

Corning® Nonbinding Surface (NBS™) Microplates for Fluorescent HTS Assays

Application Report

Innovative Techniques in Drug Discovery



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Since the detection of proteolytic activity or its inhibition remains the subject of many clinical investigations (1-6), the efficacy of the Corning Nonbinding Surface (NBS) Microplate was evaluated for use in a homogeneous fluorescence polarization protease assay. Fluorescently labelled casein substrate in digestion buffer was incubated with dilutions of *Streptomyces griseus* protease or Trypsin type IX in black opaque 96 well microplates and Corning NBS Microplates. Protease activity was detected as a decrease in polarized fluorescence resulting from the release of fluorescently labelled peptides emitting fluorescence out of the excitation plane of polarization. More than a two-fold greater decrease in fluorescence polarization was detected for *S. griseus* protease in the Corning NBS Microplates when compared to the assay plates without the nonbinding surface. This suggests that the sensitivity of this protease assay was enhanced in the Corning NBS Microplates.

Introduction

Researchers seeking therapeutic agents for treatment of a multitude of afflictions as well as facilities involved with the generation of products (7,8) require the ability to detect proteolysis or its inhibition. Presently, there are a broad variety of assays available for detecting the activity of proteases through changes in the absorbance, fluorescence, luminescence and radioactivity of protease substrates (3-5). Fluorescent assays have been particularly beneficial in high throughput screening because of readily available fluorophores and their adaptability to the homogeneous format. These assays include fluorescence polarization, time-resolved fluorescence, fluorescence resonance energy transfer, and fluorescence intensity. Crucial to the success of these assays is a significant difference between the specific signal and the background signal or “noise.” Typically, black opaque microplates with low autofluorescence are used for their ability to reduce light scattering by the surface of the microplate as well as their ability to reduce well-to-well crosstalk. Further enhancement of assay sensitivity can now be achieved for some fluorescent applications through the use of Corning NBS Microplates.

Numerous assays have been developed that utilize fluorescence polarization because this method of detection is especially conducive to the homogeneous high throughput screening format (9). Fluorescence polarization is based on the principle that fluorescently-labelled molecules excited with plane polarized light, will emit polarized light into the same plane if there is minimal molecular movement between the time of excitation and emission. Polarization is thus related to molecular rotation, which is proportional to molecular volume if viscosity and temperature remain constant. Any biochemical reaction that leads to an alteration in the molecular volume of a fluorescently labelled molecule, such as that which occurs through cleavage, binding or a conformational change, can be monitored by fluorescence polarization. In the case of this protease assay, cleavage of the large fluorescently labelled casein substrate molecules results in a protease concentration-dependent decrease in fluorescence polarization as the more mobile protease digestion products are released, and rapidly rotate to emit fluorescence out of the excitation plane of polarization.

Materials and Methods

For protease activity assays, 1X digestion buffer and BOD-IPY FL casein substrate were diluted according to product instructions (Kit #E-6658, Molecular Probes) and added to microplates of black opaque 96 well assay microplates (Costar® Cat. No. 3915) and NBS microplates (Corning Cat. No. 3650 and Corning Cat. No. 3651) at a final substrate concentration of 1.0 µg/mL for 200 µL total reaction volumes. Dilutions (weight/volume) of *S. griseus* protease (4.3 units of activity/mg, Cat. No. P-6911, Sigma® or

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Trypsin type IX (15,200 units of activity/mg, Cat. No. T-0134, Sigma®) in 1X digestion buffer were added to all but control microplates, in triplicate. Protease activity was detected in millipolarization units (mP) using the standard fluorescence polarization protocol in an LJL Biosystems Analyst™ as follows: Digital conversion, attenuator out, units = mP, Plate = Costar® 96 well solid, static polarizer (s) = excitation, dynamic polarizer (p) = emission, polarizer settling time = 30ms, z height = 2.2 mm, integration time: 100 ms, excitation filter 485 nm, emission filter 530 nm.

