



Implementation of NanoBRET™

Assay Technology in the

Spark® multimode reader.

Application Note

INTRACELLULAR INTERACTION STUDIES USING MULTICOLOR LUMINESCENCE



INTRODUCTION

Dynamic interactions between proteins are key mediators of multiple cellular functions and are involved in almost all biological processes. Accordingly, they are valuable targets for the development of novel drug therapies. In this context, bioluminescence resonance energy transfer (BRET), developed by Promega, is a well-established detection technology for the investigation of protein-protein interactions in living cells. However, its application has so far been somewhat hampered by its limited dynamic range and sensitivity.

With the recent employment of the new, extremely bright NanoLuc[®] luciferase and its combination with a means for tagging intracellular proteins with a long-wavelength fluorophore Promega has taken the BRET technology to the next level: NanoBRET[™].

Because of its small size (19 kDa), high emission intensity, and relatively narrow spectrum (460 nm peak intensity) NanoLuc[®] Luciferase is ideally suited as an energy donor. A highly efficient red-emitting acceptor fluorophore (635 nm peak intensity) enables an overall spectral separation of more than 175 nm for BRET (see figure 1). This combination of greater light intensity with improved spectral resolution results in markedly improved detection sensitivity and a higher dynamic range compared to other BRET assay technologies [1].

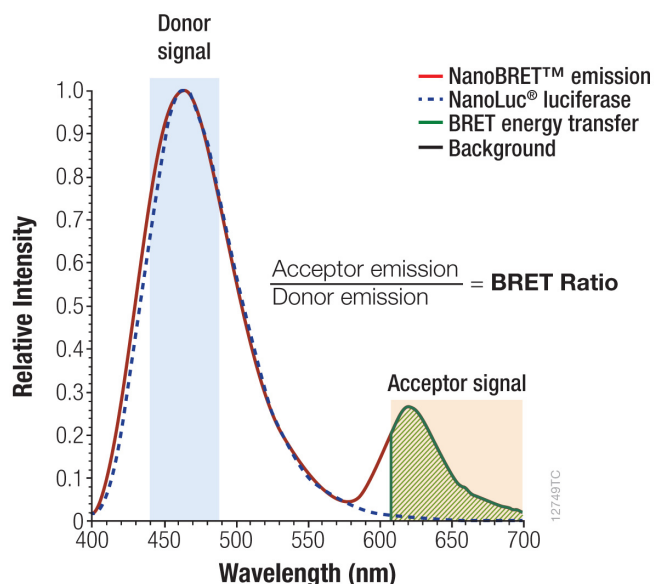


Figure 1: Spectral characteristics of the NanoBRET assay technology

The NanoBRET[™] Target Engagement Intracellular HDAC Assay measures compound binding at select target HDAC proteins within intact cells by analyzing the affinity of test compounds by competitive displacement of a NanoBRET[™] tracer reversibly bound to a NanoLuc[®]

fusion protein in cells. In the first step of the assay, a fixed concentration of tracer is added to cells expressing the desired NanoLuc[®] fusion protein. Introduction of competing compounds results in a dose-dependent decrease in NanoBRET[™] energy transfer for estimating intracellular affinity at the target protein. The NanoBRET[™] TE Intracellular HDAC Assay assesses compound binding to a histone deacetylase (HDAC)-NanoLuc luciferase fusion protein [2]. In this application note, the use of the assay for evaluating compound binding to HDAC6, a histone deacetylase enzyme associated with cancer cell metastasis and tumorigenesis, was demonstrated on the Spark multimode reader.

