Botulinum Neurotoxin – Detection and Bioavailability Research

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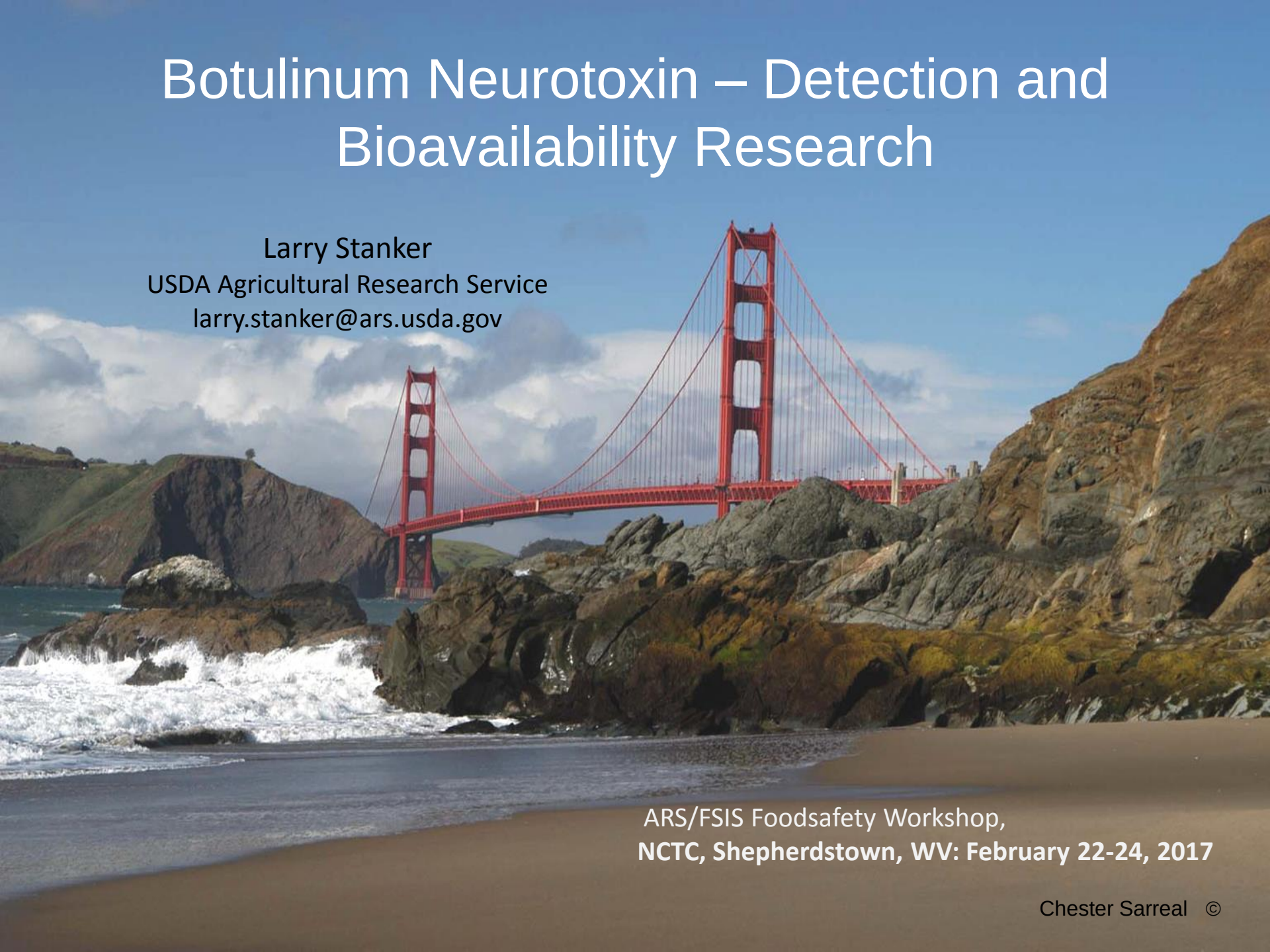


ARS/FSIS Foodsafety Workshop,
NCTC, Shepherdstown, WV: February 22-24, 2017

Chester Sarreal ©

Monoclonal Antibodies & Immunoassays

Antigen Development

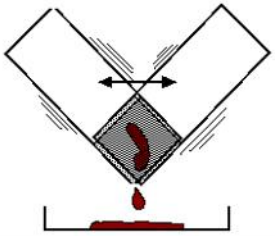
- Purification
- Toxoid
- Recombinant

→

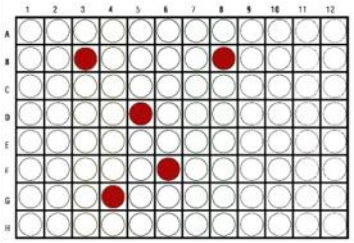
Immunization

→

Hybridoma Technology

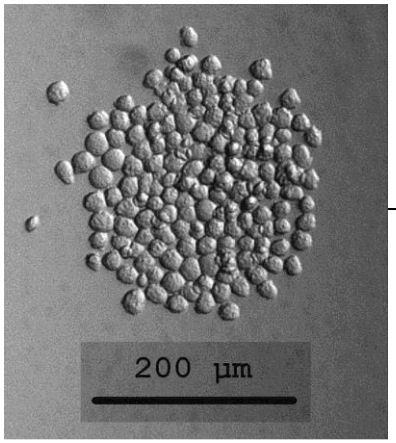


Fusion



Screen & Select

Hybridoma Colony



Clonal Cells

→

Monoclonal Antibodies (mAbs)

Characterization

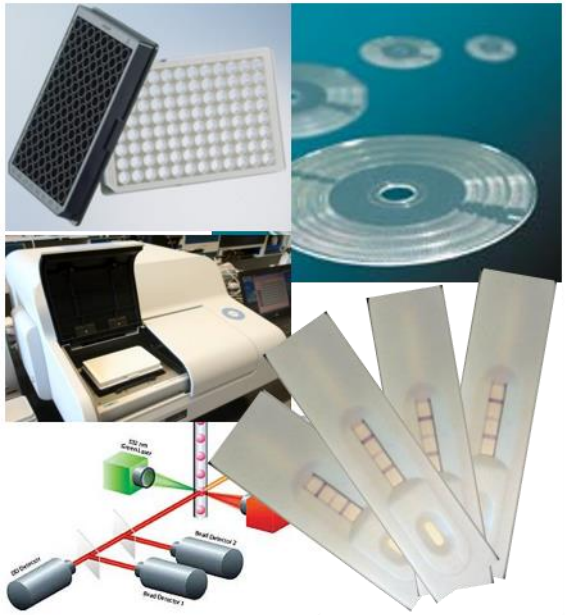
- Isotype
- Affinity
- Epitope Mapping
- Gene Sequencing
- Immunoassay selection

