Enhancement of BRET² Assay Robustness and Handling Utilizing the PerkinElmer EnVision 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.

For the Better

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 GFP^2 (mutant of Green Fluorescent Protein). If both partners stably interact, RLuc and GFP^2 are in close vicinity and BRET^2 can be measured as a function of GFP^2 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 GFP^2 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.
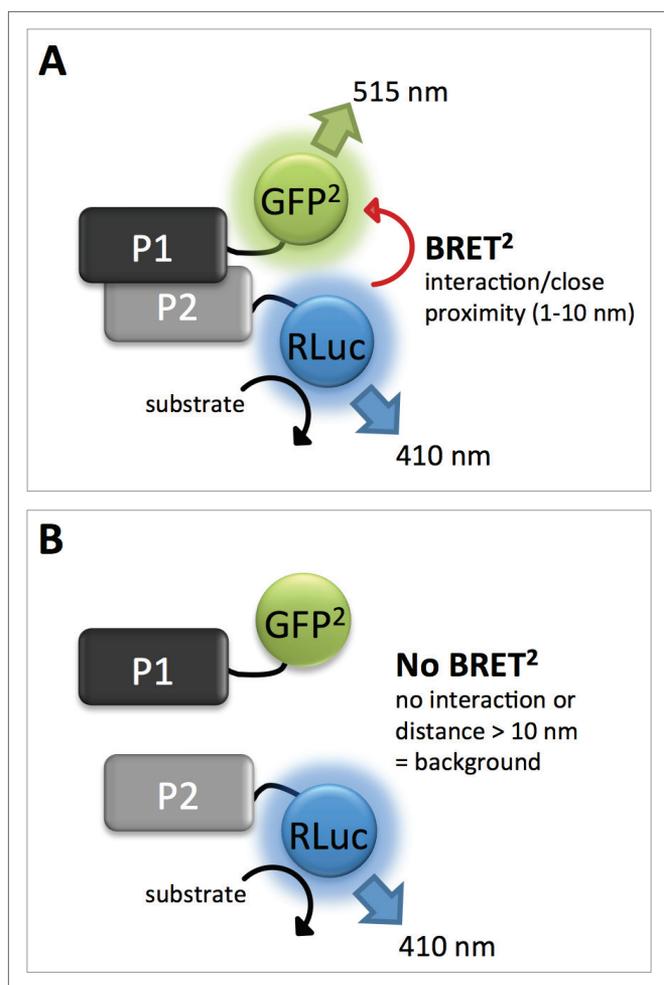

Figure 1. Schematic illustration of a BRET^2 sensor pair consisting of interacting protein 1 and 2 (P1 and P2). A: RLuc and GFP^2 are fused to the interacting proteins, respectively. After addition of the luciferase substrate Coelenterazine 400a, BRET^2 occurs between the energy donor (RLuc) and the energy acceptor (GFP^2) 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 BRET^2 signal or no BRET^2 signal (non-interacting proteins can be used as background/ negative/low control).

The fusion proteins used here are two BRET^2 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 BRET^2 signal. This sensor has been extensively used for structure-function and cAMP analog studies [Wojtal et al., 2009; Diskar et al., 2010].

The second BRET^2 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, BRET^2 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

BRET^2 Pairs

The following two BRET^2 pairs were used for the experiments in a variety of cell types:

- BRET^2 pair 1: RIα-RLuc/RLuc8 and GFP^2-Cα = human PKA type I holoenzyme or PKA-Iα (both human full length proteins)

- BRET^2 pair 2: human regulatory subunit R of PKA (R-GFP^2), and the HCV Core protein (RLuc8-HCV Core)

As BRET^2 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. BRET^2 pair 2 was used for the screening campaign.

Cell Preparation for BRET^2 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% CO_2. Cells were passaged routinely twice a week. BRET^2 experiments were performed as described in citations above. In brief, 1.5×10^4 cells per well were seeded in white 96-well microplates and transfected the following day with

0.1 μg of plasmids encoding BRET^2 pair 1 or 2. The transfection of the RLuc construct without its GFP^2-tagged interaction partner served as low control. 48 hours post-transfection and prior to BRET^2 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 BRET^2 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 BRET^2 was assessed as described above.

