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How to

# Assessment of ELISA as endpoint in neuronal cell-based assay for BoNT detection using hiPSC derived neurons☆

Sabine Pellett[a,*], William H. Tepp[a], Eric A. Johnson[a], Dorothea Sesardic[b]

[a] Department of Bacteriology, University of Wisconsin, 1550 Linden Dr., Madison, WI 53706, United States
[b] Division of Bacteriology, National Institute for Biological Standards and Control (NIBSC), A centre of Medicines and Healthcare products Regulatory Agency; Hertfordshire EN6 3QG, UK

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ABSTRACT

*Introduction:* Botulinum neurotoxins (BoNTs), the causative agents of botulism, are widely used as powerful bio-pharmaceuticals to treat neuro-muscular disorders. Due to the high potency and potential lethality of BoNTs, careful monitoring of the biologic activity of BoNT-based pharmaceuticals is required to ensure safe usage. For decades, the only approved method for potency determination of pharmaceutical BoNTs was the mouse bioassay (MBA), but in recent years improvements in cell-assay technologies have enabled MBA replacement by cell-based assays for specific product evaluations. This project details a method for quantitative and sensitive detection of biologic activity of BoNT/A1 in human induced pluripotent stem cell (hiPSC) derived neurons using an ELISA as a method to determine SNAP-25 cleavage by BoNT/A1 following toxin exposure.
*Methods:* HiPSC derived neurons from two different sources were exposed to serial dilutions of BoNT/A1, and quantitative detection of toxin activity was evaluated and optimized in cell lysates using ELISA to detect cleaved SNAP-25.
*Results:* The results from this study indicate that an ELISA using ultra TMB as a substrate quantitatively detects cleaved SNAP-25 in cell lysates of BoNT/A1 exposed hiPSC-derived neuronal cells with similar or greater sensitivity as Western blot (EC50 ~ 0.3 U/well).
*Discussion:* This study demonstrates a human specific and sensitive cell-based detection platform of BoNT/A1 activity using ELISA as an endpoint for quantitative detection of the SNAP-25 cleavage product. This assay is applicable to moderate to high-throughput formats and importantly employs non-cancerous human-specific neuronal cells for potency evaluation of a bio-pharmaceutical for human use.

## 1. Introduction

Botulinum neurotoxins (BoNTs) are the causative agent of botulism, but are also widely employed as powerful pharmaceutical agents to treat a variety of neuromuscular disorders as well as other neuronal syndromes such as hyperhidrosis, chronic migraines, neurogenic bladder, and more. (Dressler, 2012). Traditionally, the potencies of pharmacological preparations of BoNTs have been assessed by the in vivo mouse bioassay to determine toxin activity for product stability, lot to lot consistency, and product release (Hatheway, 1988; Schantz & Kautter, 1978). However, due to ethical and biosafety concerns regarding the use of large numbers of mice, the Food and Drug Administration (FDA) and manufacturers of pharmaceutical BoNTs have been putting forth efforts to replace the mouse bioassay (Adler et al., 2010; Sesardic & Jones, 2007). Neuronal cell-based assays (NCB assays) have emerged as a feasible alternative in recent years, due to the sensitive and specific detection of holotoxin activity in several neuronal cell models and the ability to standardize these assays (Fernandez-Salas et al., 2012; Hubbard et al., 2012; Kiris et al., 2014; Pellett, 2013; Whitemarsh et al., 2012). Unlike assays that determine individual steps in the BoNT intoxication process, NCB assays determine all four steps which is essential for API final characterization. The largest manufacturer of pharmaceutical BoNTs in the USA, Allergan developed a cell based assay for product release in 2012 using the human neuroblastoma cell line SIMA cells (Fernandez-Salas et al., 2012). Other efforts have utilized human induced pluripotent stem cell (hiPSC) derived neurons for sensitive detection of BoNT activity in a human non-cancerous cell model (Whitemarsh et al., 2012).

A useful method to determine the endpoint for a cell-based assay for BoNT/A activity is Western blot detecting cleaved and uncleaved SNAP-

☆ Contact for antibody reagents from NIBSC: yvonne.liu@nibsc.org.