Advantages

- Stable Renewable Commodity
- Patent Protect and License

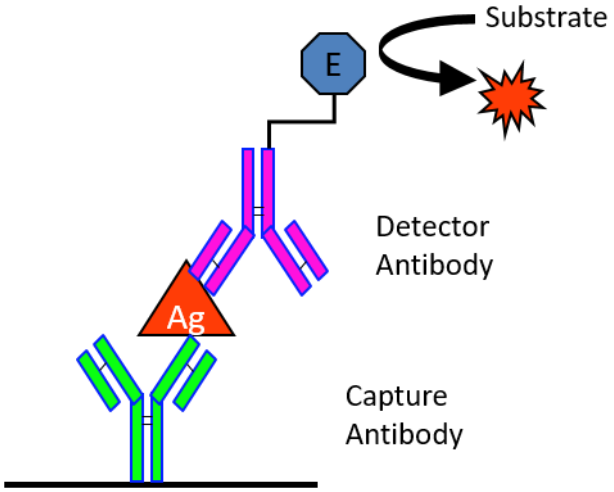
→

Immunoassay Platforms

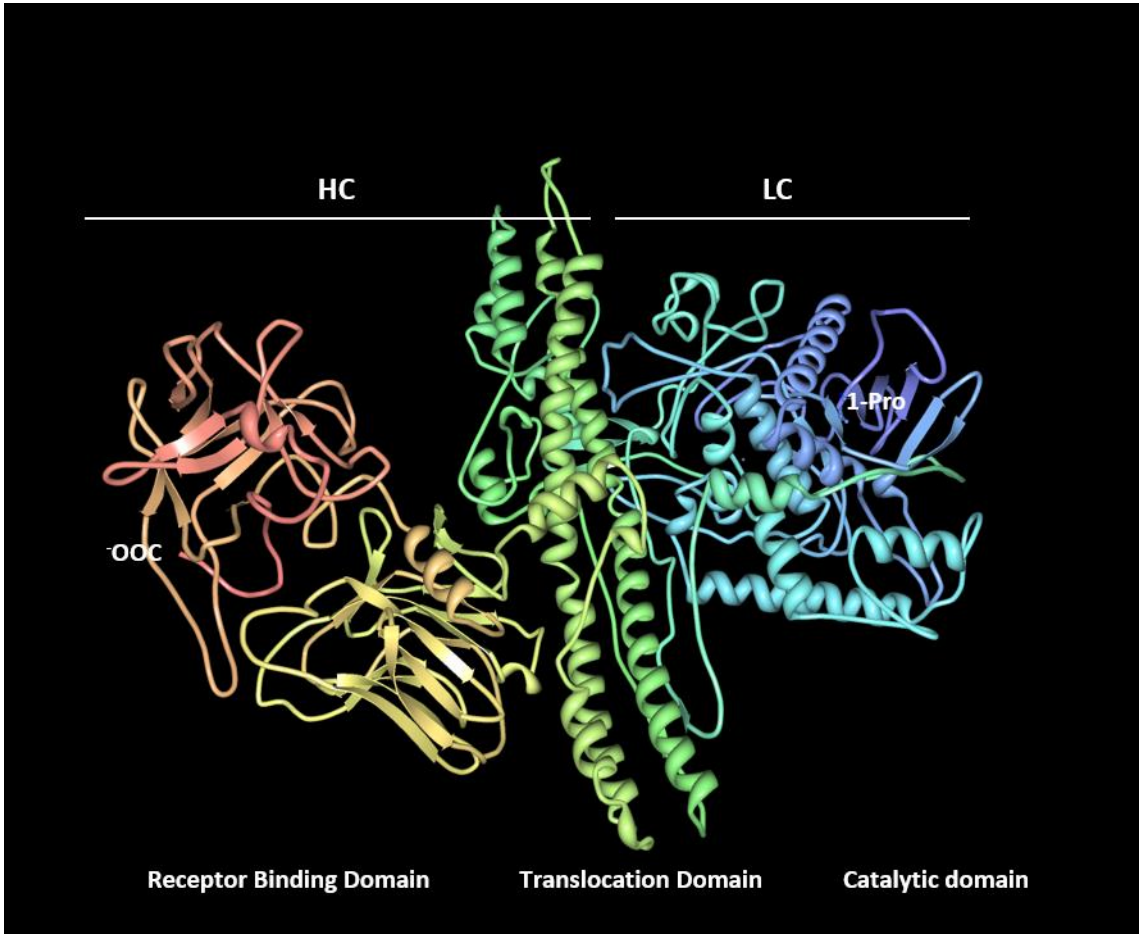


Antibody Based Detection Approaches

- Developed Monoclonal Antibodies (mAbs) to different toxin serotypes and accessory proteins
- Formatted these mAbs onto different platforms
  - ELISA
  - Luminex (bead based multi-analyte)
  - Electrochemiluminescent Measurement (MSD)
  - SpinDX assay
  - Lateral Flow Device
  - Immuno-PCR
- Stakeholders
  - Technology Transfer
    - Patents
    - License Agreements
    - Collaborations



Botulinum Neurotoxin



- Dichain polypeptide Toxin
- Synthesized by *Clostridium bolulinum*
- Synthesized as an ~ 150,000 dalton precursor protein, holotoxin
  - 100 kDa heavy chain
  - 50 kDa light chain
- Joined by a single disulfide bond
- Light chain is a Zn^{++} containing endopeptidase, cleaves SNARE Proteins
  - Blocks acetylcholine-containing vesicles from fusing with the terminal membrane of the motor neuron
  - Results in flaccid paralysis
- 8 distinct toxin serotypes describe (A-H)

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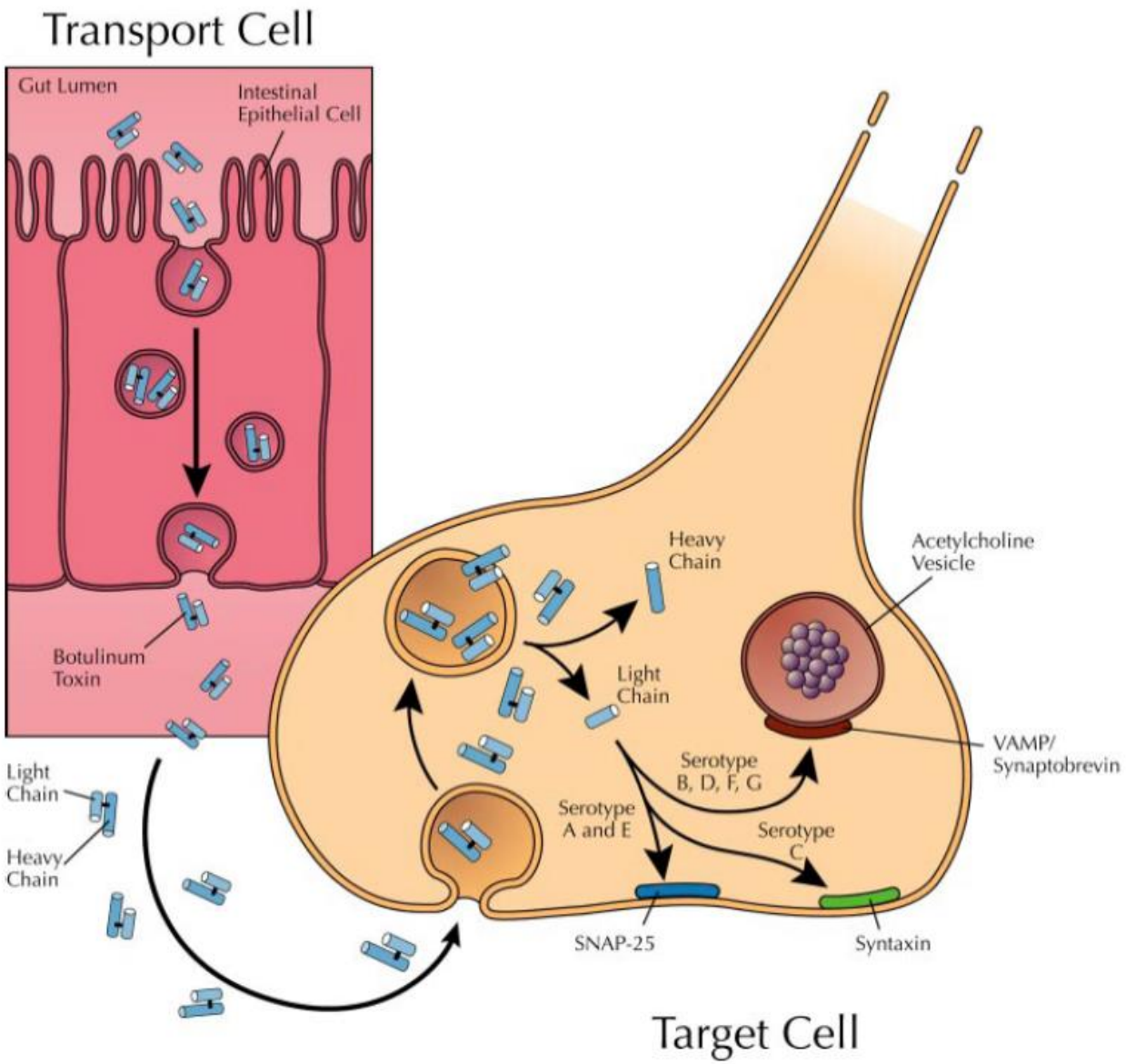
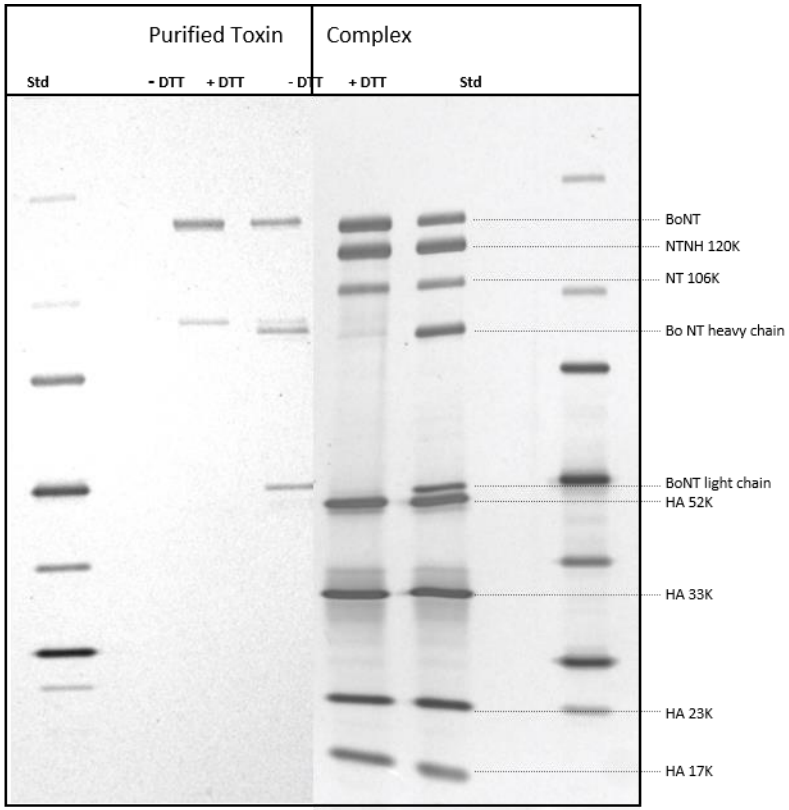
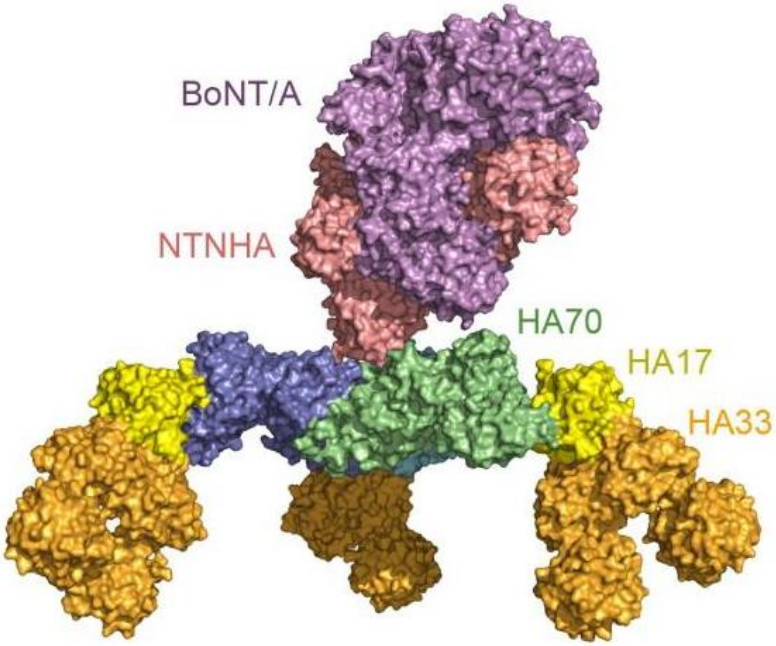
Major Steps in Toxin Action



