Video Article

A High-throughput-compatible FRET-based Platform for Identification and Characterization of Botulinum Neurotoxin Light Chain Modulators

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Abstract

Botulinum neurotoxin (BoNT) is a potent and potentially lethal bacterial toxin that binds to host motor neurons, is internalized into the cell, and cleaves intracellular proteins that are essential for neurotransmitter release. BoNT is comprised of a heavy chain (HC), which mediates host cell binding and internalization, and a light chain (LC), which cleaves intracellular host proteins essential for acetylcholine release. While therapies that inhibit toxin binding/internalization have a small time window of administration, compounds that target intracellular LC activity have a much larger time window of administrations, particularly relevant given the extremely long half-life of the toxin. In recent years, small molecules have been heavily analyzed as potential LC inhibitors based on their increased cellular permeability relative to larger therapeutics (peptides, aptamers, etc.). Lead identification often involves high-throughput screening (HTS), where large libraries of small molecules are screened based on their ability to modulate therapeutic target function. Here we describe a FRET-based assay with a commercial BoNT/A LC substrate and recombinant LC that can be automated for HTS of potential BoNT inhibitors. Moreover, we describe a manual technique that can be used for follow-up secondary screening, or for comparing the potency of several candidate compounds.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50908/>

Introduction

Botulinum neurotoxin A (BoNT/A), the most potent toxin currently known ($LD_{50} \sim 1 \text{ ng/kg}$)¹, is a potent neurotoxin that is produced by the bacterium *Clostridium botulinum*. Within an affected host, BoNT/A disrupts neurotransmission at the neuromuscular junction by binding motor neurons, internalizing into the cytosol, and ultimately cleaving neuronal proteins that are essential for acetylcholine exocytosis. Once inside a neuron, BoNT/A can persist for as long as several months². Long-term inhibition of acetylcholine release hampers normal muscle contraction and results in flaccid paralysis, which, in severe cases, may result in cardiac and/or respiratory failure. Because of its extreme potency, ease of production, and long-term effects within the host, the CDC has labeled all BoNT serotypes as high-risk bioterrorism agents.

The mechanism of action of the toxin involves numerous steps, including binding to neuronal surface receptors, cellular uptake via receptor-mediated endocytosis, and translocation into the neuron cytosol. BoNT/A is comprised of two chains, a heavy and light chain, and both chains are required for toxicity. The heavy chain (HC) contains binding and translocation domains, while the light chain (LC) is a zinc-dependent metalloprotease that translocates from the endosome to the cytoplasm. Once inside the cytosol, the LC/A metalloprotease localizes to the inner cytoplasmic membrane and cleaves the membrane-bound host protein SNAP-25. SNAP-25 is a member of the SNARE (Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor) protein family, which plays a crucial role in acetylcholine exocytosis (reviewed in reference³). LC/A cleavage of SNAP-25 impairs SNARE complex function, which inhibits acetylcholine neurotransmitter release and impairs muscle contraction.

Currently, treatment for botulism is limited and often includes administration of an equine neutralizing antibody⁴; however, because the toxin is rapidly internalized into neurons, the antibody has a narrow time window of administration. Thus, many researchers believe that the BoNT/A LC may be a better therapeutic target. Because the LC is a zinc-dependent metalloprotease, one approach to inhibit LC/A activity has been to develop compounds that chelate the active-site zinc ion. For example, hydroxamate compounds chelate the active-site zinc and have excellent *in vitro* potency (K_i of the best BoNT/A LC small molecule inhibitor to date is 77 nM)⁵. However, many small molecules fail to advance as therapeutics due to various problems *ex vivo* or *in vivo*, including poor aqueous solubility, rapid metabolism, and/or high cytotoxicity. Therefore, new compounds with improved pharmacological and pharmacokinetic properties are needed. Small molecule compound identification often involves high-throughput screening (HTS) to identify novel scaffolds. Initial methods for BoNT/A LC activity screening were based on HPLC detection of short peptide substrate cleavage, which is time-consuming and not amenable to HTS applications⁶⁻⁸. Subsequently, Schmidt and colleagues⁹ developed a high-throughput BoNT/A LC activity assay that utilizes a fluorescein-labeled peptide substrate covalently attached to a microtiter plate. The BoNT/A LC cleaves the substrate and releases fluorescein, which can be quantified with a fluorometer. The plate format of this assay allows numerous compounds to be screened simultaneously; however, the assay requires labeling synthetic peptides with fluorescein