Instrument Settings

Emissions at 410 nm (±40 nm bandpass; RLuc/RLuc8) and 515 nm (±15 nm bandpass; GFP^2) 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 (BRET^2 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 (GFP^2) for all samples. The relative luminescence (BRET^2 ratio) was calculated according to:

$$\text{BRET}^2 \text{ ratio} = \frac{Emission_{515\,nm}}{Emission_{410\,nm}}$$

High and low controls were included in each experiment. High controls consisted of cells expressing the corresponding BRET^2 pair. Low controls were composed of cells expressing the luciferase alone. Signal-to-background ratio (S/B) was determined by:

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

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

$$Z' = 1 - \frac{3 \cdot (Std_{high} + Std_{low})}{Avg_{high} - Avg_{low}}$$

with *Avg* being the mean and *Std* the standard deviation of the BRET^2 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 BRET^2 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 BRET^2 ratios for the extensively studied BRET^2 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.
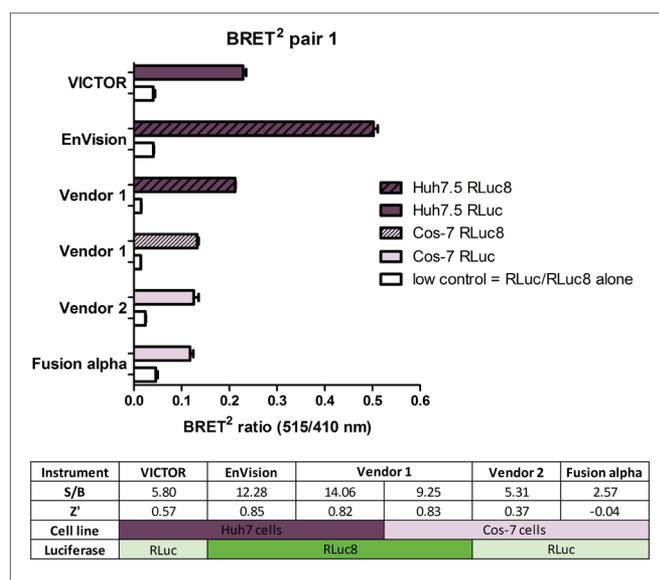

Instrument	VICTOR	EnVision	Vendor 1		Vendor 2	Fusion alpha
S/B	5.80	12.28	14.06	9.25	5.31	2.57
Z'	0.57	0.85	0.82	0.83	0.37	-0.04
Cell line	Huh7 cells			Cos-7 cells		
Luciferase	RLuc	RLuc8			RLuc	

Figure 2. Comparison of BRET^2 ratio of BRET^2 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 BRET^2 pair 1 leads to lower BRET^2 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 BRET^2 ratios are more than two times higher than for the vendor 1 instrument. The table in Figure 2 shows that the expression of BRET^2 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.
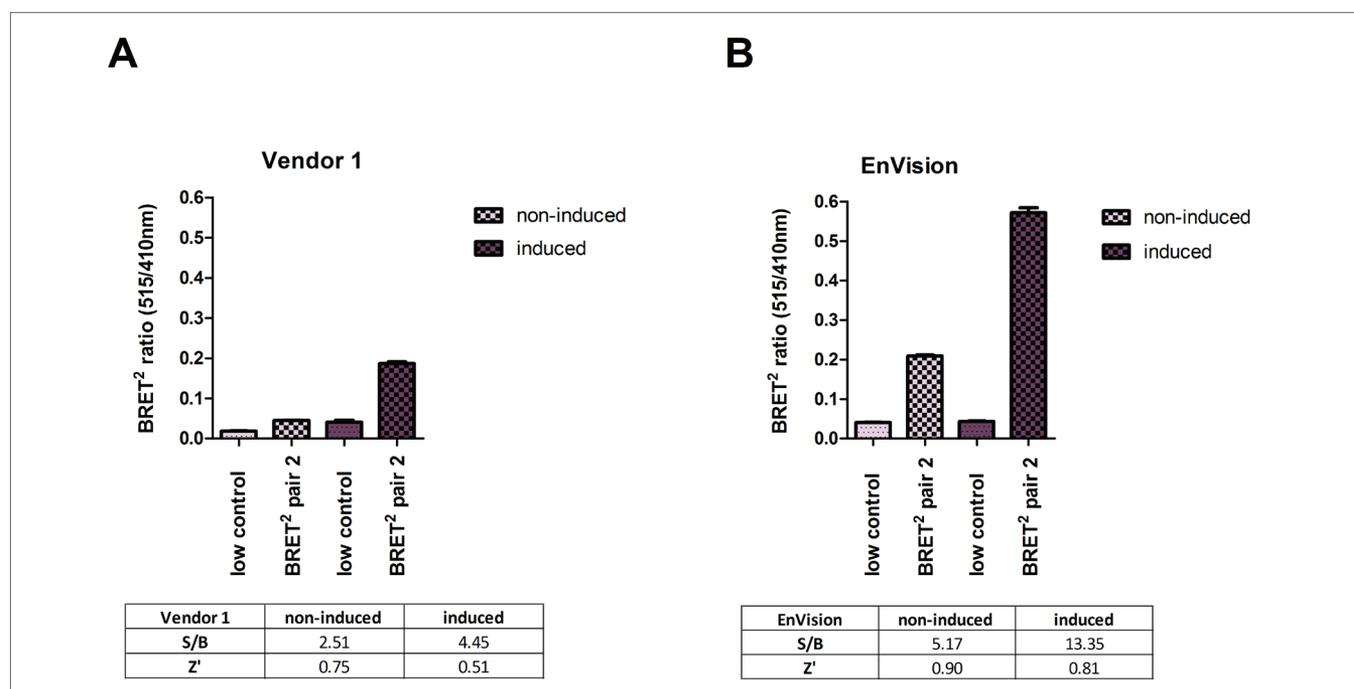