Simpson L.L., Annu. Rev. Pharmal. 2004

Electrophoresis of BoNT type A Toxin and Complex





Silver stain 4-20% Gel

Toxin/Complex Source: Hall Strain
Metabiological

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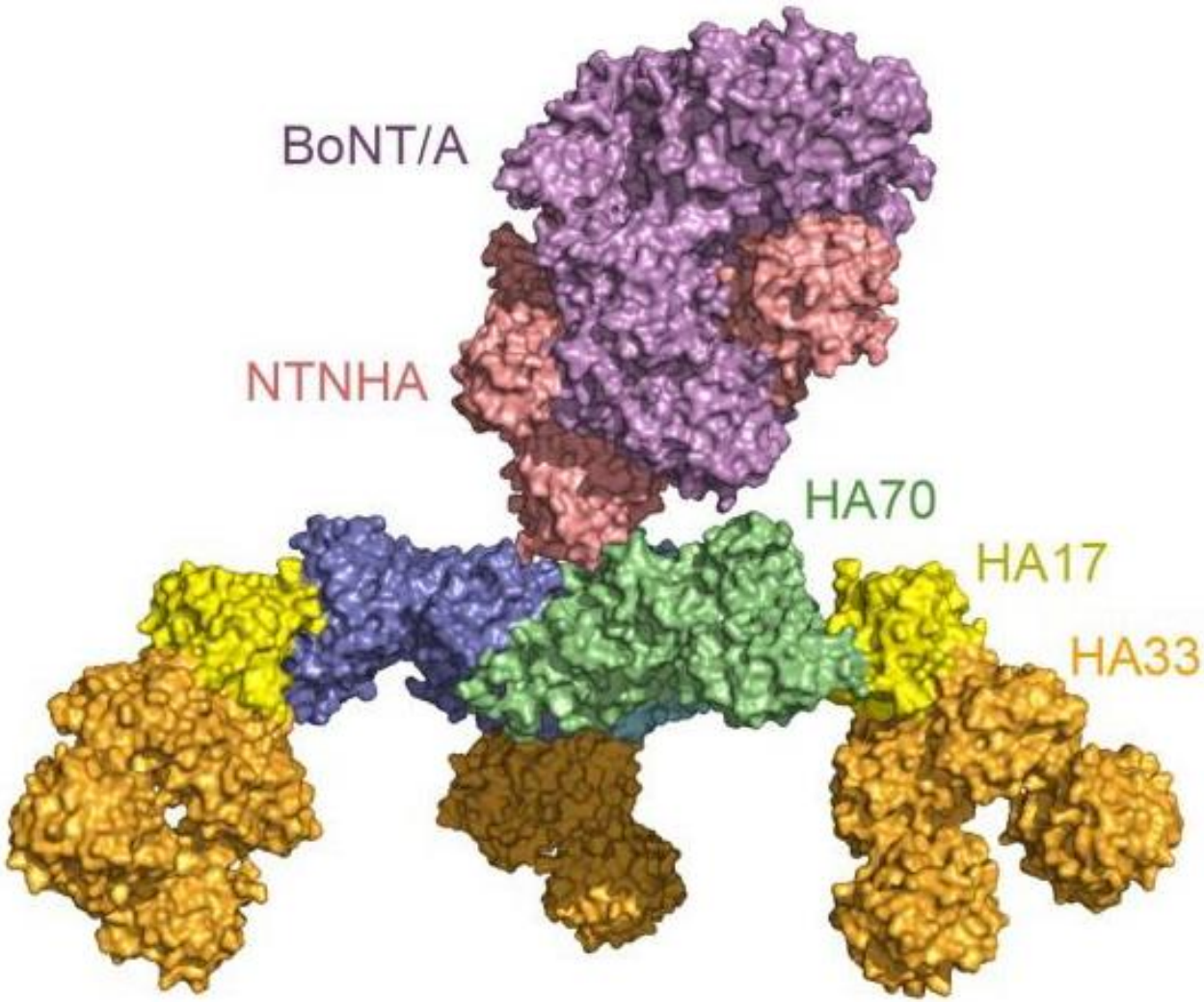
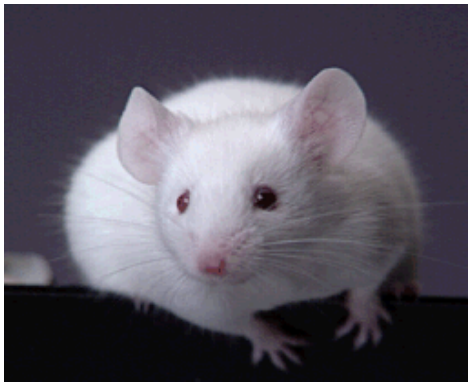
SDS-PAGE of BoNT/A complex