and coating the assay plates with derivatized substrate molecules, which are cumbersome techniques. A much simpler method for detecting BoNT/A LC activity at low concentrations was later described by Schmidt *et al.*, where a series of fluorogenic substrates were utilized to monitor BoNT LC activity in real time¹⁰. Additional techniques described in the literature include a depolarization after resonance energy transfer-based assay to detect and quantify BoNT activity in crude extracts; this method can be used for high-throughput applications^{10,11}, although it requires sophisticated equipment to measure fluorescence resonance energy transfer (FRET) and polarization signals. Finally, several cell-based models for BoNT intoxication have been reported (reviewed in reference¹¹) that will enable researchers to study the often limiting properties of compounds previously mentioned, including cytotoxicity, cell permeability, and stability. However, most of the existing cell-based assays are not amenable to HTS, and are labor and time intensive.

Herein, we describe a detailed protocol for a HTS method that utilizes the commercially available FRET-based BoNT/A LC substrate. The substrate is based on the SNAP-25 cleavage sequence and is a synthetic 13-mer peptide that contains a terminal fluorophore and quencher. BoNT/A cleavage separates the fluorophore and quencher, abolishing FRET and increasing measured fluorescence, which can be continually measured in a fluorometer plate reader. The assay is used routinely in our, as well as other laboratories, to identify new classes of BoNT/A LC inhibitors or to determine the relative potency of previously identified compounds^{5,12-15}. This assay is suitable for HTS because of its simplicity, automation potential, low cost of materials, and ability to screen numerous compounds simultaneously (see reference¹⁶; Caglič *et al.*, submitted; Bompiani *et al.*, in preparation). In addition to HTS, this assay can be used to compare the relative potency of compounds by determining the IC₅₀ value (concentration required to inhibit 50% of BoNT/A LC activity) of a compound. The assay can either be performed manually in a 96-well format (Manual Screening section of the Protocol Text) or can be automated in a 384-well format for HTS (Automated Operation section of the Protocol Text).

Protocol

Manual Screening or IC₅₀ Determination

This protocol can be used to determine the relative potency of a compound (IC₅₀ value) by preparing a dilution series of the compound, or to manually screen for small-molecule inhibitors at a single concentration.

1. Preparation of Buffers, Reagents, and Required Instrumentation

1. Prepare 50 ml of assay buffer (40 mM HEPES, pH 7.4 and 0.01% Tween-20) and filter sterilize. The buffer can be stored at room temperature (RT) for several months.
2. Prepare a 70 nM working dilution of recombinant botulinum neurotoxin/A light chain (LC/A (1-425))¹⁷ in assay buffer, gently vortex, and store on ice. Testing of each compound requires at least 120 μ l of LC/A working dilution.
3. Prepare 7 μ M FRET-based BoNT/A LC substrate working dilution in assay buffer containing 10% DMSO. Initially dilute a 5 mM stock solution of the substrate in an appropriate volume of 100% DMSO then slowly (drop-wise) add the assay buffer while gently mixing. Wrap in foil and store on ice. Testing of each compound requires at least 130 μ l of the substrate working dilution.
4. Configure a fluorescence microtiter plate reader to shake the plate at medium speed for 10 sec, then monitor the fluorescence at excitation and emission wavelengths of 490 nm and 523 nm, respectively, every 5 min (kinetic mode) for 90 min at RT. Note that product formation (*i.e.* the increase in fluorescence) is linear throughout this period of time (**Figure 1A**).