Vendor 1	non-induced	induced
S/B	2.51	4.45
Z'	0.75	0.51

EnVision	non-induced	induced
S/B	5.17	13.35
Z'	0.90	0.81

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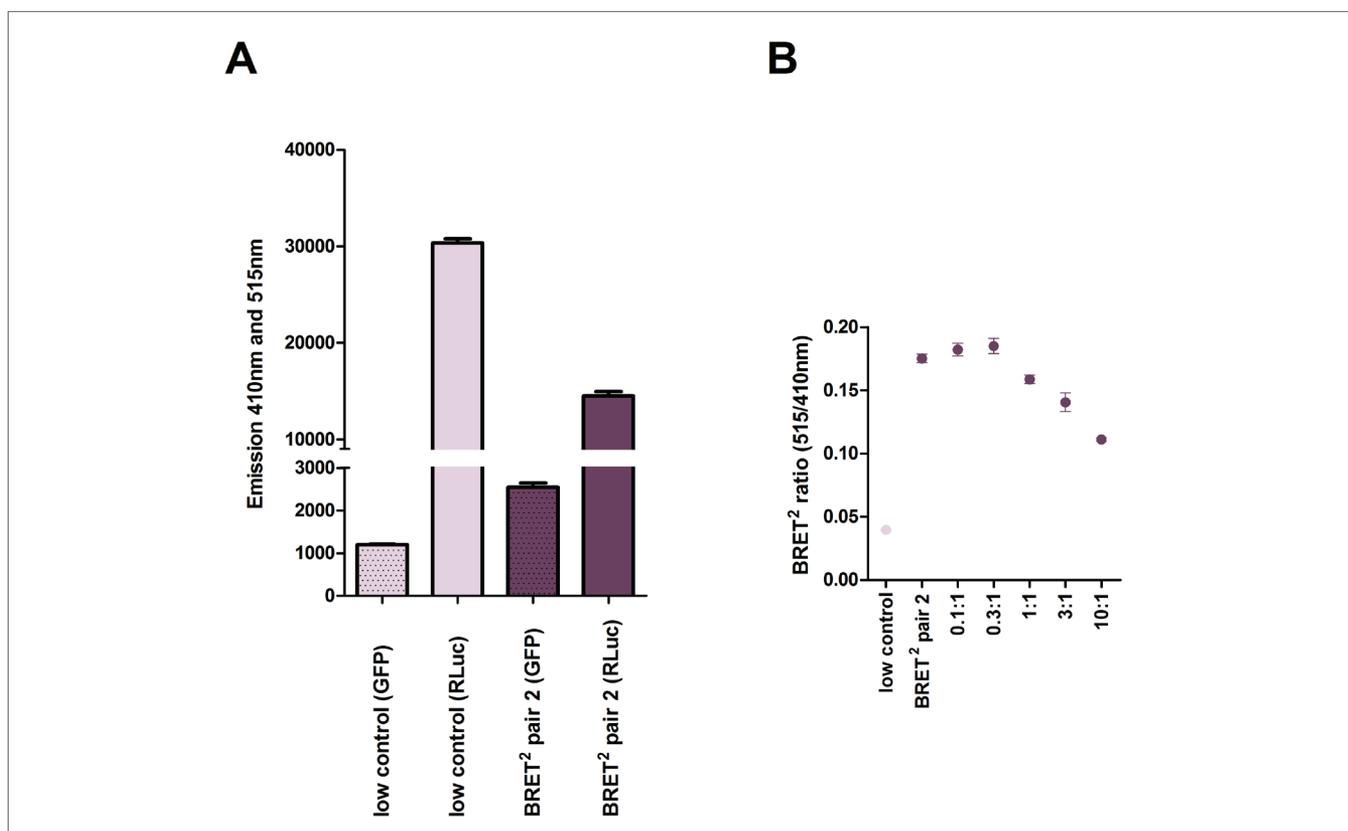
Figure 4. Competition assay performed on the EnVision instrument by titrating an unlabeled binding competitor for BRET^2 pair 2 in non-induced cells. A: EnVision raw values for each emission channel, GFP^2 and RLuc, are shown for low control (RLuc8 only) and BRET^2 pair 2. B: BRET^2 ratios resulting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

