Overview of Cell-Based Assays For Botulinum Neurotoxin Product Release

Session 3B

ICCVAM/NICEATM/ECVAM Scientific Workshop on Alternative Methods to Refine, Reduce, and Replace the Mouse LD50 Assay For Botulinum Toxin Testing

13 November 2006

Agenda

- ***Objective***
- ***Essential steps of botulinum neurotoxin mechanism***
- ***Literature review***
- ***Current status***

Currently, a cell-based assay is not available for botulinum toxin-based product release

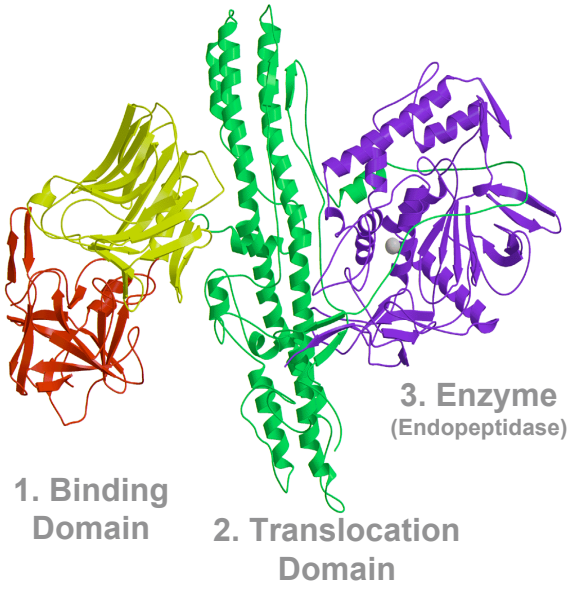
Objective: Replacement

- ***Cell-based potency assay for botulinum toxin-based product release***
- ***Potency assessment in vitro corresponding with approved in vivo assay***
- ***Amenable for use by release QC laboratory and regulatory agency laboratories***
- ***Meets global regulatory requirements for replacement***

Essential Steps For Botulinum Neurotoxin Action

- ***Binding***
- ***Internalization***
- ***Cleavage of substrate***
- ***Inhibition of neurotransmitter***

All Modules Must Be Assessed

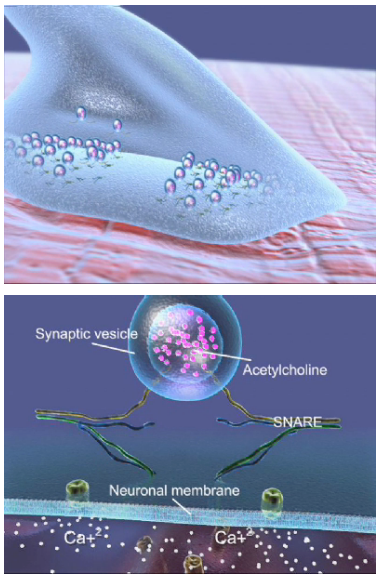


1. Binding domain allows protein to attach to nerve
2. Translocation domain moves enzyme from one compartment to interior of nerve
3. Enzyme continues to work inside nerve

