

Enhancement of BRET² Assay Robustness and Handling Utilizing the PerkinElmer EnVision Plate Reader

EnVision Multimode Plate Reader

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Introduction

Robust assay performance is a vital requirement for screening campaigns. Assay performance measures such as signal-to-background ratio (S/B) and Z' factor are commonly used to compare the reliability of an assay on an experiment-to-experiment and day-to-day basis [Iversen et al., 2006]. These measures are dependent on many factors and can easily be affected by a change in assay conditions. Especially when working with biological samples as in cell-based assays, assay robustness is frequently affected if the cell line is changed or if the biochemistry is modified. In these cases, it is particularly important that the measurement device provides high-quality and trustworthy data.

In this technical note, we show how a well-known cell-based BRET² assay was transferred from a university lab to a screening facility to be utilized in a screen for Hepatitis C virus inhibitors. The assay had been performed on a number of readers before transfer. The screening facility uses the PerkinElmer EnVision® Multilabel Plate Reader. Use of the EnVision reader significantly improved the assay robustness and reliability, making it an ideal device for screening applications.

Background

Resonance energy transfer (RET) techniques apply a naturally occurring phenomenon first described by Theodor Förster, where the energy derived from a bioluminescent reaction caused by a luciferase (BRET) or a fluorophore (FRET) can be used to excite a fluorescent energy acceptor if they are in close proximity [Förster, 1948].

For the assay described here, cells are transfected with genetically encoded fusion proteins that express two interacting proteins (P), one fused to RLuc (Renilla luciferase) and the other to GFP² (mutant of Green Fluorescent Protein). If both partners stably interact, RLuc and GFP² are in close vicinity and BRET² can be measured as a function of GFP² emission. This interaction can be disturbed by ligands leading to dissociation of the complex or a conformational change and a subsequent decrease or total absence of GFP² emission (Figure 1). Fusion proteins containing non-interacting proteins, constructs that lead to a higher distance than 10 nm or RLuc alone are frequently used as negative controls.