Detection Approaches

Mouse Bioassay

- gold standard



Advantages

- Exquisitely sensitive

10 pg/mouse

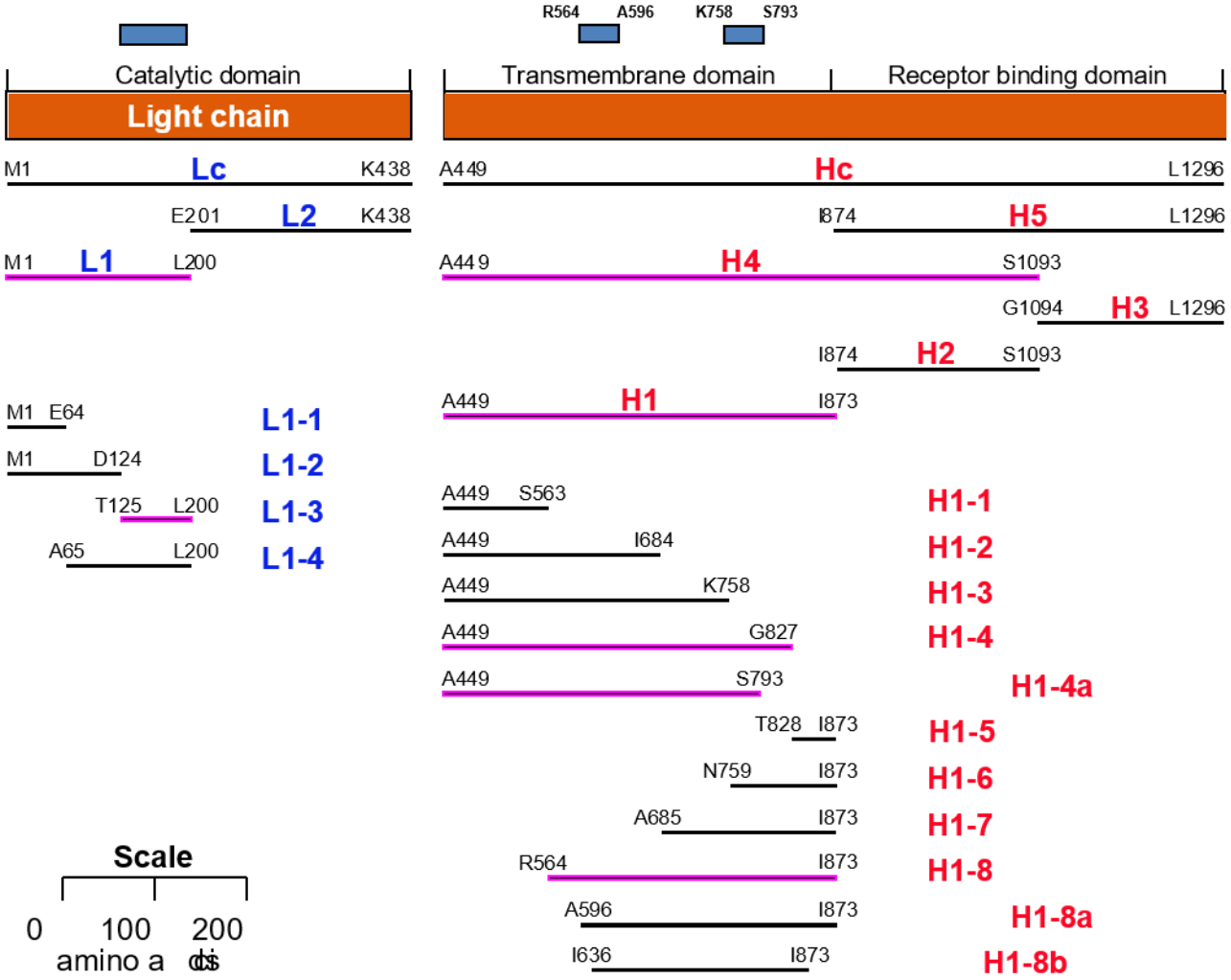
Disadvantages

- Hazardous
- Extensive animal use
- Non-specific
- Only 17 test sites in the U.S.

Anti-Botulinum Neurotoxin/A1

Name	Antigen	Myeloma	Binding Domain	Epitope	Isotype	ELISA	Capture		Serotype							Westerns	
									A	B	C	D	E	F	G		
F1-2-	BoNT/A	SP2/0 (davis)	H1	R564-A596 & K758-S793	IgG1	+	+	C	+	-	-	-	-	-	-	+	
F1-5	BoNT/A	SP2/0 (davis)	H1	R564-A596 & K758-S793	IgG1	+	+	C	+	-	-	-	-	-	-	+	purified ascites
F1-40	BoNT/A	SP2/0 (davis)	L1 / L1-3	Q138 – S145	IgG1	+	+		+	-	-	-	-	-	-	+	purified ascites
F1-23	BoNT/A	SP2/0 (davis)	Lc		IgG1	+			+			nd				nd	
F1-51	BoNT/A no binding peptides of types A,B,or E	SP2/0 (davis)	Hc		IgG1	+	+	D	+			nd				neg	purified ascites
F2-43	BoNT/A	SP2/0 (davis)	Lc, H1	R564 to S793	IgG1	+	+		+	-	-	-	-	-	-	+ native	purified ascites
F2-46	BoNT/A	SP2/0 (davis)	Hc		IgG1	weak			+	-	-	-	-	-	-	+	

Recombinant BoNT/A Peptides



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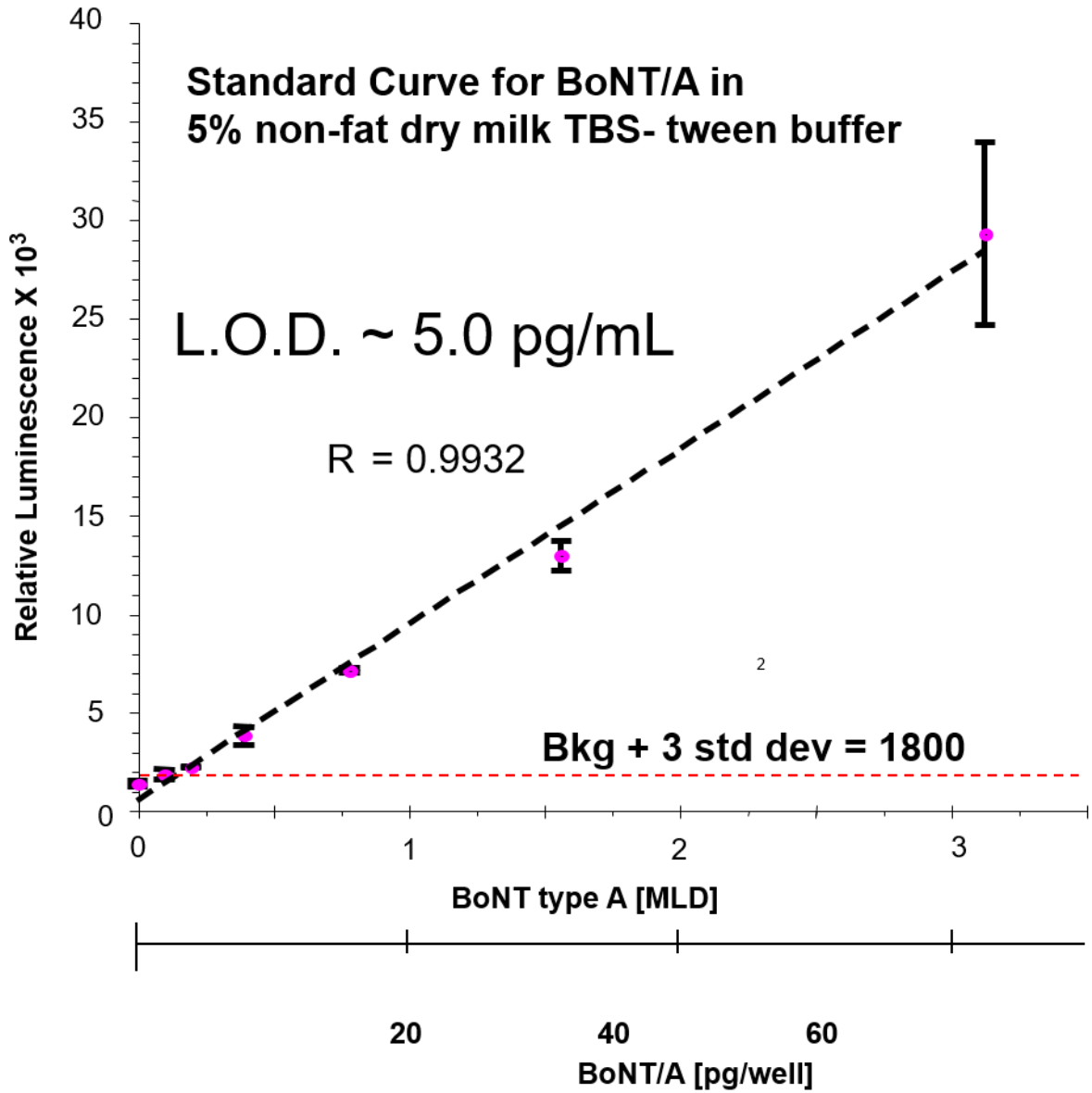
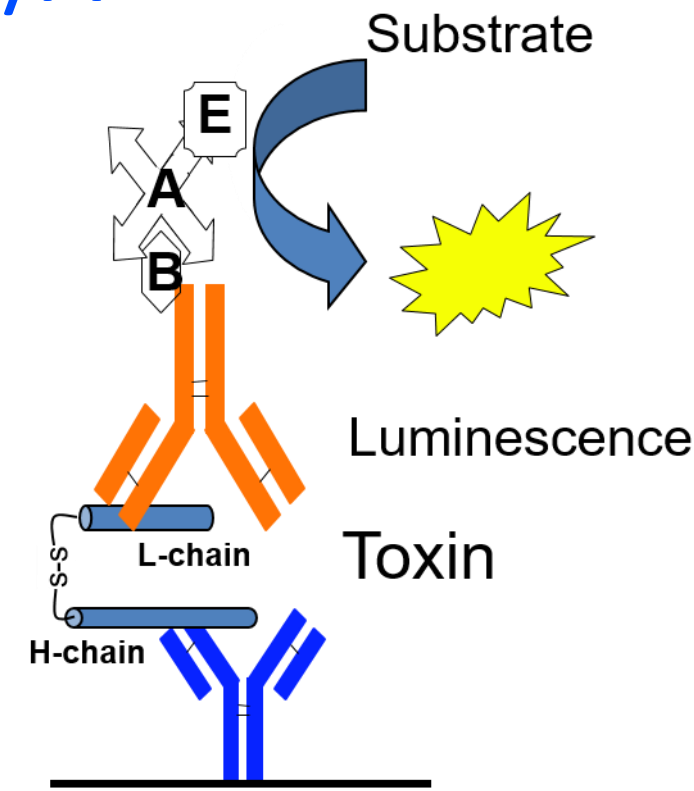
Sandwich ELISA for BoNT/A