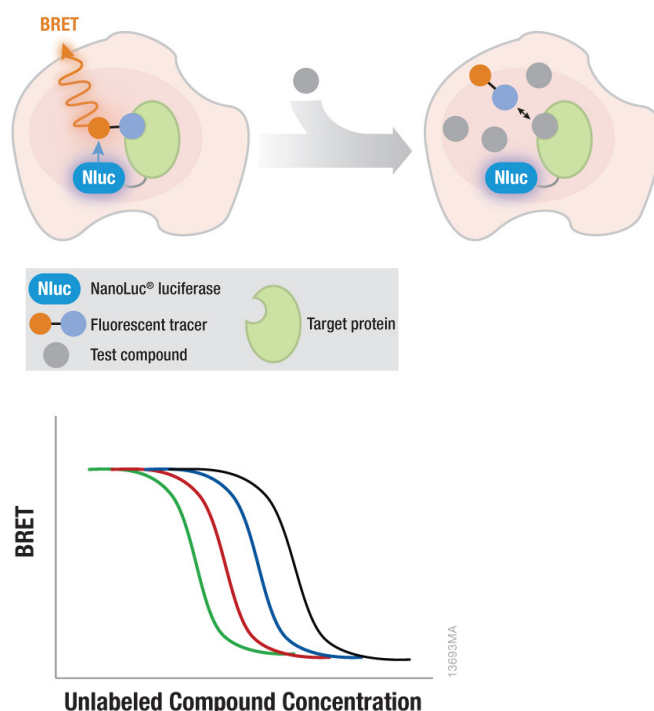


Figure 2: NanoBRET[™] TE Intracellular HDAC Assay principle

The Spark microplate reader, Tecan's multimodal detection platform, provides exceptional performance for luminescence-based applications, including flash and glow luminescence and multicolor luminescence and luminescence scanning [3,4]. A dedicated photomultiplier tube (PMT) allows for single photon counting, eliminating the conflict between sensitivity and dynamic range commonly associated with instruments that use a single PMT for fluorescence and luminescence measurements. This unique optical combination results in superior sensitivity rivaling that of a stand-alone luminometer. The reader is equipped with optimized luminescence filters for NanoBRET applications.

This application note summarizes the results of test series performed with the Spark reader and the NanoBRET assays technology, using the NanoBRET™ Target Engagement Intracellular HDAC Assay.

MATERIALS AND METHODS

- HEK293 cells stably expressing HDAC6-NanoLuc® fusion protein
- NanoBRET™ TE Intracellular HDAC Assay (Promega, Cat# N2080)
 - includes NanoBRET™ Nano-Glo® Substrate, Tracer Dilution Buffer, NanoBRET™ Intracellular TE HDAC Tracer, and Extracellular NanoLuc® Inhibitor
- Opti-MEM® I Reduced Serum Medium, no phenol red (Fisher, cat# 11058021)
- SAHA (suberoylanilide hydroxamic acid)/Vorinostat (Sigma, cat# SML0061, prepared as 10mM DMSO stock)
- Panobinostat (Selleck Chemicals, cat# S1030, prepared as 10mM DMSO stock)
- Costar® #3917 96-Well White Solid, Tissue Culture Treated Plates with Lid (Fisher, cat# 07-Spark-628)
- Assorted single and multichannel pipettors and tips, pipettor troughs

HEK293 cells stably expressing HDAC6-NanoLuc® fusion protein were resuspended to a density of 2×10^5 cells/mL in Opti-MEM without serum or phenol red. 85 µl of cell suspension (17,000 cells/well) was transferred to experimental wells of a Corning assay plate.

20X NanoBRET™ Tracer Reagent was created by combining 80 µl of NanoBRET™ Intracellular TE HDAC Tracer and 320 µl of Tracer Dilution Buffer.

5 µl of NanoBRET™ Tracer Reagent was added to all experimental wells.

10mM stocks of Panobinostat and suberoylanilide hydroxamic acid (SAHA) test compounds were diluted to 200 µM in Opti-MEM, then serially titrated 1:3 in Opti-MEM to create an 11-pt dose response titration at 10X concentration. 10 µl of diluted compound was added to experimental wells in replicates of three. A no-compound control was also included by adding 10 µl medium to the assay well.

The assay plate was mixed on an orbital shaker for 15 seconds at 700 rpm, then placed into a 37 °C/5% CO₂ incubator for 85 minutes.

Following incubation, the assay plate was removed from the incubator and allowed to equilibrate to room temperature for 15 minutes.

During room temperature equilibration, 3X Complete Substrate plus Inhibitor Solution was created by combining 30 µl of NanoBRET™ Nano-Glo® Substrate and 10 µl of Extracellular NanoLuc® Inhibitor with 4.96 ml of Opti-MEM without serum or phenol red. 50 µl of 3X Complete Substrate plus Inhibitor Solution was added to all experimental wells.