- Potent and long duration of effect

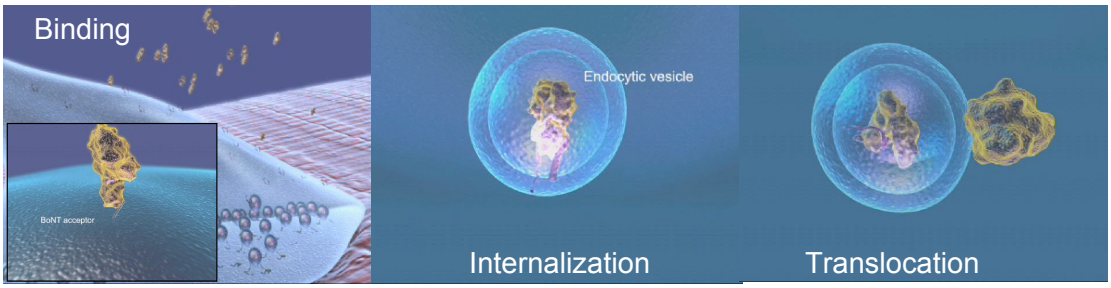
Motor Nerve Neurotransmitter Release

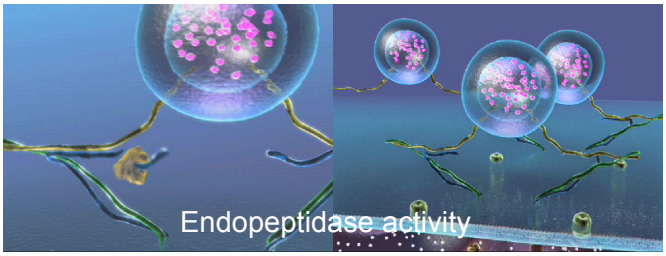
- ***Calcium-dependent response to action potential***
- ***Vesicle docking, fusion and release***
- ***Mediated by specialized proteins***



Adapted from animations provided by www.neurotoxininstitute.org

Botulinum Toxin Type A Action In Motor Neuron

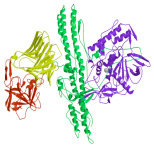




Adapted from animations provided by www.neurotoxininstitute.org

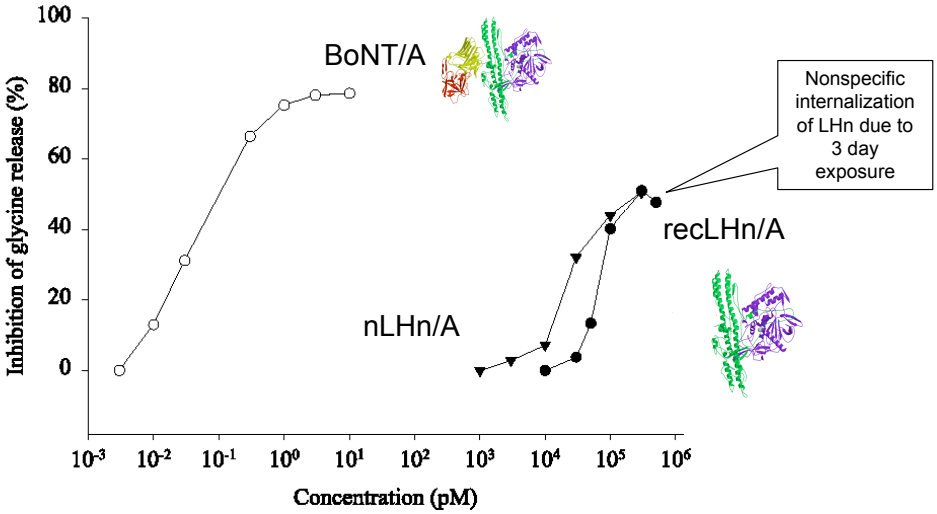
Cell-Based Assay Requires Intact Botulinum Toxin (BoNT)

- ***Literature example***
  - Chaddock et al, 2002
- ***Fetal rat spinal cord cells (primary culture)***
- ***Inhibition of potassium stimulated ^{3}H-glycine release***
- ***Very sensitive to BoNT/A***
- ***Requires intact dichain molecule***



Chaddock et a.l Expression and purification of catalytically active, non-toxic endopeptidase derivatives of Clostridium botulinum toxin type A. Protein Expression and Purification 25 (2002) 219–228

Cell-Based Assay Measures Intact 150 kDa Dichain Activity In Vitro



Chaddock et al Protein Expression and Purification 25 (2002) 219–228

Challenges With Measurement of Formulated Product Potency

- ***Quantity of botulinum toxin (ng level ~ pM range)***
  - Assay limit of detection
  - Losses due to surface adsorption
- ***Formulation excipients***
  - Interference with analytical methods
- ***Complex multi-step mechanism of action***

The 3 R's

- *Reduce*
  - Number of animals used in assays
- *Refine*
  - Non-lethal in vivo assay assessments
- ***Replace***
  - **In vitro assays**
    - **Cell-based (potency, neutralizing antibody levels)**
    - **Biochemical (substrate cleavage)**
    - **Immunological (identity, quantity, neutralizing antibody)**

Cell-Based Assays Overview

- ***Cell source***
  - Primary culture
  - Cell lines
  - Modified cells

Stable cell line required for routine use in a product release assay

- ***Efficacy measure***
  - Loss of intact substrate/appearance of cleavage product
  - Exocytosis
  - Neuronal networks