2. Preparation of Compound Dilution Series

1. Determine the compound concentration range (*i.e.* number of dilutions in the dilution series and dilution factor); the concentration range is chosen based on the expected IC₅₀ value to ensure an optimal curve fit. For compounds with an unknown IC₅₀, use undiluted compound stock as the highest compound concentration.
2. Label tubes with the appropriate compound name and concentration.
3. Aliquot 100% DMSO to all of the tubes to prepare them for compound serial dilution. A minimum of 2 μ l final dilution volume is recommended, where 1 μ l of each concentration is used per well in the assay plate. For example, a standard 1:3 dilution series requires mixing 2 μ l of compound with 4 μ l DMSO per tube.
4. Prepare serial dilutions of compounds and vortex or manually mix thoroughly in between each dilution; change pipette tips in between each dilution. For the standard 1:3 dilution series, add 2 μ l of the undiluted compound to 4 μ l of DMSO in the first tube of the dilution series and mix well. With a new pipette tip, remove 2 μ l of the first dilution and add to 4 μ l of DMSO in the second tube; mix well. Repeat for the remaining dilutions. Make sure to mix the compound dilutions well to ensure that the compound concentrations are accurate.
5. Cover the compound tubes and set aside at RT.

3. Plate Preparation and Spotting (Table 1)

1. Obtain a flat-bottom, opaque, black 96-well plate. See the recommended plate setup in **Table 1**, where each compound dilution series is formatted in rows of a 96-well plate. Manually dispense 1 μ l of the respective compound dilution directly to the bottom of each corresponding well (wells 1-9). Dispense 1 μ l of the highest concentration of compound to be tested to well 11 (compound intrinsic fluorescence control).
2. Manually dispense 1 μ l of a known potent inhibitor to well 12 (positive control), and 1 μ l 100% DMSO to well 10 (negative control).
3. With an automated pipettor, dispense 79 μ l of assay buffer to the side of wells 1-10 and 12 (compound dilutions and positive control), and 89 μ l to the side of well 11 (compound intrinsic fluorescence control). Make sure not to touch the tip to the bottom of the well where compound is present so that you do not cross-contaminate the wells.
4. Vortex the LC/A working dilution and use an automated pipettor to add 10 μ l to the side of each well, except for well 11 (compound intrinsic fluorescence control). Gently tap the plate to ensure that all of the added enzyme goes to the bottom of the well.

5. Gently mix, cover, and incubate the plate for 5 min at RT. This incubation provides a pre-equilibrium step for the compounds to bind to the LC/A prior to substrate addition.

4. Substrate Addition and Fluorescence Measurement

1. Gently vortex the BoNT/A LC substrate, and using an automated pipettor add 10 μ l of substrate to the other side of each well. Be sure to add the substrate to the side of the well opposite of where the enzyme was added earlier.
2. Gently tap the plate to mix the reagents, immediately place in plate reader and begin fluorescence measurement with the settings previously specified in step 1.4.
3. The enzyme velocities are calculated from the slope of the line generated by graphing the relative fluorescence unit (RFU) vs. time during the linear period of the reaction.

Table 1. Suggested assay plate layout.