Non-specific binding of substrate to microplates was examined by incubating dilutions (w/v) of BODIPY FL casein substrate in 1X digestion buffer at a volume of 200 µL/well in wells of the above microplates for 30 min at room temperature. Control wells were incubated with 1X digestion buffer only. Substrate solution was aspirated and wells washed 3 times with PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4). 200 µL of 1X digestion buffer was added to wells, and the fluorescence intensity detected on an LJL Biosystems Analyst set to the following parameters: Digital conversion, high attenuator, units = cps, Plate = Costar 96 well solid, plate settling time = 150 ms, z height = 2.2 mm, integration time = 100 ms, excitation filter = 485 nm, emission filter = 530 nm.

Results and Discussion

In many assays using fluorescent methods of detection, a low signal to noise ratio can reduce the sensitivity of the assay, compromising the information gleaned from the data that has been generated. When evaluating protease activity or its inhibition, this can result in missing potential candidates for therapeutic intervention or lead to the release of contaminated products. Signals can be strengthened by increasing the concentration of reagents used in these assays, but in the high throughput screening format, these adjustments can be costly. Another way to increase assay sensitivity without increasing reagent concentration is to decrease the loss of these reagents due to non-specific adsorption to the walls of the microplates in which the reactions are conducted. Results from this fluorescence polarization assay for protease activity indicate greater than a two-fold increase in sensitivity *S. griseus* protease when the assay is carried out in Corning® NBS™ Microplates compared to assays conducted in microplates without the nonbinding surface. Incubation of BODIPY FL casein substrate with *S. griseus* protease in the Corning NBS Microplates resulted in approximately 17% greater reduction in polarized fluorescence at 0.5 µg/µL concentration than the reduction detected in the microplates without the nonbinding surface (Figure 1). This reduction in polarized fluorescence increased to approximately 42% greater than the reduction that was observed in the microplates without the nonbinding surface in a protease concentration-dependent manner. Similarly, the activity of Trypsin type IX at all concentrations was approximately 23% greater in the Corning NBS Microplates than in the microplates without the nonbinding surface (Figure 2).

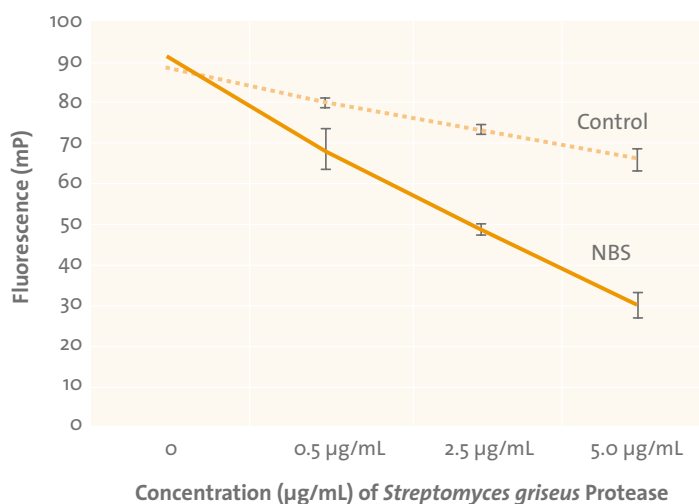


Figure 1. BODIPY FL casein substrate at a concentration of 1.0 µg/mL in digestion buffer was incubated with dilutions of *Streptomyces griseus* protease in black opaque Corning Nonbinding Surface Microplates (NBS) and in microplates without the nonbinding surface (Control). Protease activity was detected as a protease concentration-dependent decrease in polarized fluorescence on an LJL Biosystems Analyst.