Sensitivity to Botulinum toxin required:

Short exposure,

Low concentrations

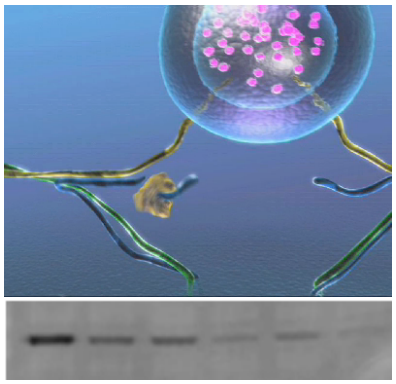
Literature Review: Botulinum Cell-Based Assays Western Blot Readout

Cell Type	Read-out	Substrate	BoNT tested	Concentrations Tested *	Reference
PC12 cells (serum-free, 24 hr)	Cleavage detected with antibody to SNAP25	Endogenous SNAP25	BoNT/A	5 µg/mL for 48 hr	Eubanks ML, et al. FEBS Lett. 2005 Oct 10;579(24):5361-4.
Neuro-2A (serum-free, 24 hr)	Cleavage detected with antibody to SNAP25	Endogenous SNAP25	BoNT/A	1-2.5 µg/mL for 48 hr	Eubanks ML, et al. FEBS Lett. 2005 Oct 10;579(24):5361-4.
Differentiated Neuro-2A cells	Detection of cleaved $SNAP25_{197}$ by specific antibody	Endogenous SNAP25	BoNT/A	0.002 to 20 nM for 16 hr exposure	Fernandez-Salas E et al., ABS-29; Steward LE et al., ABS-76, Proc. of the Int. Conference 2005, Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins.

* Molar concentrations calculated when appropriate information available

Strengths and Limitations of Western Blot Readout

- ***Strengths***
  - **Changes to BoNT substrate**
    - **Loss or appearance (Ab dependent)**
  - **No further alteration of cell line required**
- ***Limitations***
  - **Requires appropriate antibody**
  - **WB increases variability of assay**
  - **Limited throughput**
  - **Non-linear relationship between substrate cleavage and inhibition of exocytosis**



Adapted from animations provided by www.neurotoxininstitute.org

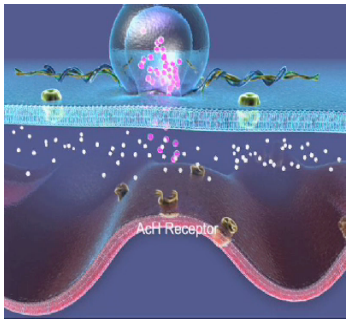
Literature Review: Botulinum Cell-Based Assays Neuronal Network or Release

Cell Type	Read-out	Substrate	BoNT tested	Concentrations Tested*	Reference
Embryonic murine spinal cord and frontal cortex	Reduction of spontaneous spiking and bursting	Endogenous SNAP25	BoNT/A	50 ng/mL (100pM) 10% at 1.7 hr, 50% at 3.2 hr, and 90% reduction at 5 hr. 10 ng/mL 90% at 20 hr	Gross GW. Et al. 2003, Abstract 122.9 Society for Neurosciences Keefer EW and Gross GW. Et al. 2001, Abstract 1302 Society for Neurosciences
Embryonic murine spinal cord	Increase of spontaneous spiking and bursting	Endogenous synaptobrevin	Tetanus toxin	0.5-2 nM in 0.5 hr persisting for 1.2 hr followed by total loss of activity by 3 hr	Keefer EW and Gross GW. Et al. 2001, Abstract 1302 Society for Neurosciences
Embryonic frontal cortex neurons	Reduction of spontaneous spiking and bursting	Endogenous synaptobrevin	Tetanus toxin	1.5 hrs to total shut-off	Keefer EW and Gross GW. Et al. 2001, Abstract 1302 Society for Neurosciences
Embryonic spinal cord neurons	[3H]-glycine release	Endogenous SNAP25	BoNT/A	IC50 = 2.95± 0.9 pM after 16 hr exposure	Chaddock et al Protein Expression and Purification 25 (2002) 219–228 Hall Y.H.J. et al., J Immunol Methods. 2004 May;288(1-2):55-60.