B = Biotin
A = Streptavidin
E = Horseradish Peroxidase

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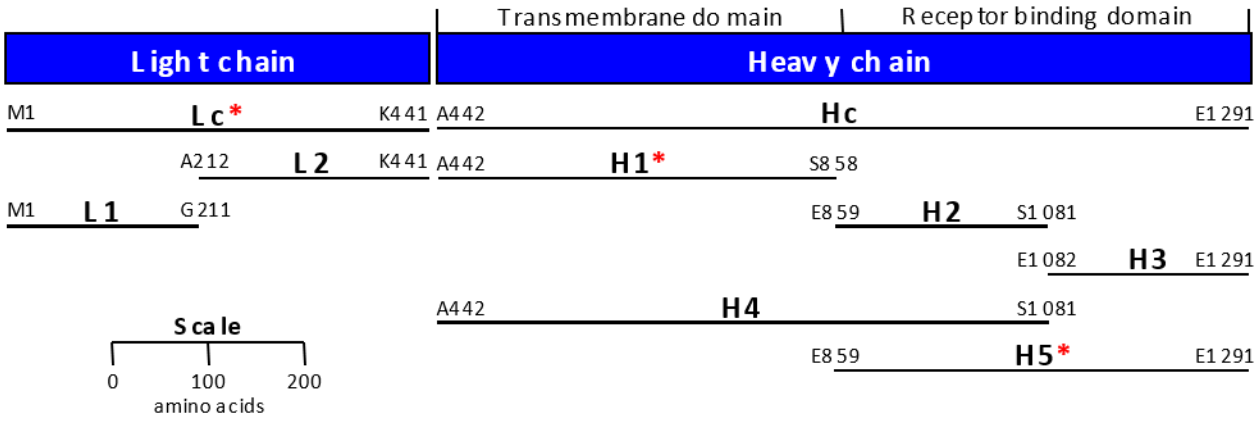
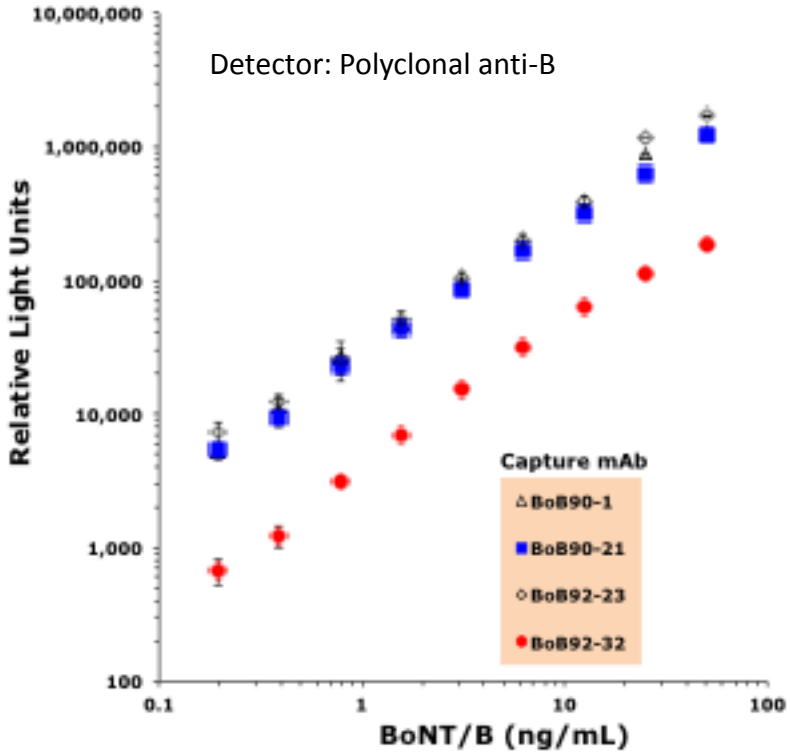
Recombinant BoNT/B *Domains*