The assay plate was mixed on an orbital shaker for 15 seconds at 700 rpm, then incubated at room temperature for 2 minutes prior to measuring NanoBRET.

The NanoBRET Target Engagement Intracellular HDAC Assay was prepared and pipetted according to the kit instructions for use [2] in white 96-well flat bottom Costar microplates in a total assay volume of 150 µl. The measurements on the Infinite Spark were performed with the settings summarized in table 1.

Parameter	Setting
Plate	COS96fw
Measurement Mode	Multicolor Luminescence (well-wise)
Wavelength range 1	445-470 nm
Integration time	300 ms
Wavelength range 2	610-700 nm
Integration time	300 ms
Settle time	0 ms
Results output	counts/s

Table 1: Measurement settings for NanoBRET assays in SparkControl™ software

RESULTS AND CONCLUSIONS

Sequential reads of donor and acceptor wavelengths were performed on a per well basis using the Spark. BRET ratios were computed manually and results multiplied by 1000 to convert the ratios to milliBRET units (mBU).

Both Panobinostat and SAHA, known HDAC inhibitors, exhibit binding to HDAC6 resulting in dose-dependent changes in the BRET ratio. High concentrations of both compounds displace the intracellular HDAC tracer, resulting in a decrease in BRET [3]. The measured EC50 values were 1.42 µM for Panobinostat and 0.54 µM for SAHA, respectively (see figure 3). The competition for tracer binding is less apparent as the drug becomes more dilute. Lower concentrations of compound displace less tracer, allowing energy transfer between the donor NanoLuc® Luciferase and the tracer to occur.



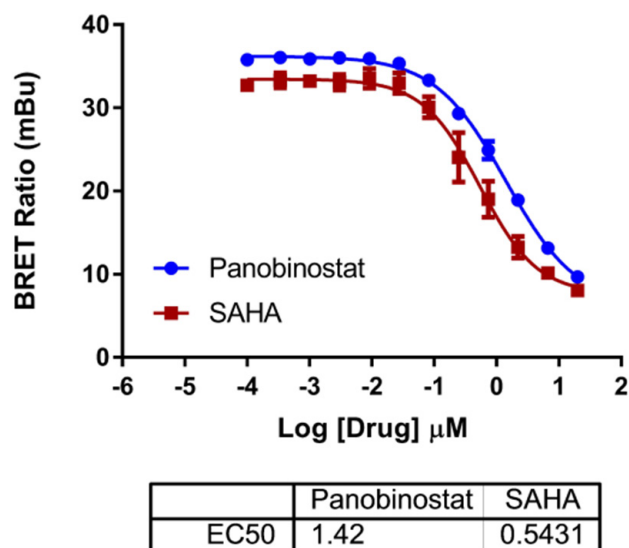


Figure 3. Panobinostat and SAHA exhibit binding to HDAC6 and a dose-dependent displacement of tracer with corresponding change in BRET.

In conclusion, filtered luminescent signal was detectable at both donor (NanoLuc® Luciferase) and acceptor (tracer) wavelengths. The Spark multimode reader is therefore shown to be compatible with detection of NanoBRET™ technology.

ABBREVIATIONS

BRET	bioluminescence resonance energy transfer
DMSO	dimethyl sulfoxide
HDAC	histone deacetylase
Luc	luciferase
MEM	minimal essential medium
TE	target engagement

REFERENCES

- 1) Machleit et al. NanoBRET—A novel BRET platform for the analysis of protein-protein interactions. ACS Chem. Biol., 2015, 10 (8), pp 1797–1804
- 2) NanoBRET™ Target Engagement Intracellular HDAC Assay. Technical Manual/Instructions for Use. 6/16, TM483 (Promega)
- 3) Unparalleled flexibility for luminescence detection with the Spark™ multimode reader. Technical Note. 398597 V2.0. 01-2017 (Tecan)
- 4) Spark® multimode reader - luminescence sensitivity. Optimizing the detection limits for flash and glow luminescence. Technical Note. 398751 V2.0. 01-2017 (Tecan)
- 5) Implementation of NanoBRET assay technology the Infinite 200. Application Note. 400193 V1.0 04-2017 (Tecan)

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