* Molar concentrations calculated when appropriate information available

Strengths and Limitations of Exocytosis Readout

- ***Strengths***
  - **Cellular target reflecting activity of all three modules**
  - **Potentially amenable to screening assay**
- ***Limitations***
  - **Usually requires tracer (radioactive, FL, etc)**
  - **Expensive equipment and resources to maintain cells (neural network uses primary cells)**
  - **Average of all cells (sensitivity)**



Adapted from animations provided by www.neurotoxininstitute.org

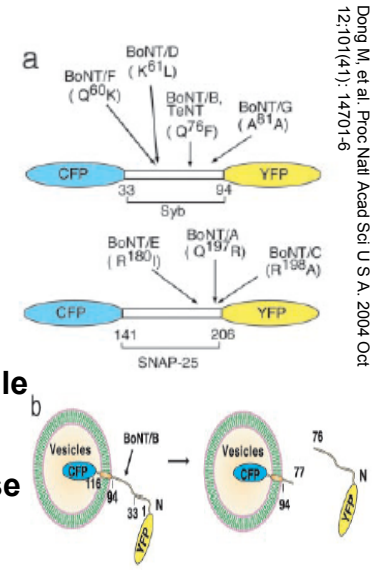
Literature Review: Botulinum Cell-Based Assays Fluorescent Sensors

Cell Type	Read-out	Substrate	BoNT tested	Concentrations Tested*	Reference
Wild-type PC12 cells	FRET	CFP-SNAP25(FL)-YFP	BoNT/A	50nM for 48 hr and 96 hr	Dong M, et al. Proc Natl Acad Sci U S A. 2004 Oct 12;101(41): 14701-6
PC12 cells expressing synaptotagmin II	Redistribution of YFP fluorescence from vesicles to cytoplasm and nucleus	YFP-Syb(FL)-CFP	BoNT/B	50nM for 48 hr	Dong M, et al. Proc Natl Acad Sci U S A. 2004 Oct 12;101(41): 14701-6
Differentiated Neuro-2A cells	FRET	SNAP25- GFP	BoNT/A	0.002 nM to 10 nM for 16 hrs or 0.0002 nM to 1 nM for 3 days	Steward LE et al., ABS-76, Proc. of the Int. Conference 2005, Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins.

* Molar concentrations calculated when appropriate information available

Strengths and Limitations of Fluorescent Readouts

- ***Strengths***
  - **Potential gain in sensitivity and throughput (FRET)**
  - **Positive (transfected) cells respond**
- ***Limitations***
  - **Extensive cloning/cell modification potential – more difficult to create stabile cell line**
  - **Equipment costs (detector, etc) increase**
  - **Potential license fees**



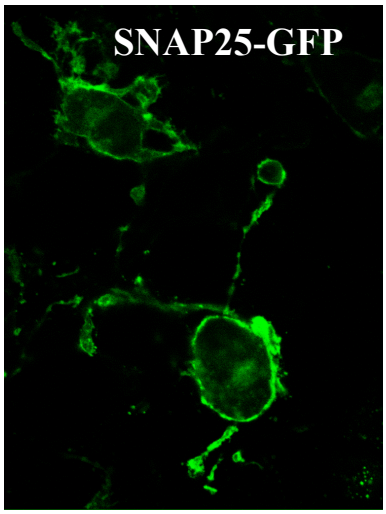
Dong M, et al. Proc Natl Acad Sci U S A. 2004 Oct 12;101(41): 14701-6

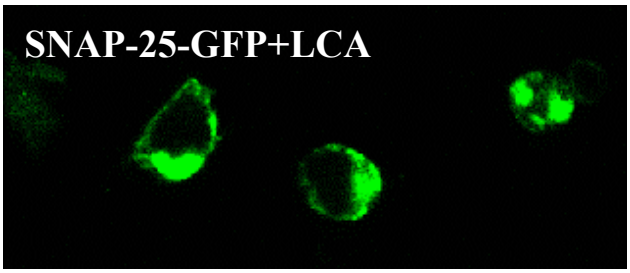
Progress: Research Cell-Based Assay

- ***Differentiated cell line***
  - Transient transfection with SNAP25-GFP
- ***FRET* loss when BoNT/A activity positive***
  - Overnight incubation
  - Addition of membrane selective dye
- ***Finished product activity detection possible***
- ***Refinements in progress***