Well #	Well description	Compound	Assay Buffer	Substrate	LC	DMSO
1	Compound dose 1	1 μ l	79 μ l	10 μ l	10 μ l	---
2	Compound dose 2	1 μ l	79 μ l	10 μ l	10 μ l	---
3	Compound dose 3	1 μ l	79 μ l	10 μ l	10 μ l	---
4	Compound dose 4	1 μ l	79 μ l	10 μ l	10 μ l	---
5	Compound dose 5	1 μ l	79 μ l	10 μ l	10 μ l	---
6	Compound dose 6	1 μ l	79 μ l	10 μ l	10 μ l	---
7	Compound dose 7	1 μ l	79 μ l	10 μ l	10 μ l	---
8	Compound dose 8	1 μ l	79 μ l	10 μ l	10 μ l	---
9	Compound dose 9	1 μ l	79 μ l	10 μ l	10 μ l	---
10	Negative control (100% LC activity)	---	79 μ l	10 μ l	10 μ l	1 μ l
11	Compound intrinsic fluorescence control	1 μ l	89 μ l	10 μ l	---	---
12	Positive control (0% LC activity)	1 μ l	79 μ l	10 μ l	10 μ l	---

Automated Operation for High-Throughput Screening

5. Preparation of Buffers, Reagents, Compound Plates, Barcodes, and Required Instrumentation

1. Thaw the sealed stock compound plates at room temperature for at least 1 hr.
2. Prepare 500 ml (or more, depending on the number of compounds to screen) of the assay buffer (40 mM HEPES, pH 7.4 and 0.01% Tween-20) and filter sterilize. The filtered assay buffer can be stored at RT for several months.
3. Prepare an 8.75 nM working dilution of recombinant BoNT/A LC (1-425) in assay buffer, vortex, and store on ice. Each well in a low-volume 384-well plate requires 8 μ l. Prepare a 10-15% excess, or approximately 3.7 ml or each low-volume 384-well plate.
4. Prepare a 3.5 μ M FRET-based BoNT/A LC substrate working dilution in assay buffer containing 10% DMSO. Initially dilute the 5 mM stock solution of the substrate directly in the appropriate volume of 100% DMSO, then slowly (drop-wise) add the assay buffer while gently mixing. Wrap in foil and store on ice. Each well in a low-volume 384-well plate requires 2 μ l. Prepare a 10-15% excess, or approximately 1 ml for each low-volume 384-well plate.
5. Prepare barcodes for each black, 384-well, low-volume microtiter plate [assay plate] and adhere the label directly to the side of the plate where the barcode scanner can read it; make sure the label does not extend over the plate side, or it may interfere with plate handling and stacking. Stack the plates in numerical order and set aside. Cover the top plate. Note that this step is optional, but facilitates tracking results to corresponding compounds when multiple plates are used.
6. Centrifuge the thawed, sealed stock compound plates at 200 x g for 30 sec at RT. Carefully remove the plate seal, make sure to remove any remaining adhesive, and cover the plate with a clean plastic lid to prevent evaporation. Note that if any adhesive is present on the plate surface, it may interfere with automated lid transfer and plate stacking. Place the covered plate in a hotel attached to the liquid handler. Do not keep the plates uncovered or evaporation will occur.
7. Configure a stacked fluorescence microtiter plate reader to read the fluorescence of each assay plate at excitation and emission wavelengths of 490 nm and 523 nm, respectively, every 30 min (kinetic mode) for 3-6 cycles at RT.

6. Preparation of Pin Tool

1. Visually inspect the 384-head pin tool for any abnormalities, including warping, splintering or large debris.
2. Fill three separate trays with approximately 1 in of 100% methanol (HPLC grade), 100% isopropanol (HPLC grade), and pin tool cleaning solution diluted in ultrapure water.
3. Fill the cleaning station on a liquid handler with 100% DMSO.
4. Stack 2-3 pieces of clean, new blotting paper in a tray. It is important to calibrate the robot in the z-axis so that the pins come in contact with, but do not puncture, the blotting paper.
5. Secure a fan attachment to the working area for drying the pins after washing.
6. Attach the pin tool to robotic arm of the liquid handler and preclean the pins by dipping in DMSO, drying on blotting paper, soaking for 2 min in cleaning solution, drying on blotting paper, dipping in isopropanol, drying on blotting paper, dipping in methanol, drying on blotting paper, and finally drying over the fan.