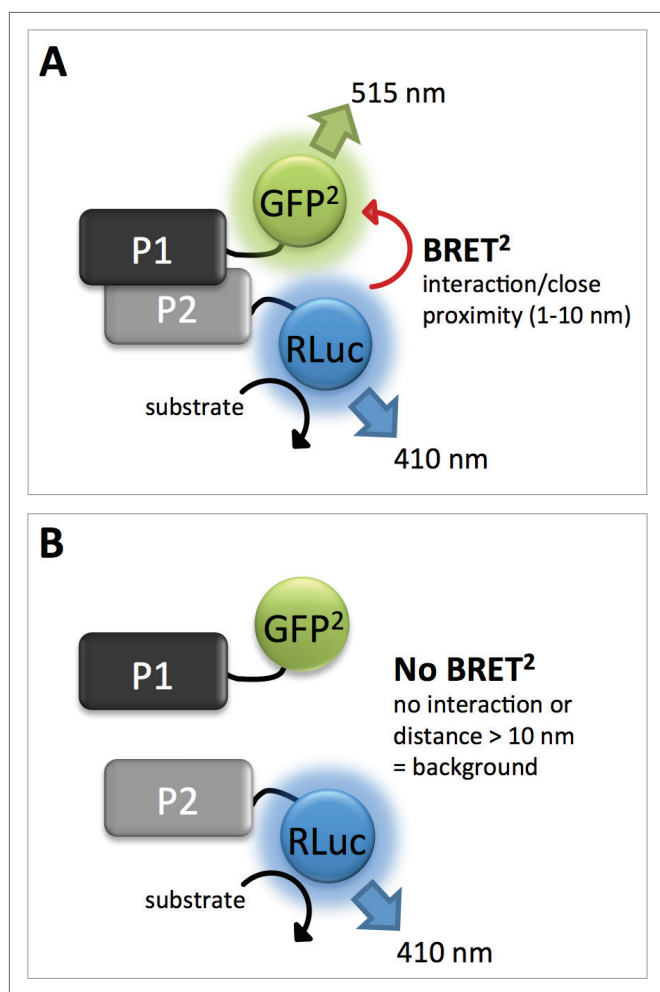


Figure 1. Schematic illustration of a BRET² sensor pair consisting of interacting protein 1 and 2 (P1 and P2). A: RLuc and GFP² are fused to the interacting proteins, respectively. After addition of the luciferase substrate Coelenterazine 400a, BRET² occurs between the energy donor (RLuc) and the energy acceptor (GFP²) provided that the two reporters are kept in close proximity (1–10 nm) by the interaction. B: Disruption of the interaction by either a ligand, no interaction or if the distance between the interacting proteins is bigger than 10 nm results in a decrease in BRET² signal or no BRET² signal (non-interacting proteins can be used as background/negative/low control).

“The EnVision is a great and trustworthy instrument.

I would love to have used it at university, because it made my assay so much more reliable and easier in handling, and further optimization steps were unnecessary.”

Mandy Diskar

The fusion proteins used here are two BRET² pairs: the first consists of the regulatory and catalytic subunit of the cAMP-dependent protein kinase A type I alpha (PKA-I) and serves as an internal assay control. PKA is a Ser/Thr kinase, regulated by cyclic AMP (cAMP) levels. It is involved in biological processes such as cell growth and division, cell differentiation, as well as metabolism and immune responsiveness [reviewed in Tasken and Aandahl, 2004]. Increasing cAMP leads to dissociation of the PKA holoenzyme complex, activation of the catalytic subunit and the loss of BRET² signal. This sensor has been extensively used for structure-function and cAMP analog studies [Wojtal et al., 2009; Diskar et al., 2010].

The second BRET² pair is based on the discovery that PKA plays an essential role in the assembly of infectious hepatitis C virus (HCV) particles [Farquhar et al., 2008]. Recent findings have shown that HCV Core, the central component of the virus particle, physically interacts with the regulatory subunit of PKA [Diskar et al., manuscript in preparation]. To reconstitute this protein-protein interaction in cells and to set-up a high-throughput assay to identify small molecules that disrupt the complex, BRET² pair 2 was engineered and optimized (see Material and Methods). Substances that disrupt the interaction might be exploitable as anti-viral agents in the future.

Materials and Methods

BRET² Pairs

The following two BRET² pairs were used for the experiments in a variety of cell types:

- BRET² pair 1: R α -RLuc/RLuc8 and GFP²-C α = human PKA type I holoenzyme or PKA-I α (both human full length proteins)
- BRET² pair 2: human regulatory subunit R of PKA (R-GFP²), and the HCV Core protein (RLuc8-HCV Core)

As BRET² pair 1 is a well characterized construct it was used as an assay development control tool. It is described in more detail in Prinz et al., 2006 and Chepurny et al., 2013. BRET² pair 2 was used for the screening campaign.

Cell Preparation for BRET² Measurements

Huh7.5 and Cos-7 cells were cultured in phenol red-free DMEM (Biochrom) supplemented with 10% FCS (Sigma) and 1% penicillin and streptomycin (Sigma) at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were passaged routinely twice a week. BRET² experiments were performed as described in citations above. In brief, 1.5 x 10⁴ cells per well were seeded in white 96-well microplates and transfected the following day with

0.1 µg of plasmids encoding BRET² pair 1 or 2. The transfection of the RLuc construct without its GFP²-tagged interaction partner served as low control. 48 hours post-transfection and prior to BRET² measurement, cells were washed with D-PBS (Lonza) and the luciferase substrate Coelenterazine 400a (Biotrend) was added at a final concentration of 5 µM in a total volume of 30 µl PBS per well. For DMSO induction experiments, 48 h post-transfection, instead of a BRET² read-out, the cells were treated for another 7d with 1.5% DMSO (Hybri-Max™, Sigma) in DMEM (medium was exchanged twice during that time) and BRET² was assessed as described above.

Instrument Settings

Emissions at 410 nm (±40 nm bandpass; RLuc/RLuc8) and 515 nm (±15 nm bandpass; GFP²) were detected using a PerkinElmer EnVision HTS Multilabel Plate Reader (for details, see Table 1) or using other readers as indicated, either sequentially or simultaneously, depending on the reader's ability. Relative Luminescence (BRET² ratio) was calculated automatically for all samples.