* Corresponding author.
*E-mail addresses:* Sabine.pellett@wisc.edu (S. Pellett), whtepp@wisc.edu (W.H. Tepp), eric.johnson@wisc.edu (E.A. Johnson), Thea.Sesardic@nibsc.org (D. Sesardic).

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25 (synaptosomal associated protein 25). While this endpoint is appropriate and useful for research purposes, the semi-quantitative nature of Western blots and the laborious and costly steps involved make this end-point less well suited for a product release assay. Alternatively, quantitative and scalable ELISA platforms for detecting intracellularly cleaved SNAP-25 in cell lysates have been developed (Fernandez-Salas et al., 2012; Nuss et al., 2010). However, ELISA platforms are dependent on the availability of an antibody that detects only BoNT/A cleaved SNAP-25, and an optimized protocol for the cell model used. In a recent report, an NCB-assay combined with an ELISA endpoint for detection of cleaved SNAP-25 has been described using mouse embryonic stem cell derived neurons (Yadirgi, Jones, Fleck, Rasetti-Escargueil, & Sesardic, 2013). However, the same method was not directly transferrable to hiPSC derived neurons due to low detection levels. Here a modification of the method is described that allows the sensitive detection of BoNT/A holotoxin activity in hiPSC derived neurons using quantitative ELISA based detection of cleaved SNAP-25 as an endpoint. Assessment of the NCB assay-ELISA method in two different hiPSC derived cell models showed that this method is as or more sensitive in detecting BoNT/A holotoxin activity as an NCB assay using Western blot to detect the cleaved SNAP-25.

## 2. Materials and methods

### 2.1. Botulinum neurotoxin

Isolated pure 150 kDa BoNT/A1 toxin heterodimer was isolated from the *Clostridium botulinum* strain Hall A-hyper essentially as previously described (Malizio, Goodnough, & Johnson, 2000). Specific activity was determined by mouse bioassay (MBA) (Hatheway, 1988; Schantz & Kautter, 1978) to be $1.25 \times 10^8$ mouse $LD_{50}S$ (Units)/mg.

### 2.2. Reagents and antibodies

For the ELISA, the Polystyrene Maxisorp plates (Nunc) or Immulon Immunoassay plates (Thermo Scientific) were used as indicated. The antibodies were all provided by the Sesardic lab. The BoNT/A cleavage site-specific anti-peptide antibody against SNAP25 190-197 was as previously described (Ekong, Feavers, & Sesardic, 1997) and was affinity purified. This antibody has previously been shown to bind only BoNT/A cleaved SNAP-25 in both the ELISA and Western blot assays ((Ekong et al., 1997), Jones, Ochiai, Liu, Ekong, & Sesardic, 2008). Anti SNAP25 detecting and signal amplification antibodies were made in sheep against SNAP25 1-57 and SNAP25 111-157, respectively (Liu, Rigsby, Sesardic, Marks, & Jones, 2012). Rabbit anti-sheep HRP was from Thermo Scientific. All SDS-PAGE gel reagents and Western blot reagents were purchased from Life Technologies, and other chemicals and reagents were purchased from SIGMA unless otherwise noted. The BCA assay was purchased from Pierce (Thermo Fisher) and used according to manufacturer's instructions.