7. Preparation of Low-volume Liquid Dispenser

1. Attach a low-volume (1-10 μ l) cassette to the liquid dispenser, and connect a waste line to an appropriate vessel.
2. Visually inspect the dispensing nozzles of the cassette and check that they are not warped, torn or blocked. It's important to keep the channels and nozzles clean as precipitation or debris will influence liquid distribution.
3. Prime the channels by running 30 ml of ultrapure water, 30 ml of 70% ethanol, then 30 ml of ultrapure water again directly into waste vessel.
4. Position a low-volume 384-well assay plate into the liquid dispenser and add a known volume of water to each well. Visually inspect to make sure each well is receiving the correct volume and there is no spilling or solution dispensed in between the wells.
5. Prime the tubes with air for approximately 5 sec to remove the water and dry the channels.

8. Dispensing BoNT/A Light Chain into Assay Plate

1. Submerge the liquid dispenser intake line into BoNT/A LC working solution, prime to remove any air, and dispense 8 μ l into each well of a low-volume 384-well assay plate. Note that the plates can be manually positioned on the working area of the liquid handler, or automatically positioned and restacked using an attached plate hotel.
2. Cover each plate with a clean plastic lid and stack in a different column of the plate hotel attached to the liquid handler. Do not keep plates unsealed or evaporation will occur.
3. Clean the liquid dispenser by running 30 ml of ultrapure water, 30 ml of 70% ethanol, 30 ml of ultrapure water again directly into waste vessel.
4. Prime the tubes with air for approximately 5 sec to dry the channels.

9. Stamping Compounds into Assay Plate

1. Assign areas on the liquid handler platform for the compound plates, assay plates and containers with cleaning solutions.
2. Program the liquid handler to place the compound plates and the assay plates in the designated positions and remove the plate lids prior to stamping.
3. Stamp 50 nl of the compound directly into the assay plate containing 8 μ l of BoNT/A LC. When calibrating the stamping program, make sure that the pins are submerged, but do not scratch the bottom of the assay plate. Note that the addition of compounds in 100% DMSO may create a local zone of enzyme denaturation as it disperses. Care should therefore be taken to assess the level of enzyme denaturation by comparing the DMSO only control to the untreated control (*i.e.* enzyme without the addition of DMSO).
4. Clean the pins after each plate by dipping in DMSO, drying on blotting paper, dipping in isopropanol, drying on blotting paper, dipping in methanol, drying on blotting paper, and finally drying over the fan.
5. After the compounds have been stamped in the assay plate with enzyme, separately stack the stock compound plates and the assay plates. Cover the top assay plate in the stack to prevent evaporation and set aside. Make sure to incubate LC with compounds for at least 5 min at RT; longer incubation times can be used when stamping a large number of plates.

10. Dispensing Substrate into the Assay Plate

1. Submerge the intake line of the liquid handler into the BoNT/A LC substrate working solution and protect from light. Check the dispensing nozzles to ensure that they are dry and not blocked. Prime the channels to remove all air bubbles.
2. Un-stack the assay plates and dispense 2 μ l of working dilution of the substrate into each well. Check that the substrate is being dispensed directly into each well. Stack the plates and place one empty assay plate on top of the first plate and one empty plate under the bottom plate in the stack to prevent evaporation during reading and restacking in plate reader.

11. Fluorescence Measurements

1. Immediately load the stack of assay plates into a fluorescence plate reader.
2. Begin the fluorescence measurement with the settings previously specified in step 5.7. The assay rate for each compound is calculated from a graph of the relative fluorescence unit (RFU) over time. Note that it is important to include a negative control (DMSO) to obtain an uninhibited rate; a positive control with a known inhibitor can also be added to each plate. A representative output from a single time point is shown in **Figure 2**.

12. Cleaning up

1. Seal each compound stock plate with a foil seal using a roller, and store at -80°C .
2. After each assay plate has been read, discard the plates, any remaining LC/A or substrate dilutions in the biohazard waste.