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

High BRET^2 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 BRET^2 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.

References

Chepurny, O. G., Bertinetti, D., Diskar, M., Leech, C. A., Afshari, P., Tsalkova, T., & Holz, G. G. (2013). Stimulation of proglucagon gene expression by human GPR119 in enteroendocrine L-cell line GLUTag. Molecular Endocrinology, 27(8), 1267-1282.

Diskar, M., Lone, A.M., Farquhar, M.J., Meredith. L.W., Hu, K., Lissauer, S., Wilson, G.K., Balfe, P., Taskén, K., & McKeating, J.A. Protein Kinase A (PKA) defines HCV particle infectivity: HCV Core interacts with the regulatory subunit of PKA, manuscript in preparation.

Diskar, M., Zenn, H. M., Kaupisch, A., Kaufholz, M., Brockmeyer, S., Sohmen, D., Berrera, M., Zaccolo, M., Boshart, M., Herberg, F.W. & Prinz, A. (2010). Regulation of cAMP-dependent Protein Kinases: the human protein kinase x (PrKX) reveals the role of the catalytic subunit aH-aI loop. Journal of Biological Chemistry, 285(46), 35910-35918.

Farquhar, M. J., Harris, H. J., Diskar, M., Jones, S., Mee, C. J., Nielsen, S. U., Brimacombe, C. L., Molina, S., Toms, G. L., Maurel, P., Howl, J., Herberg, F. W., van Ijzendoorn, S. C. D., Balfe, P. & McKeating, J. A. (2008). Protein kinase A-dependent step(s) in hepatitis C virus entry and infectivity. Journal of Virology, 82(17), 8797-8811.

Förster, T. (1948). Zwischenmolekulare Energiewanderung und Fluoreszenz. Annalen der Physik, 437(1-2), 55-75.

Iversen, P. W., Eastwood, B. J., Sittampalam, G. S., & Cox, K. L. (2006). A comparison of assay performance measures in screening assays: signal window, Z' factor, and assay variability ratio. Journal of Biomolecular Screening, 11(3), 247-252.

Loening, A. M., Fenn, T. D., Wu, A. M., & Gambhir, S. S. (2006). Consensus guided mutagenesis of Renilla luciferase yields enhanced stability and light output. Protein Engineering Design and Selection, 19(9), 391-400.

Morley, P., & Whitfield, J. F. (1993). The differentiation inducer, dimethyl sulfoxide, transiently increases the intracellular calcium ion concentration in various cell types. Journal of Cellular Physiology, 156(2), 219-225.

Pal, R., Mamidi, M. K., Das, A. K., & Bhonde, R. (2012). Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells. Archives of Toxicology, 86(4), 651-661.

Prinz, A., Diskar, M., Erlbruch, A., & Herberg, F. W. (2006). Novel, isotype-specific sensors for protein kinase A subunit interaction based on bioluminescence resonance energy transfer (BRET). Cellular Signalling, 18(10), 1616-1625.

Tasken, K., & Aandahl, E. M. (2004). Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiological Reviews, 84(1), 137-167.

Wojtal, K. A., Diskar, M., Herberg, F. W., Hoekstra, D., & van IJzendoorn, S. C. (2009). Regulatory subunit I-controlled protein kinase A activity is required for apical bile canalicular lumen development in hepatocytes. Journal of Biological Chemistry, 284(31), 20773-20780.

Acknowledgement

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