Table 1. Instrument settings for Dual Detector Measurement on EnVision reader. Measurement height was set to 6.5 mm and measurement time to 0.5 s.

	Mirror module	Emission Filter 1 (Acceptor)	Emission Filter 2 (Donor)
Name	Optical Module BRET ² Dual Emission (BRET ² Enhancer)	BRET ² 515	BRET ² 410
Description	BRET ² -/D475 dual mirror	M515 CWL = 515 nm	M410 CWL = 410 nm
Barcode	#659	#219	#213

Data Analysis

Data was analyzed with EnVision Manager 1.12 (PerkinElmer). Primary readouts were luminescence signals at 410 nm (RLuc) and 515 nm (GFP²) for all samples. The relative luminescence (BRET² ratio) was calculated according to:

$$\text{BRET}^2 \text{ ratio} = \frac{\text{Emission}_{515 \text{ nm}}}{\text{Emission}_{410 \text{ nm}}}$$

High and low controls were included in each experiment. High controls consisted of cells expressing the corresponding BRET² pair. Low controls were composed of cells expressing the luciferase alone. Signal-to-background ratio (S/B) was determined by:

$$S/B = \frac{\text{Avg}_{\text{high}}}{\text{Avg}_{\text{low}}}$$

Z' factor was calculated as described in Iversen et al., 2006 using the following equation:

$$Z' = 1 - \frac{3 \cdot (\text{Std}_{\text{high}} + \text{Std}_{\text{low}})}{\text{Avg}_{\text{high}} - \text{Avg}_{\text{low}}}$$

with Avg being the mean and Std the standard deviation of the BRET² ratio for high or low control. A Z' of 0.5 is commonly used as a threshold above which an assay qualifies for screening.

Experiments were repeated at least three times with 6-12 replicate wells per experimental condition. Statistical evaluation (One-way ANOVA followed by a Newman-Keuls or Dunnett's Post Test) was carried out using Graph Pad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

Assay Reliability and Robustness are Dependent on Cell Line, Luciferase and the Measurement Device

Changing BRET² assay conditions by using different cell lines, modified luciferases, alternative plate types, or transferring the assay to different measurement devices can have a considerable effect on assay performance. Figure 2 summarizes BRET² ratios for the extensively studied BRET² pair 1 and the luciferase alone as low control either expressed in Huh7.5 or in Cos-7 cells. Two different luciferases, RLuc or RLuc8 [Loening et al., 2006], were used as indicated and the assay was measured on different plate readers from PerkinElmer and other vendors.

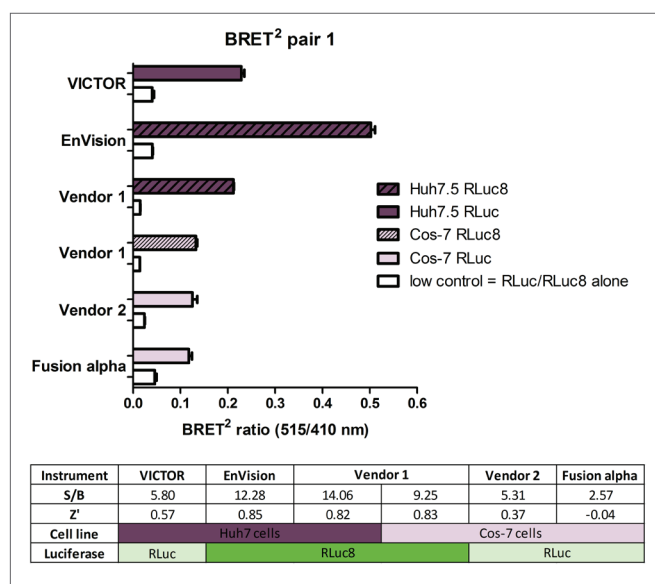


Figure 2. Comparison of BRET² ratio of BRET² pair 1 on different PerkinElmer and alternative instruments, in different cell types (Huh7.5 and Cos-7 cells) and using different luciferases (RLuc and RLuc8). The assay reliability and robustness is demonstrated by the S/B ratio and Z'. The diverse assay conditions are indicated as colored bars for the cell line and luciferase that was used.