Representative Results

The FRET-based BoNT/A LC assay can be performed manually in a low-throughput manner to characterize known inhibitors or screen small libraries; alternatively, the protocol can be scaled-down and automated for high-throughput screening (HTS) with large libraries with the aim of identifying novel BoNT/A LC inhibitors. Regardless of the approach taken, an increase in fluorescence should be observed over time when BoNT/A LC is incubated with the substrate (**Figure 1A** shows a representative plot of fluorescence over time). If serial dilutions of a compound are tested, then a series of lines with varying slopes is often obtained (**Figure 1B**). Well 10 in the plate layout described here serves as a negative control as vehicle only (DMSO) is added; the rate of this reaction is defined as "100% enzyme activity" (or uninhibited activity), and rates in the presence of compound can be normalized to this rate to obtain the relative rate, or percent inhibition. Wells 11 (compound intrinsic fluorescence control) and 12 (positive control) should show horizontal curves (*i.e.* slopes close to 0).

When the assay is performed with serial dilutions of a compound, a graph of the rate of the reaction (assay slope) versus the inhibitor concentration can be used to determine the IC_{50} (**Figure 1C**). The dilution range should be chosen so that the plot appears sigmoid-shaped for optimal curve fitting. Most scientific graphing or statistical analysis software packages enable nonlinear fitting of the sigmoid curve to calculate the concentration of an inhibitor required to inhibit 50% of enzymatic activity (*i.e.* IC_{50} value). The IC_{50} value is a relative measure of potency best described as an apparent K_i value, as it depends upon the concentration of enzyme present in the assay. However, the potency of several compounds can be quickly compared and ranked with the protocol outlined here. It is important to note that in the assay described here one is capturing competitive inhibitors or the competitive component of noncompetitive inhibitors. This assay will not capture uncompetitive inhibitors or the uncompetitive component of noncompetitive inhibitors since these bind to the enzyme substrate complex, the concentration of which is vanishingly low when substrate concentration is much lower than the K_m .

Proper mixing of the plate after adding each component is crucial to obtain a smooth linear increase in fluorescence over time, which is necessary to accurately determine the initial velocities. **Figure 1D** shows a representative experiment where there was insufficient mixing and the rate of product formation is not linear over time, confounding accurate data analysis.

Figure 1. A) Representative graph showing an increase in fluorescence (RFU, relative fluorescence units) upon cleavage of the FRET substrate by BoNT/A LC over time. Under the conditions described in the Protocol Text, increase in fluorescence is linear for greater than 60 min. The slope of the linear portion of the data corresponds to initial velocity. B) Serial dilution of a hydroxamate-based compound results in a dose-dependent increase in the amount of product formed over time. The concentrations shown are final concentrations of the compound in μM . C) Initial velocities (in RFU/min) are plotted against the inhibitor concentration, resulting in a sigmoidal dose-response curve. The concentration of an inhibitor required to reduce the initial velocity to 50% of its uninhibited value is the IC_{50} value. D) Representative image of a low-quality data set. Note the spikes that are present at $t=30$ min are due to insufficient mixing; these nonlinear data make determining the initial velocities challenging. [Click here to view larger image.](#)

Figure 2. Representative data output from a fluorescence plate reader. The image shows a heat-plot diagram of one time point measurement of a 384-well plate from a HTS campaign. The numbers in the wells represent fluorescence in RFU. [Click here to view larger image.](#)

Discussion

The FRET-based BoNT/A LC assay described here represents an attractive method for identifying and characterizing small molecules that modulate BoNT/A LC activity. The solution-based nature of the assay makes this protocol amenable to high-throughput screening described in the Automated Operation section of the Protocol Text. The Z-factor is often used to determine the suitability of an assay for HTS campaigns¹⁸. Determined as $Z = 1 - 3(\sigma_p + \sigma_n)/(\mu_p - \mu_n)$, a Z-factor of 1 is ideal, between 0.5-1 is excellent, and less than 0.5 is marginal. The Z-factor of the HTS protocol as described here is 0.89 (standard deviation of the positive and negative controls are $\sigma_p = 6.67$ RFU/min and $\sigma_n = 0.788$ RFU/min, respectively; mean velocity of negative and positive controls are $\mu_p = 196.1$ RFU/min and $\mu_n = -7.67$ RFU/min, respectively), which indicates that this assay is well suited for HTS.

