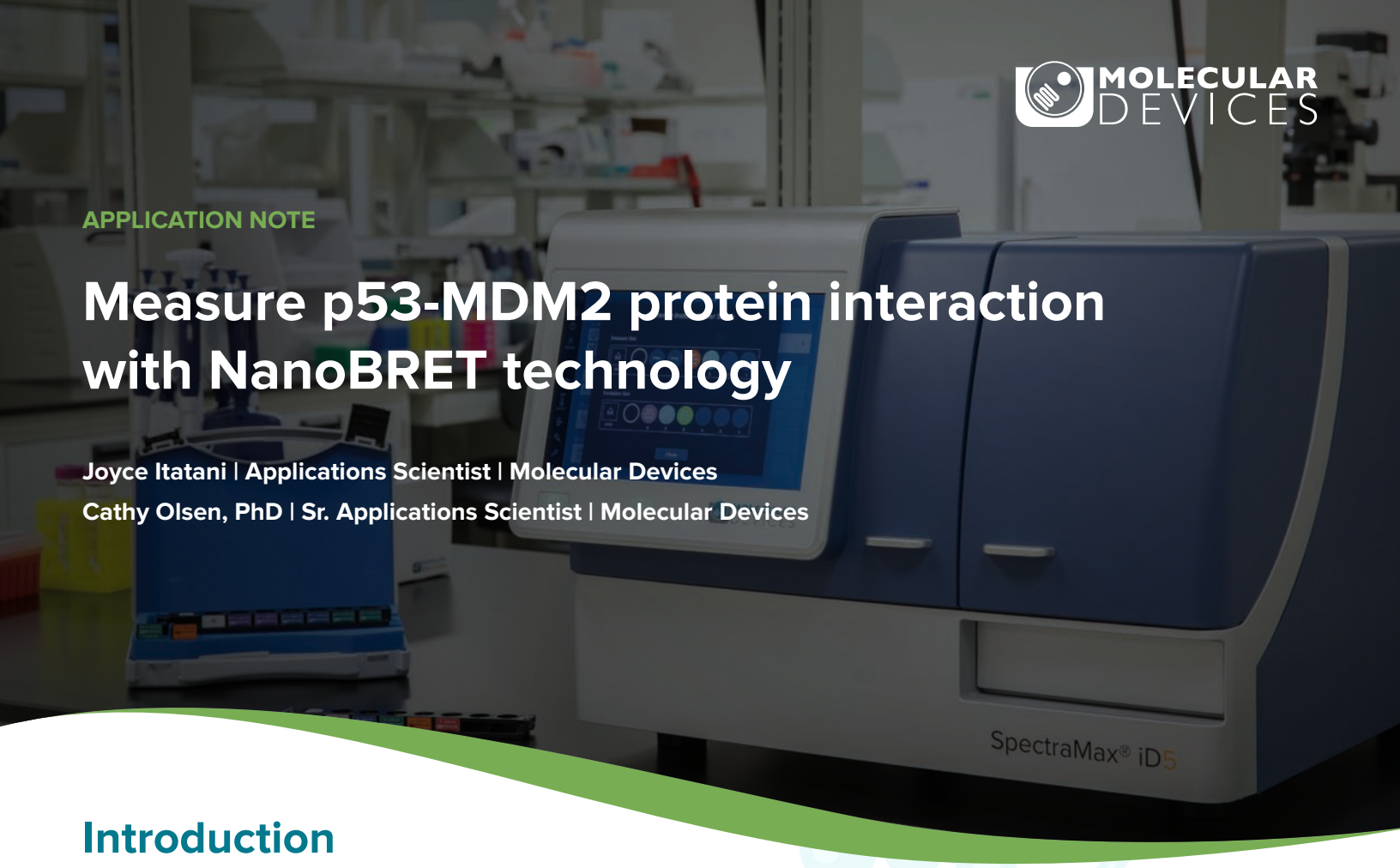

APPLICATION NOTE

Measure p53-MDM2 protein interaction with NanoBRET technology

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Introduction

BRET (bioluminescence resonance energy transfer) is a technique for measuring protein-protein or protein-ligand interactions that involves the interaction of a bioluminescent donor and a fluorescent acceptor. When donor and acceptor are closer than 10 nm to each other, the donor excites the acceptor, which then emits fluorescence. By tagging one protein of interest with the donor and its binding partner with the acceptor, one can measure protein interactions by using a microplate reader to detect light emitted by the donor and acceptor.

NanoBRET™ technology from Promega improves upon earlier generations of BRET, including BRET1 and BRET2, by incorporating a brighter luminescence donor (NanoLuc luciferase), optimized energy acceptor (HaloTag®-NCT), and a wider separation between donor and acceptor wavelengths (Figure 1). These improvements offer increased signal, better sensitivity, and lower background, enabling detection of protein interactions within the context of living cells[1].