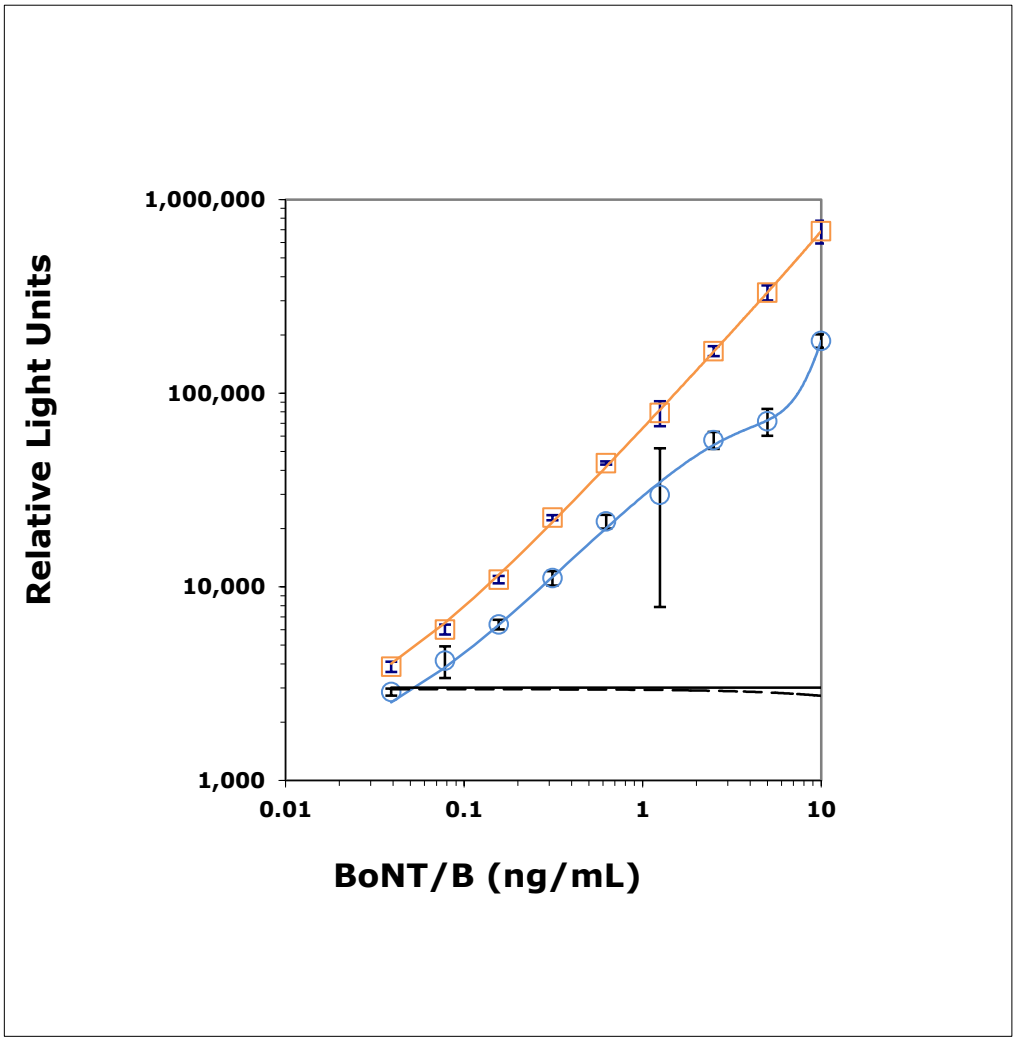
Anti-Botulinum Neurotoxin/B1

Monolonal	Antigen	Binding Domain	Epitope	Isotype	ELISA	Capture	capture /detector	Histo.	Serotype							Westerns	
									A	B	C	D	E	F	G		
MCS 6-27	BoNT/B HcP5	H5	E859-E1291	IgG1	poor	+	C		-	+	-	-	-	+			purified ascites
F24 4-2-4-3	BoNT/B Lc	Lc	nd		strong	-										nd	
F24 1-2-6-5	BoNT/B Lc	L2	A212-K441		strong	-			-	+	-	-	-	+		+	
F26 18-1-5-5	BoNT/B HcP5 peptide	H2	E859-E1291		strong	-						nd					
F26 16-5-3	BoNT/B HcP5 peptide	H4	E1082-E1291	IgA	strong	-			-	+	-	-	-	-		+	
F27 33	BoNT/B Lc	L2	A212-K441	IgG1	strong	-			-	+	-	-	-	-		+	
F28 69	BoNT/B HcP1 peptide	H1	A442-S858		strong	-						nd					
F28 80	BoNT/B HcP1 peptide	H1	A442-S858		strong	-						nd					
F28 85	BoNT/B HcP1 peptide	H1	A442-S858		strong	-						nd					
F28 90	BoNT/B HcP1 peptide	H1	A442-S858		strong	-						nd					
F29 38	BoNT/B HcP5 peptide	H5	E859-E1291	IgM	strong	-			-	+	-	-	-	-		nd	
F29 40	BoNT/B HcP5 peptide	H5	E1082-E1291	IgG1	strong	-			-	+	-	-	-	-		+	
BoB 92-23-23-7	BoNT/B HcP5 peptide	H5	E859-E1291	IgG2b	poor	+	C					nd				+ Weak	purified ascites
BoB 92-32-1-10	BoNT/B HcP5 peptide	H5	E859-E1291	IgG1	poor	+	D					nd				+ Weak	purified ascites
BoB 90-1-5-1	BoNT/B HcP5 peptide	H5	E859-E1291	IgG1	poor	+						nd				+ Weak	purified ascites
BoB 90-21-9-2	BoNT/B HcP5 peptide	H5	E859-E1291	IgG1	poor	+						nd				+ Weak	purified ascites