The assay tolerates up to 2% DMSO in the final volume without significant degradation of the assay signal or impact on enzyme function. Previous studies have also shown that 0.01% Tween 20 increases the mean velocity of the assay¹². The velocity can be further increased by performing the assay at 30-32 °C, although typically the assay is performed at room temperature for convenience. The between-plate and day-to-day variation of the velocity of the negative control (uninhibited reaction) is less than 10% (coefficient of variation, CV = σ_p/μ_p).

It is important to note there are several critical parameters to consider when using this assay. Inherent properties of certain small-molecule compounds may interfere with the fluorescence signals. For example, some small molecules may exhibit auto-fluorescence and thereby impact the assay signal. The inclusion of the intrinsic compound fluorescence control (well 11 in the Manual Screening section of the Protocol Text) can determine whether a compound is auto-fluorescent. If a compound auto-fluoresces and interferes with the fluorescent readout of the assay, the signal in well 11 will appear higher than the signal for DMSO, and the compound may appear less potent. One way to compensate for compound auto-fluorescence is to subtract the signal from this control well (well 11) from the raw fluorescent kinetic data. On the other hand, a compound that quenches the fluorescence signal of the assay will decrease the apparent signal and the compound will consequently appear more potent. To determine if a molecule is quenching product signal, the FRET substrate can first be cleaved with a high concentration of BoNT/A LC (1-425) (e.g. 1 μ M or higher) until no further increase in fluorescence is observed. Compound is then added to the fully cleaved substrate, and the change in fluorescence is recorded. A quenching compound will decrease the fluorescence signal, and such compounds can be analyzed in an alternative BoNT/A LC activity assay that does not utilize a fluorescent substrate or cell-based assay.

Although this FRET-based assay can be utilized to rapidly identify new scaffolds or compounds through HTS, or quickly compare the potency of several compounds, the assay cannot be utilized to determine more rigorous kinetic constants such as the K_i . Because the K_M of the FRET substrate is well above 1 mM, any assay with this substrate is performed under substrate-limiting conditions¹⁹. To address these shortcomings, a more sensitive assay amenable to steady-state kinetics has been reported¹⁹. However, because this assay utilizes liquid chromatography coupled with mass spectrometry to detect cleavage products, it is not amenable for high-throughput applications. In our laboratory, we frequently use FRET-based screening as a primary triage of large compound libraries, with LC-MS-based peptide cleavage assays serving as secondary screens. Another FRET-based assay that is amenable to steady-state kinetics is based on a 66-mer FRET substrate and was shown to be effective for HTS (Z' values > 0.9)²⁰. A clear advantage of the system is the ability to produce the FRET substrate by heterologous expression in *E. coli*, thereby reducing the operational costs, but to our knowledge has not been applied to a HTS campaign.

In summary, the protocol described here utilizes a FRET-based, commercial substrate to monitor botulinum neurotoxin light chain enzymatic activity. This assay allows for rapid, high-throughput screening of large libraries of small molecules to identify novel compounds that modulate

enzymatic activity. This method can be used for both primary and secondary screening, as well as to compare the relative potency of identified compounds and rank compounds of interest based on their activity. Once initially characterized with this protocol, compounds can be further rigorously tested in the additional assays previously described (LC-MS, cell-based assays, *etc.*).

Disclosures

The authors have nothing to disclose.

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