Detection of NanoBRET signals, and analysis of the resulting data, requires sensitive instrumentation and advanced software. The SpectraMax® iD5 Multi-Mode Microplate Reader with SoftMax® Pro Software lets researchers acquire NanoBRET data using an optimized filter set, and apply analysis including curve fitting to the results. Here, we describe validation of the microplate reader with the NanoBRET™ PPI Control Pair, consisting of the interacting protein partners p53 and MDM2, on the SpectraMax iD5 reader. The p53 pathway activator nutlin-3 was used to disrupt the p53-MDM2 interaction in a concentration-dependent manner, and the results were analyzed and graphed using SoftMax Pro Software.

Benefits

- Improve sensitivity over other BRET techniques with brighter NanoLuc signal and greater spectral separation
- Detect protein interactions at physiological levels in live cells with high sensitivity
- Calculate NanoBRET ratios and graph results automatically with SoftMax Pro Software

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Materials

- NanoBRET PPI Control Pair (p53, MDM2; Promega cat. #N1641)
- NanoBRET Nano-Glo® Detection System (Promega cat. #N1661)
- ViaFect™ Transfection Reagent (Promega cat. #E4981)
- Nutlin-3 (Millipore-Sigma cat. #6287)
- Opti-MEM™ Reduced Serum Medium, no phenol red (ThermoFisher cat. #11058021)
- 293 (HEK-293) cells (ATCC cat. #CRL-1573)
- Eagle's Minimum Essential Medium (EMEM, Corning cat. #10-010-CV)
- BenchMark™ Fetal Bovine Serum (Gemini Bio-Products cat. #100-106)
- Penicillin-streptomycin (10,000 U/mL, ThermoFisher cat. #15140122)
- 6-well clear microplate (VWR cat. #1006-892)
- 96-well white, flat bottom polystyrene microplate (Corning cat. #3917)
- SpectraMax iD5 Multi-Mode Microplate Reader (Molecular Devices cat. #iD5-STD), equipped with the following:
  - Donor filter: 447 nm BW 60 nm (Molecular Devices cat. #6590-0088)
  - Acceptor filter: 610 nm LP (Molecular Devices cat. #6590-0117)

Methods

HEK-293 cells were suspended in cell culture medium (EMEM + 10% fetal bovine serum + 1% penicillin/streptomycin) at 400,000 cells per mL and plated into a 6-well plate at 2 mL per well, or 800,000 cells per well. Cells were allowed to attach to the wells for 4 to 6 hours at 37°C, 5% CO_2. They were then transfected with 2 µg p53-HaloTag Fusion Vector DNA and 0.2 µg NanoLuc-MDM2 Fusion Vector DNA in 100 µL Opti-MEM Reduced-Serum Medium, without phenol red, and a 3:1 ViaFect Transfection Reagent:DNA ratio. Cells were incubated overnight for 20 to 24 hours at 37°C, 5% CO_2.

HEK-293 cells were harvested by spinning down at 1,000 rpm for five minutes, and the culture medium was discarded. Cell density was adjusted to 2.2 x 10^5 cells per mL in Opti-MEM + 4% FBS and divided into two 15-mL conical tubes. One tube was treated with 0.1 µM HaloTag 618 Ligand, and the other was treated without ligand. The cells were plated into a 96-well white microplate at 2 x 10^4 cells per well and immediately treated with either a 1:3 serial dilution of nutlin-3 (n = 4 replicates per concentration) or 0.5% DMSO. The cells were incubated overnight at 37°C, 5% CO_2.

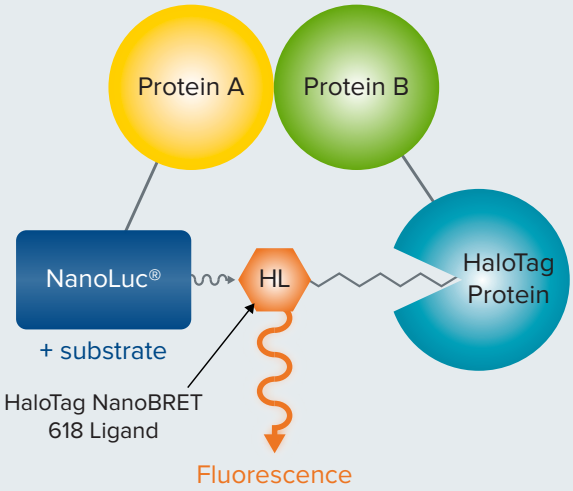

Figure 1. *NanoBRET assay. When a NanoLuc-Protein A fusion (energy donor) interacts with a fluorescently labeled HaloTag-Protein B fusion (energy acceptor), donor and acceptor are brought close together and energy is transferred.*