Since a variety of parameters were changed between experiments, not all results can be compared with each other. Nevertheless, some general observations can be made based on Figure 2. Expression of BRET² pair 1 leads to lower BRET² ratios in Cos-7 than in Huh7.5 cells independent of the instrument that was used. A striking and surprising effect observed when using the EnVision system is that the BRET² ratios are more than two times higher than for the vendor 1 instrument. The table in Figure 2 shows that the expression of BRET² pair 1 in Cos-7 cells also leads to lower S/B ratios than in Huh7.5 cells, which were compared directly on the vendor 1 instrument using the luciferase RLuc8. Both cell lines provide very good Z' values of more than 0.8 on the same device. Using the EnVision reader results in a similar S/B ratio and an even higher Z' value of 0.85 for Huh7.5 and RLuc8 compared to the vendor 1 instrument.

In contrast, using the VICTOR™ Multilabel Plate Reader, the vendor 2 instrument and the Fusion™ Universal Microplate Analyzer (Fusion alpha) the assay quality is reduced with S/B < 6 and Z' values of 0.57 at maximum for the VICTOR reader. This is caused by the instrument performance and by the luciferase RLuc, which emits smaller amounts of light than RLuc8 and increases the assay variability (data not shown).

These results verified that the EnVision reader enables excellent assay robustness needed for screening campaigns and was therefore further utilized for optimization of the screening assay applying BRET² pair 2.

High Signal-to-Background Ratio and Excellent Assay Performance of EnVision Simplifies Cell Handling

The initial assay optimization for BRET² pair 2, in the university laboratory, used a multilabel reader from vendor 1. At this time, the principal measure of assay performance was the signal-to-background ratio of the relative luminescence. As the S/B ratio for non-induced Huh7.5 cells was low (S/B = 2.5) on the vendor 1 instrument, a long-term DMSO treatment to differentiate the hepatoma cells had been necessary to achieve an S/B signal increase up to 4.5 (Figure 3, A). DMSO is known to differentiate a multitude of diverse cell types [Morley & Whitfield, 1993; Pal et al., 2012]. This laborious differentiation step was not needed when using the PerkinElmer EnVision Multilabel Plate Reader (Figure 3, B). With the EnVision system, the same assay resulted in S/B of approx. 5 for non-induced cells and an excellent S/B of approx. 13 for induced cells. Moreover, the EnVision fulfilled the requirements of a High-Throughput-Screening (HTS) assay in regard to Z' factor. It provided good assay performance with Z' values of more than

0.8 for induced and excellent Z' values of 0.9 for non-induced cells compared to the instrument from vendor 1 (Figure 3, Table). Performing the assay with the EnVision reader without the induction of cells decreased the cell preparation effort dramatically, resulting in significant time and cost-savings for screening campaigns.

To further optimize the assay for the screening campaign, a competition assay, commonly used as a BRET² control experiment, was performed. It utilizes an unlabeled binding partner (here R-HA) which is titrated in competition with the GFP²-labeled binding partner. Figure 4B shows that the binding between the interacting proteins of BRET² pair 2 can be disrupted, leading to a decline in the BRET² signal with increasing R-HA concentrations. This confirms a specific binding event between the binding partners of BRET² pair 2. The low variability and high S/B ratios provided by the EnVision system enabled reliable data for this titration assay even in non-induced cells. Based on these convincing and time-saving pre-screening tests, the EnVision reader was successfully used as the reader of choice in the screening campaign.

Discussion

Transferring even a well-characterized assay from one reader to another can be challenging and may have surprising effects. Here we have shown that the EnVision reader provides very high BRET² ratios and superior S/B ratios compared to other readers. This was surprising at first: in dual detector measurements on the EnVision instrument using the dedicated BRET² filter set, the GFP² channel is optimized for low intensity signals. Hence, the detector is more sensitive for GFP², leading to higher BRET² ratios and S/B ratios, in contrast to the alternative readers tested.