Identification of anti-BoBNT/B Capture mAbs



Capture ELISA using anti-BoNT/B Mabs



Capture mAbs

MCS6-27 Blue

BoB92-23 Orange

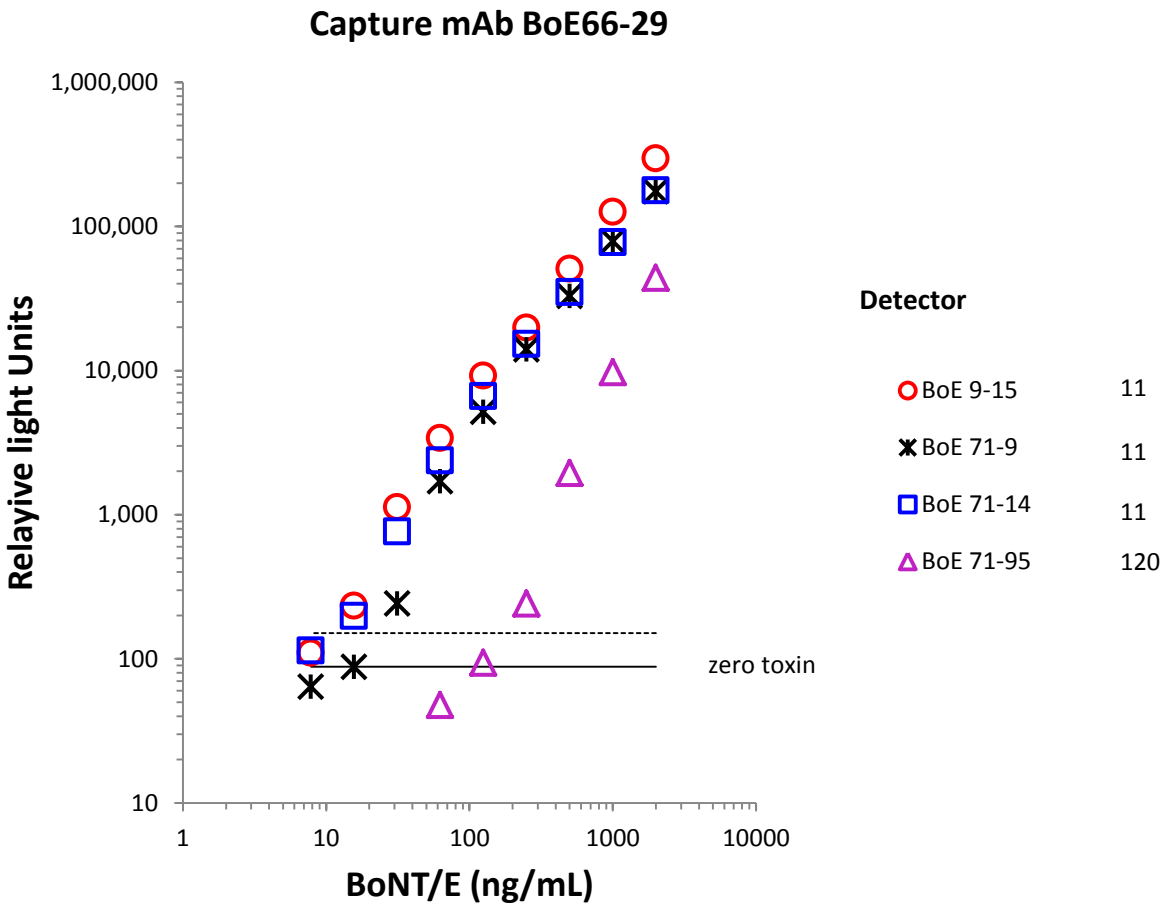
Detector mAb

BoB92-32

Anti Botulinum Neurotoxin Serotrype E

Monolonal	old name	sister	Antigen	Mouse	Myeloma	Binding Domain	Epitope	Isotype	ELISA	Capture	capture /detector	Histo.	Serotype A	B	C	D	E	F	G	Westerns
BoE 9-15			BoNT/E HcP1	Bal/c	SP2/0 davis	H1,H4	R422-I847	IgG2b	+ poor	+			-	-	-	-	+	-	-	Hc
BoE66-29-10-6	MCS66-29-10-6		BoNT/E HcP5	Bal/c	P3XU.1	H2,H4,H5	K848-T1069	IgG1	+	+			-	-	-	-	+	-	-	Hc
BoE 66-81-27-6	MCS 66-81-27-6		BoNT/E HcP5	Bal/c	P3XU.1	H2,H4,H5	K848-T1069	IgG2b	+	+			-	-	-	-	+	-	-	Hc
BoE 66-102-5-2	MCS 66-102-5-2		BoNT/E HcP5	Bal/c	P3XU.1	H5	K847-K1253	IgG1	nd	+					nd					nd
BoE 66-106-2-8	MCS 66-106-2-8		BoNT/E HcP5	Bal/c	P3XU.1	H3,H5	N1070-K1253	IgG1	+	+			-	+	-	-	+	-	-	Hc
BoE 71-9-2-13	MCS 71-9-2-13		BoNT/E HcP5	Bal/c	SP2/0 davis	H2,H4,H5	K848-T1069	IgG1	poor	weak			-	-	-	-	+	-	-	Hc
BoE 71-14-14-4	MCS 71-14-14-4		BoNT/E HcP5	Bal/c	SP2/0 davis	H2,H4,H5	K848-T1069	IgG1	+	+			-	-	-	-	+	-	-	Hc
BoE 71-95-10-8	MCS 71-95-10-8		BoNT/E HcP5	Bal/c	SP2/0 davis	H2,H4,H5	K848-T1069	IgG1	poor	+			-	-	-	-	+	-	-	Hc
BoE 33-10-13-13	MCS 33-10-13-13		BoNT/E Lc	Bal/c	SP2/0 davis	L1	M1-K225	IgG2b	+	+			-	+	-	-	+	+	-	Lc
BoE 33-12-10-7	MCS 33-12-10-7		BoNT/E Lc	Bal/c	SP2/0 davis	L1	M1-K225	IgG1	+	+			-	+	-	-	+	+	-	Lc
BoE 33-13-17-4	MCS 33-13-17-4		BoNT/E Lc	Bal/c	SP2/0 davis	L1	M1-K225	IgG1	+	+			-	-	-	-	+	+	-	Lc
BoE 33-13-17-9	MCS 33-13-17-9		BoNT/E Lc	Bal/c	SP2/0 davis	L1	M1-K225	IgG1	+	+			-	+	-	-	+	+	-	nd
BoE 34-16-10-37	MCS 34-16-10-37		BoNT/E Lc	Bal/c	SP2/0 davis	L1		IgG2a	+	+			-	+	+	+	+	+	+	Lc
EHc1-10-2-6			BoNT/E HcP1	Balb/c	SP2/0 davis	No binding		IgG2a	+	-			-	-	-	-	+	-	-	Hc
EHc1-11-5-5			BoNT/E HcP1	Balb/c	SP2/0 davis	HcP1 only	K423-I847	IgM/IgA	+	-										Hc
EHc1-18-5-5-5			BoNT/E HcP1	Balb/c	SP2/0 davis	No binding		IgM		-										Hc
EHc1-26-4-1			BoNT/E HcP1	Balb/c	SP2/0 davis	HcP1, HcP2, HcP5	K848-T1069	IgG1	+	-										Hc
EHc1-33-5-3			BoNT/E HcP1	Balb/c	SP2/0 davis	HcP1, HcP4 (weak)	K423-I847	IgG1	+	-										Hc
EHc1-38-3-1			BoNT/E HcP1	Balb/c	SP2/0 davis	HcP1 (weak), HcP2, HcP5	K848-T1069	IgG2a	+	-										Hc
EHc1-40-3-2			BoNT/E HcP1	Balb/c	SP2/0 davis	HcP1 (weak), HcP2, HcP5	K848-T1069	IgG2b	+	-										Hc
EHc1-44-3-6			BoNT/E HcP1	Balb/c	SP2/0 davis	HcP1, HcP4 (weak)	K423-I847	IgG2a	+	-										Hc
EHc1-47-6-1			BoNT/E HcP1	Balb/c	SP2/0 davis	HcP1	K423-I847	IgG2a	+	-										Hc
EHc1-48-6-2			BoNT/E HcP1	Balb/c	SP2/0 davis	HcP1, HcP4 (weak)	K423-I847	IgG2b	+	-										Hc
EHc5-			BoNT/E HcP5	Balb/c	SP2/0 davis			?	+	?										

Capture ELISA for BoNT/E



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Antibody Pairs for BoNT Detection by cELISA

Monoclonal Antibodies			Isotype	Epitope	Affinity (KD)	LOD)
BoNT /A	F1-51	Capture	IgG1, kappa	Heavy chain	nd	
	F1-2	Detector	IgG1, kappa	TM domain	16.6×10^{-12}M	5 pg/mL
BoNT /B	6-27	Capture	IgG1, kappa	RBD	8.6×10^{-11}M	
	92-32	Detector	IgG1, kappa	Heavy chain	48.3×10^{-11}M	20 pg/mL
BoNT /E	66-29	Capture	IgG1, kappa	Heavy chain	nd	
	9-15	Detector	IgG1, kappa	Heavy chain	nd	11 ng/mL

Percentage recovery of BoNT/B spiked into different food matrices.

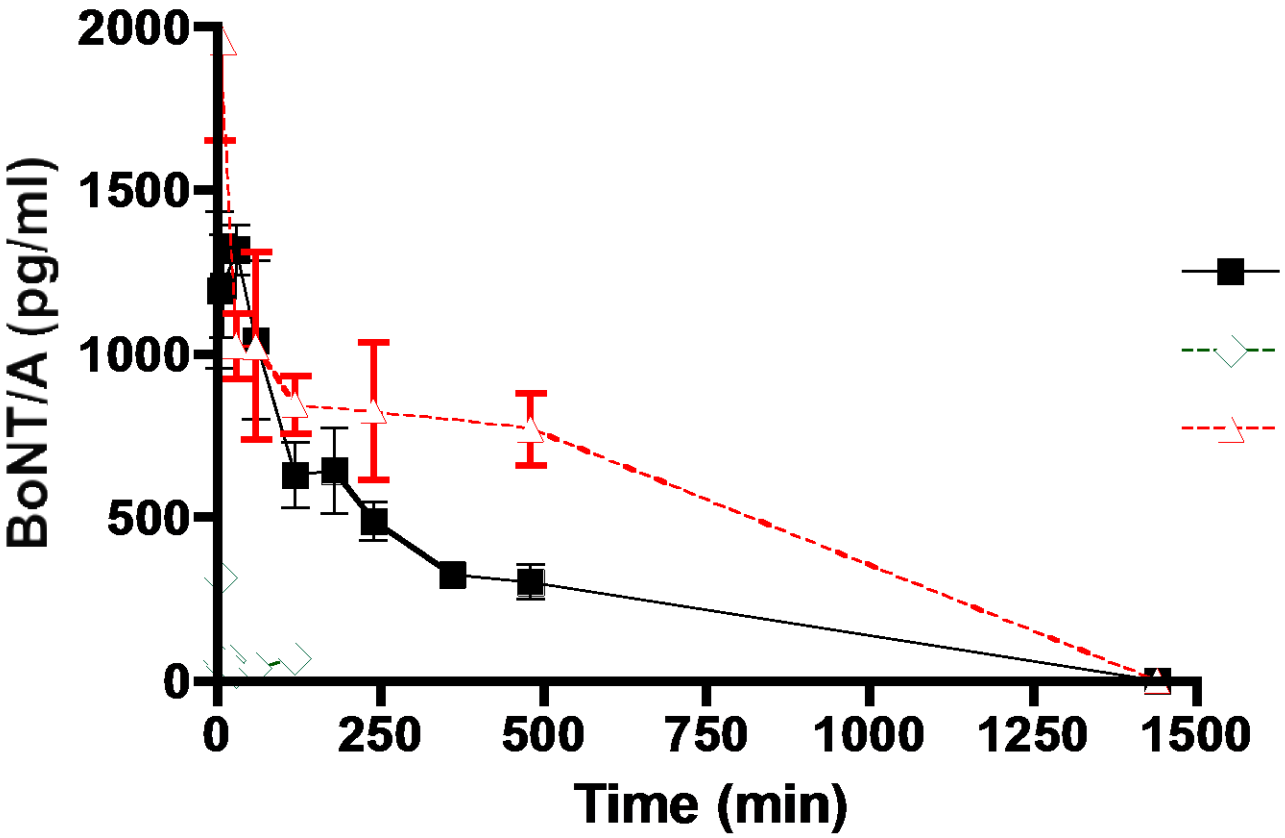
Table 2

Percentage recovery of BoNT/B spiked into different food matrices.