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A 5X solution of NanoBRET Nano-Glo substrate was made in Opti-MEM + 4% FBS, and 25 µL were added to each well. Donor emission (447 nm) and acceptor emission (610 nm) were measured on the SpectraMax iD5 reader using the settings shown in Table 1. NanoBRET ratios were calculated in SoftMax Pro Software by dividing acceptor signal by donor signal, and ratios were multiplied by 1000 to obtain whole-number milliBRET units (mBU). The mBU values were background corrected by subtracting mean mBU for samples containing no ligand. Results for nutlin-3-treated cells were analyzed and graphed using a 4-parameter curve fit in SoftMax Pro Software (version 7.0.3 and higher). Z' factors were calculated at each concentration of nutlin-3 to assess assay performance.

Results

NanoBRET ratio (mBU) vs. nutlin-3 concentration was graphed using a 4-parameter curve fit in SoftMax Pro Software (Figure 2). The calculated IC_{50} value for nutlin-3 was 1.2 µM, consistent with results shown by Promega for the NanoBRET PPI Control Pair[2].

Z' factors of at least 0.7 were obtained at all concentrations of nutlin-3 used to generate the curve shown in Figure 2. For concentrations of 0.07 µM and lower, Z' factors were equal to 0.9. These values demonstrate the robustness and low variability of this NanoBRET assay.

Parameter	Setting
Read mode	LUM (luminescence)
Read type	Endpoint
Wavelengths	⦿ Specify wavelengths ☑ Use filter [make sure filters are installed] Lm1: 447 nm Lm2: 610 nm
Plate type	96-well Costar
PMT and optics	Integration time: 1000 ms Read height: 0.67 mm*

*Read height should be optimized for the microplate used.

Table 1. *NanoBRET detection settings for the SpectraMax iD5 reader.*

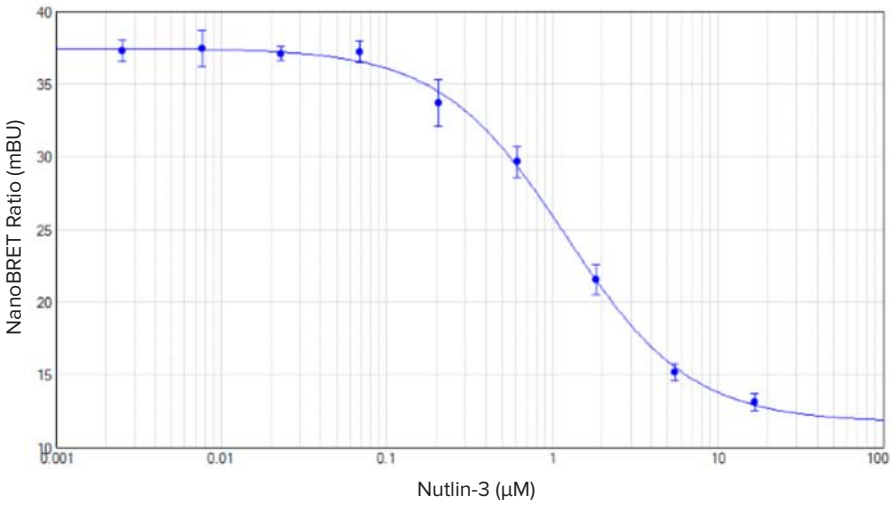

Figure 2. *Disruption of p53-MDM2 interaction by nutlin-3. The 4-parameter curve fit generated in SoftMax Pro Software had an IC_{50} of 1.2 µM (n = 4).*

Conclusion

The SpectraMax iD5 reader, equipped with optimal filters for detection of NanoBRET donor and acceptor signal, was validated for use with this assay using the NanoBRET PPI Control Pair (p53, MDM2). Nutlin-3 was used to disrupt p53-MDM2 interaction in a concentration-dependent manner, yielding an expected IC_{50} value of 1.2 µM. Z' factors calculated to assess assay performance and variability in replicates were between 0.7 and 1 for all sample concentrations tested, confirming the sensitivity of the SpectraMax iD5 reader and the robust nature of the NanoBRET assay. SoftMax Pro Software was configured to calculate NanoBRET ratios and graph the data automatically, streamlining analysis.

References

1. Machleidt T, Woodroofe CC, Schwinn MK, Mendez J, Robers MB, Zimmerman K, Otto P, Daniels DL, Kirkland TA, and Wood KV. NanoBRET—A Novel BRET Platform for the Analysis of Protein-Protein Interactions. *ACS Chem. Biol.* 2015, 10, 1797–1804.
2. Technical Manual: NanoBRET Protein: Protein Interaction System. Promega Corporation.

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