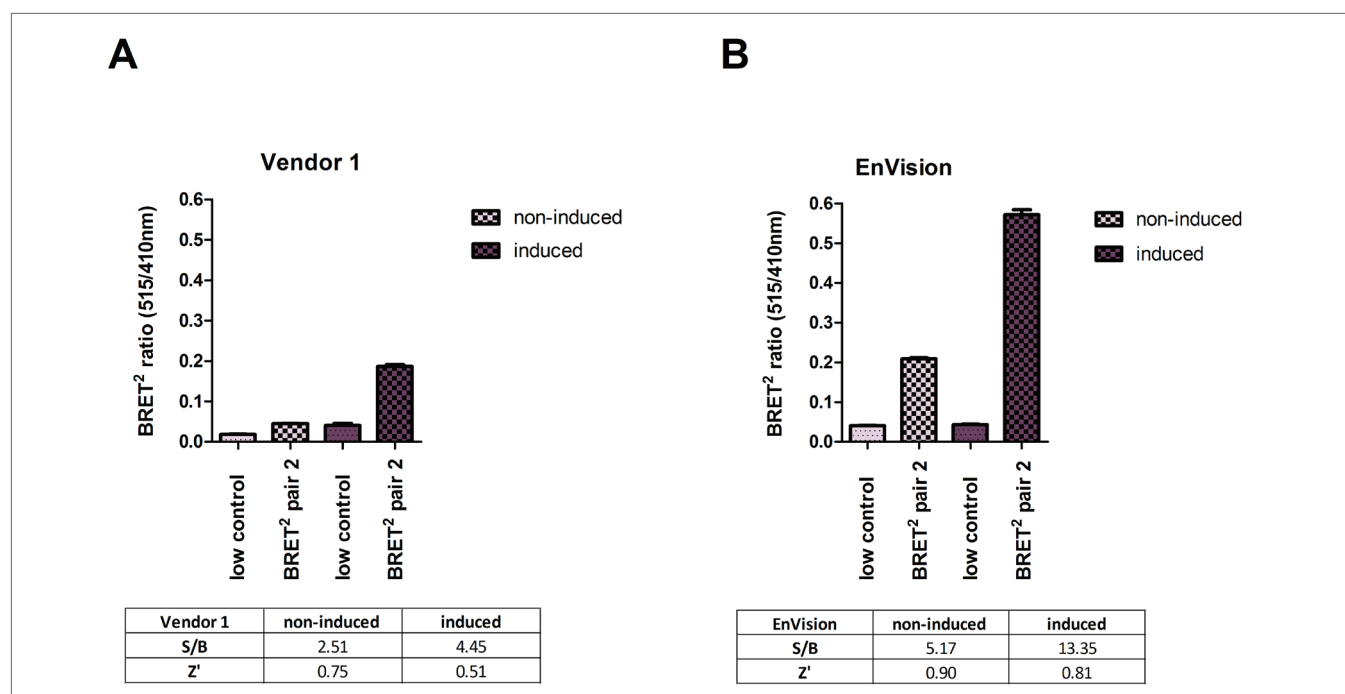


Figure 3. Comparison of induced vs. non-induced Huh7.5 cells transfected with BRET² pair 2 measured on two different instruments. A: To increase the S/B ratio on the vendor 1 instrument the cells had to be differentiated with DMSO for 7 days. B: In contrast, the EnVision reader gives similar S/B ratios even without induction of the cells and results in excellent assay performance of Z' = 0.9.