### 2.3. Neuronal cells

Human induced pluripotent stem cell (hiPSC)-derived neurons (iCell Neurons, Cellular Dynamics International, Madison, WI) were prepared and maintained in the manufacturer's provided serum-free medium as previously described (Pellett, Tepp, Clancy, Borodic, & Johnson, 2007; Pellett, Tepp, Toth, & Johnson, 2010; Whitemarsh et al., 2012a). Cells were seeded into 0.01% poly-L-ornithine (PLO) (SIGMA) and 8.3 μg/$cm^2$ matrigel (BD Biosciences) coated 96-well Techno Plastic Products (TPP) plates at a density of ~35,000 cells/well. The cells were used in the toxin assay at 5 days post plating. HIP™ Neurons and accompanying media were supplied from Global Stem (Rockville, MD) and grown according to manufacturer's instructions with the exception that they were plated onto PLO-laminin-coated plates before differentiation, and the differentiation medium was supplemented with 2 μM all-trans retinoic acid (Stemgent, Cambridge, MA) and 5 μM purmorphamine (CalBiochem). After 5 days of differentiation, the cells were kept in the same plate and differentiation media was replaced with maintenance media (supplied by Globalstem). The neurons were matured for 18 days prior to use in the toxin assays. Primary mouse spinal cord cells were prepared as previously described (Pellett et al., 2015; Whitemarsh et al., 2013) and cultured for 18 days prior to use in a toxin assay. All cell types were incubated at 37 °C in a humidified 5% $CO_2$ atmosphere. To produce bulk cell lysates containing cleaved SNAP-25 or only uncleaved SNAP-25 for use in ELISA optimization, the primary mouse spinal cord cells were either exposed to 10 U BoNT/A1 per well or no toxin in 50 μl culture media for 48 h. After 48 h, cells from 48 wells, respectively, were lysed for ELISA as described and the cell lysates were pooled. An aliquot of the lysates was examined by Western blot to confirm over 90% cleaved SNAP-25 in toxin exposed and uncleaved SNAP-25 in unexposed cell lysates.

For the toxin exposure, serial dilutions of BoNT/A1 were added to the cells as indicated, in a volume of 50 μl/well, and cells were incubated for 48 h before lysis for the ELISA or Western blot. Cell lysates were prepared from the BoNT/A1 exposed cells by aspirating the toxin containing media and washing the cells once with 75 μl of 10 mM Hepes buffer pre-warmed to 37 °C. Hepes was removed and 105 μl of room temperature M-PER (Mammalian protein extraction reagent) lysis buffer (Pierce) containing 154 mM NaCl was added. The plates were shaken on a plate shaker for 10 min. at 700 rpm, and 80 μl per sample were immediately frozen at −70 °C for the ELISA. The remainder of the sample was mixed with 8.3 μl of 4 × LDS sample buffer (Life Technologies) for Western blot analysis.

### 2.4. ELISA

The frozen samples were thawed at room temperature immediately before the ELISA, and any remaining cell debris was removed by centrifugation prior to conducting the assay. The ELISA was performed as described below, except where noted otherwise. To increase sensitivity, TMB ultra substrate (Pierce) was used instead of ABTS where indicated.

Ninety six-well ELISA plates (Nunc, Maxisorb) were coated with 50 μl/well of 5 μg/ml affinity purified capture antibody against SNAP25 190-197 in carbonate buffer (50 mM sodium carbonate, pH 9.6), overnight at +4 °C. After carefully removing buffer by decanting, the plates were blocked with 250 μl per well of 2.5% (w/v) skimmed milk powder (Marvel) in PBST (phosphate buffered saline with 0.05% v/v Tween-20) (M-PBST or blocking buffer) for 90 min at room temperature (RT). The plates were washed at least three times with PBST using a plate washer. Cell lysate (50 μl) was added to the plates, together with 50 μl of M-PBST, and placed on a shaker at 400 rpm for 10 min followed by resting for 90 min at RT. M-PBST (100 μl per well) was added to blank controls (negative control). In all experiments, cell lysate from cells not exposed to toxin was added as a second negative control. Internal controls for the ELISA included 'no capture antibody' and 'no detection antibody' (primary and secondary) were also included during the assay optimization. Plates were washed three times with PBST and detection of captured truncated SNAP-25 was performed by addition of an equal mixture of sheep anti-SNAP25 antibodies directed to SNAP25 1-57 and 111-157, respectively, in M-PBST at 100 μl per well. After incubation for 90 min at RT, the plates were again washed three times with PBST and the reaction visualized with 100 μl per well of commercially sourced rabbit anti-sheep HRP conjugated antibody (Thermo Scientific) in M-PBST and incubation for 90 min at RT. After washing with PBST 200 μl/well of substrate solution (50 mM citric acid pH 4.0, 0.05% w/v ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and 0.05% v/v of a 30% w/v Hydrogen peroxide solution) was added to all wells and the colour allowed to develop at RT for 30 min. Following colour development, the plates were briefly shaken and absorbance deter-