescence resonance energy transfer. Nat Biotechnol 1998; **16**:547-52.