* Fluorescence Resonance Energy Transfer. Steward et al, 2005

Cleavage of SNAP(FL)-GFP by LC/A Results in Fluorescence Release from Plasma Membrane

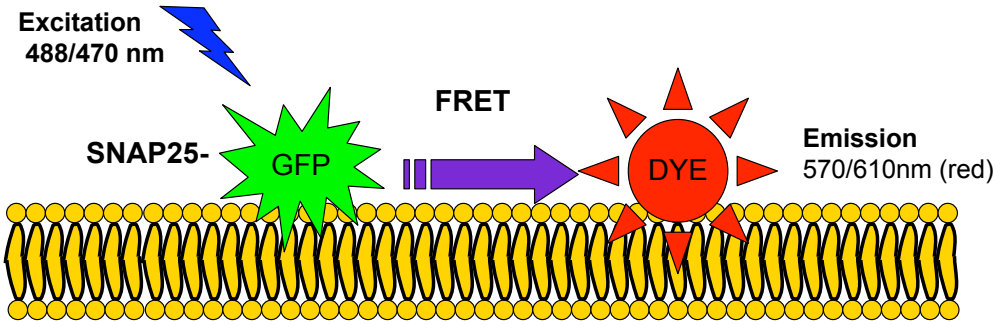




PC12 Cells. Release of GFP from plasma membrane can be used for a FRET based assay.

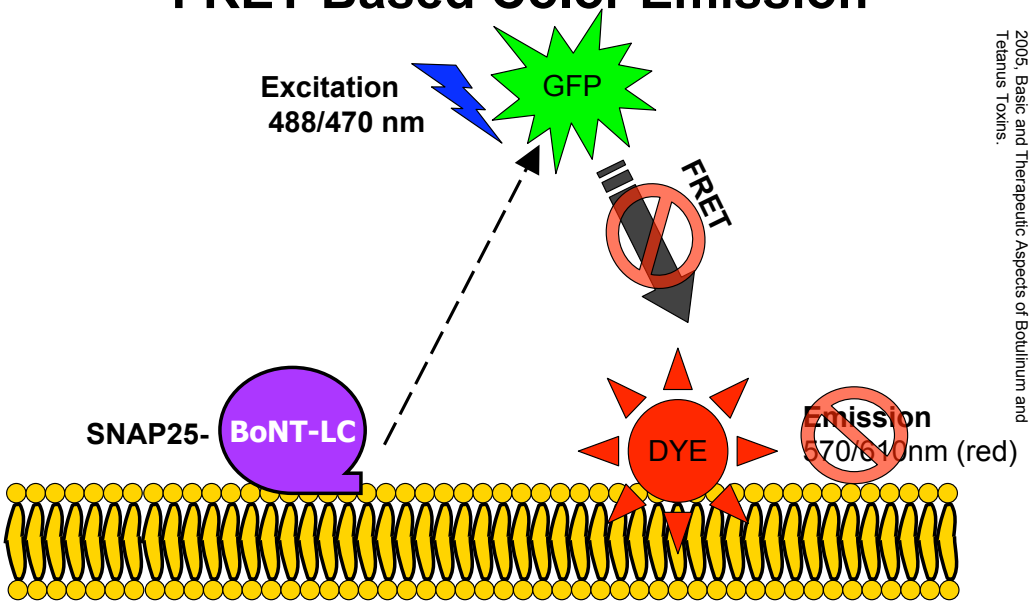
Steward LE et al., ABS-76, Proc. of the Int. Conference 2005, Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins.

Fluorescence Resonance Energy Transfer (FRET) Assay



Steward LE et al., ABS-76, Proc. of the Int. Conference 2005, Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins.

cFRET Assay: BoNT/A Releases GFP From Plasma Membrane and Prevents FRET Based Color Emission



Steward LE et al., ABS-76, Proc. of the Int. Conference 2005, Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins.