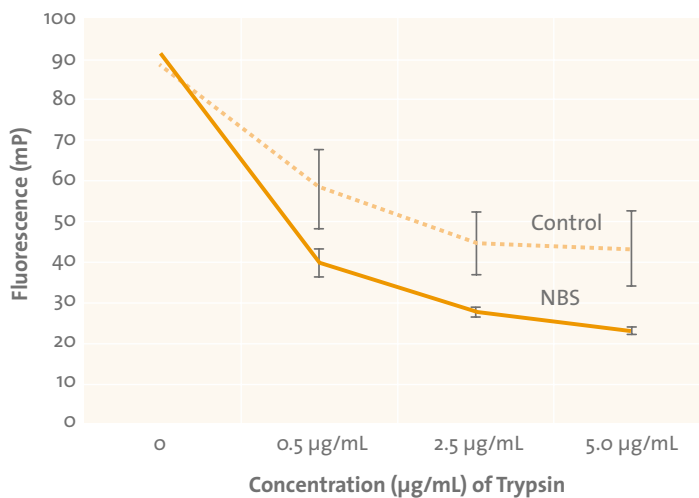


Figure 2. BODIPY FL casein substrate at a concentration of 1.0 µg/mL in digestion buffer was incubated with dilutions of Trypsin type IX in black opaque Corning Nonbinding Surface Microplates (NBS) and in microplates without the nonbinding surface (Control). Protease activity was detected as a protease concentration-dependent decrease in polarized fluorescence on an LJL Biosystems Analyst.

In an effort to evaluate the possible causes for the increased sensitivity of this fluorescence polarization protease assay in the Corning NBS Microplates, dilutions of the fluorescently labelled casein substrate were allowed to incubate in the microplate wells without the addition of protease. The wells were subsequently washed 3 times and the fluorescence intensity detected. Depending on the initial concentration of substrate added, the fluorescence emanating from the

wells of the microplates without the nonbinding surface ranged from 13 to 60% higher than in the wells of the Corning® NBS™ Microplates (Figure 3). The fluorescence observed in the wells of the Corning NBS Microplates remained at background levels of intensity because of modification of the polymer to a non-ionic, hydrophilic surface (polyethylene oxide-like) that minimizes interaction with biological molecules. Washing the microplates three times with PBS failed to remove the substrate from the walls of the wells of the microplates without the nonbinding surface. This suggests that the fluorescently labelled substrate remained inaccessible to the proteases resulting in less substrate cleavage and higher levels of polarized fluorescence. The Corning NBS Microplates appear to increase assay sensitivity by helping to retain the substrate in solution thus facilitating proteolytic cleavage.

Conclusions

- ▶ Corning NBS Microplates enhanced the sensitivity in this fluorescence polarization protease assay by preventing the non-specific adsorption of the fluorescently labelled substrate to the microplate wells.
- ▶ Corning NBS Microplates increased signal strength without increasing reagent concentrations. This can lead to cost savings through reagent conservation at high throughput screening facilities.
- ▶ Corning NBS Microplates can increase the reliability of data used in the evaluation of potential candidates for therapeutic intervention by increasing assay sensitivity.
- ▶ White opaque Corning NBS Microplates have proven to be beneficial for use in some Scintillation Proximity Assays (SPA). Now black opaque Corning NBS Microplates can be used to provide similar benefits for some fluorescent applications.

References

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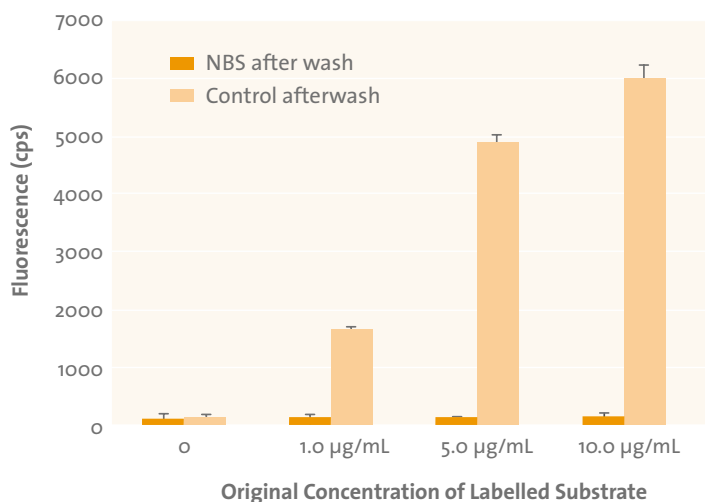


Figure 3. Dilutions of BODIPY FL casein in digestion buffer were incubated for 30 min at room temperature in black opaque Corning NBS microplates and in microplates without the nonbinding surface (Control). Control wells contained digestion buffer only. Microplates were washed 3 times with PBS, pH 7.4, and 200 µL/well of digestion buffer alone was added to the wells. Fluorescence intensity was detected on an LJI Biosystems Analyst.

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