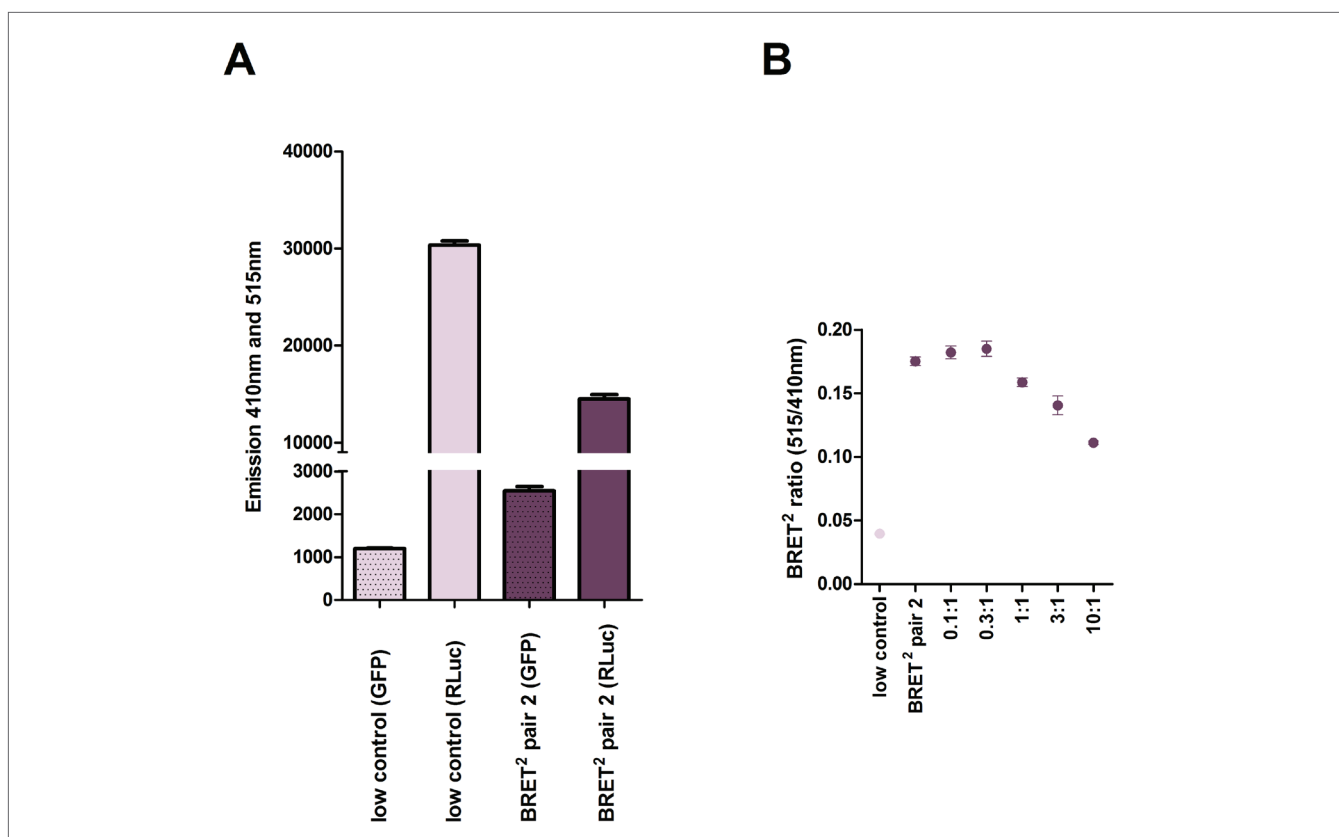


Figure 4. Competition assay performed on the EnVision instrument by titrating an unlabeled binding competitor for BRET² pair 2 in non-induced cells. A: EnVision raw values for each emission channel, GFP and RLuc, are shown for low control (RLuc8 only) and BRET² pair 2. B: BRET² ratios resulting from the raw values in A for low control, BRET² pair 2 and a titration of the binding competitor (R-HA).

This was particularly advantageous for the assay using BRET² pair 2 because no further optimization of the laborious cell differentiation step was needed.

High BRET² and S/B ratios generally indicate an assay that has been set-up well. These values are sufficient for many assays performed at low throughput, such as in academic labs. However, if the assay is to be used in screening campaigns, it is also important that the assay shows high robustness, reflected by the Z'. In contrast to the S/B ratio, the Z' also takes into account the variability of the replicates and serves as a common quality control parameter in HTS. The EnVision reader provides excellent results for the BRET² assays performed here, with Z' of at least 0.8 for all conditions tested. With a Z' of 0.9 for non-induced cells, the assay clearly fulfills the requirements of a reliable screening assay even without cell induction. Compared to all other readers tested here, the EnVision system offers the most reliable data and allows for a simplified assay workflow, leading to a cost and time-saving advantage in a screening environment.

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