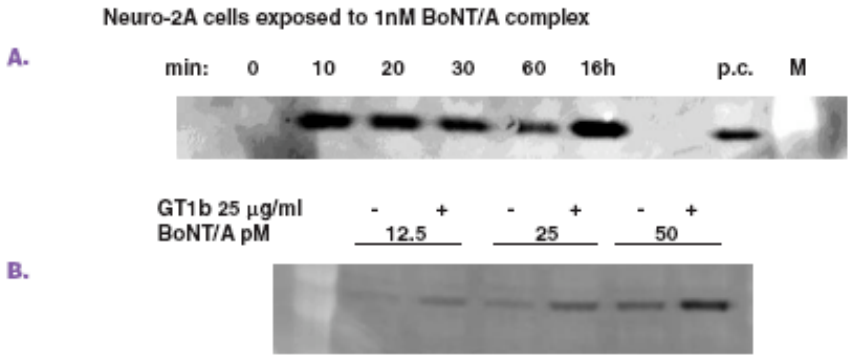
Research Cell Line Selection

- ***Sensitivity to BoNT/A action***
  - 1 nM BoNT/A complex
  - Up to 16 hours exposure
- ***18 cell lines screened***
- ***Neuro 2A cell line identified***
  - Exocytosis
  - SNAP25 cleavage (detected within 10 minutes)
- ***Transfection with SNAP25-GFP for FRET based assay***

Neuro-2A Cells Sensitive to BoNT/A

Screening of cell lines for BoNT/A uptake: Neuro-2A cells have the high affinity receptor

15 Neuronal cell lines were tested for BoNT/A uptake

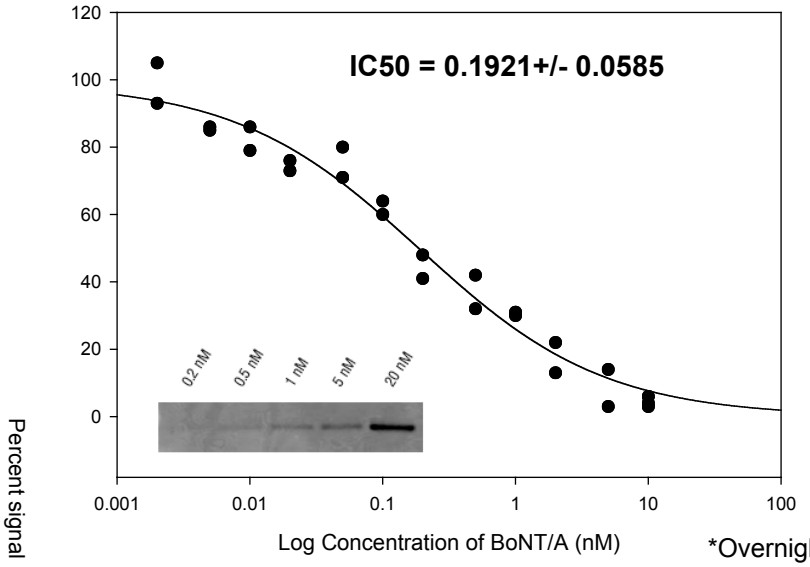


A. Time Course of SNAP25 cleavage in Neuro-2a cells treated with 1 nM BoNT/A complex analyzed by Western blot with an antibody specific for $SNAP25_{197}$. The cleavage product is detected as early as 10 minutes after toxin exposure. B. Neuro-2a cells were pre-treated with 25 µg/ml of the ganglioside GT1b and exposed to picoMolar concentrations of BoNT/A.

Steward LE et al., ABS-76, Proc. of the Int. Conference 2005, Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins.

Cell-Based FRET assay IC_{50} for BoNT/A*

Differentiated Neuro-2A cells



Steward LE et al., ABS-76, Proc. of the Int. Conference 2005, Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins.

*Overnight incubation

Preliminary Results: Activity Detected in Finished Product

The excipients in product do not interfere with the FRET assay

Sample	*Experiment Derived Concentration of BoNT/A (nM)*
Vial 1 lot A	***0.010***
Vial 2 lot A	***0.010***
Vial 1 lot B	***0.020***
Vial 2 lot B	***0.020***
Heated lot A	***0.000***
Heated lot B	***0.0005***

Steward LE et al., ABS-76, Proc. of the Int. Conference 2005, Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins.

Strengths and Limitations of Cell-Based Assay

- ***Strengths***
  - Measures essential steps of BoNT action
  - Potential replacement of product release (potency) assay
- ***Limitations***
  - Early research assay
  - Limit of detection challenges for product release
  - Cell culture facility and skills required
  - Passage number restriction
  - Transient transfection (stable cell line needed)
  - Method of BoNT activity detection dictates sensitivity and speed of assay