	Percentage Recovery				
Matrix / Spike Level	50 ng/mL	10 ng/mL	5 ng/mL	2 ng/mL	0.5 ng/mL
Non Fat Milk	108 (54)[1]	nd[2]	89 (4.45)	nd	131 (0.655)
2% Milk	103 (48.5)	nd	76 (33.5)		131 (0.365)
Whole Milk	101 (50.5)	nd	105 (5.25)	nd	116 (0.58)
Ground Beef 95% Lean	77 (31.9)	124.1 (5.8)	nd	100 (2)[3]	nd

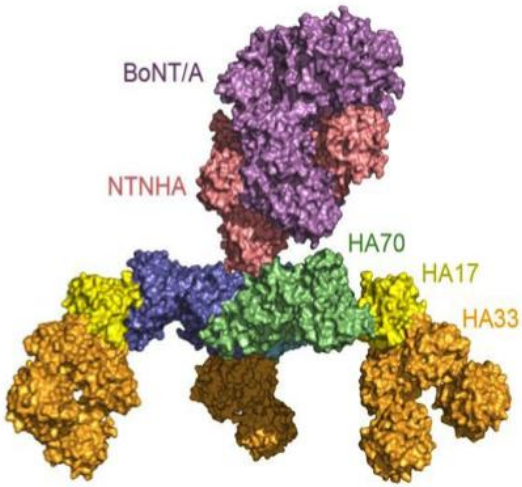
- Numbers in brackets refer to toxin level measured at each spike concentraion using the buffer standard curve.
- nd = not done.
- signal from beef sample spiked at 2 ng/mL was just at the L.O.D. for standard.

Clearance of BoNT/A in sera following mAb treatment



Botulinum toxins and their complex associated proteins

Serotype A	Serotype B	Serotype E
BoNT	BoNT	BoNT
NTNH	NTNH	NTNH
HA 70 (HA55/ HA19/20)	HA70	
HA 33/35	HA 33/35	
HA 17	HA17	



NAPs have been shown to protect BoNT from degradation in the digestive tract and HA proteins have binding affinities for epithelial cells. NAPs are not required for toxicity as holotoxin and BoNT/E are still absorbed.

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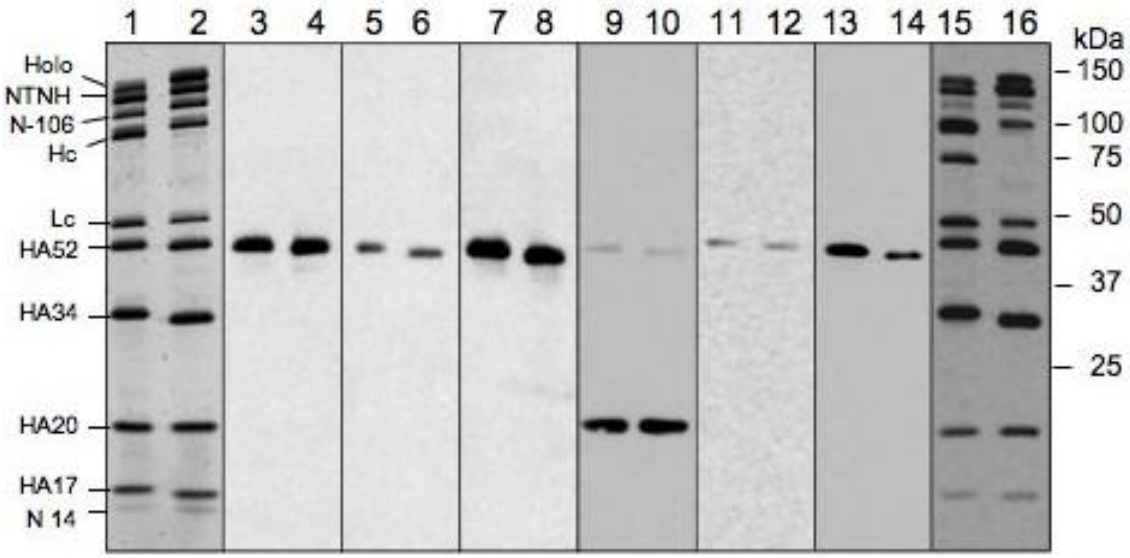
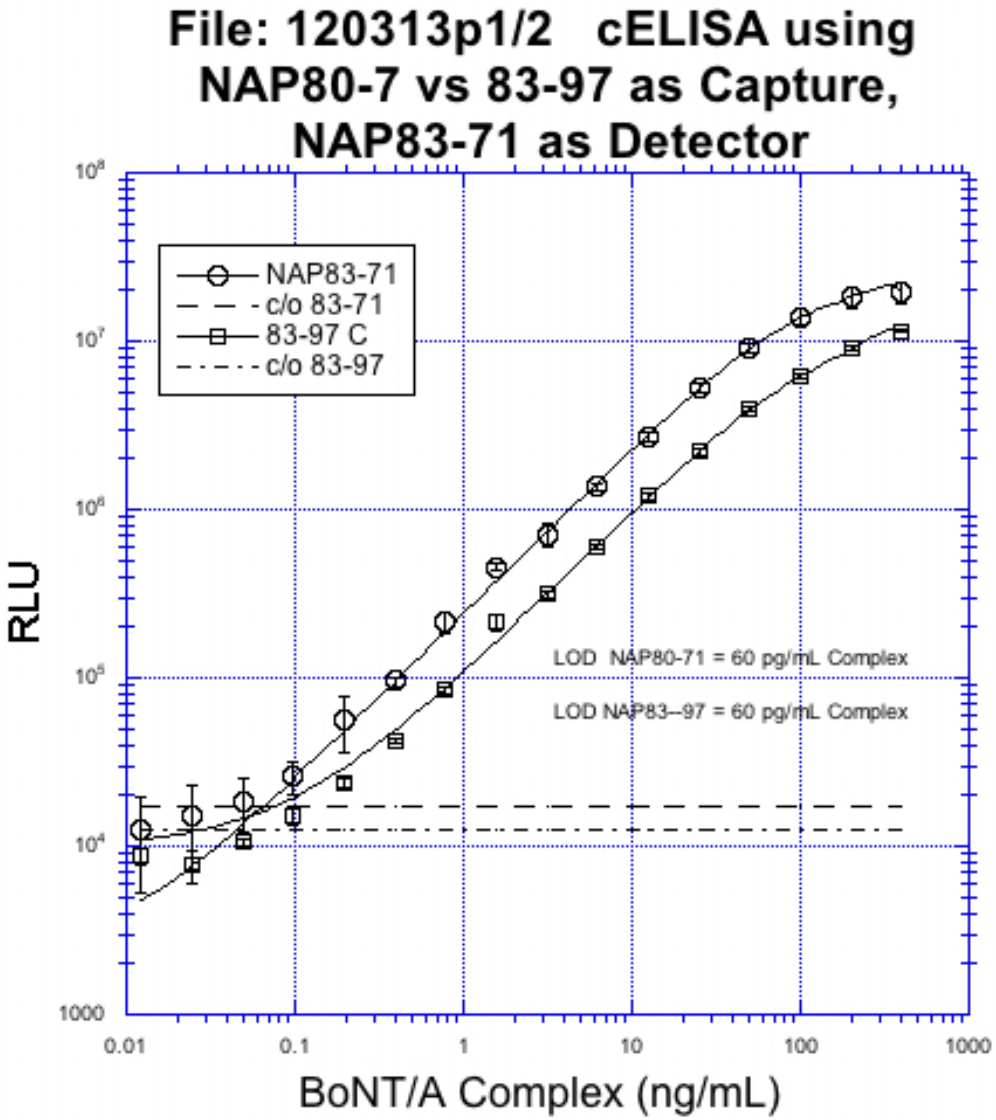
Western Blot using anti –HA70 mAbs



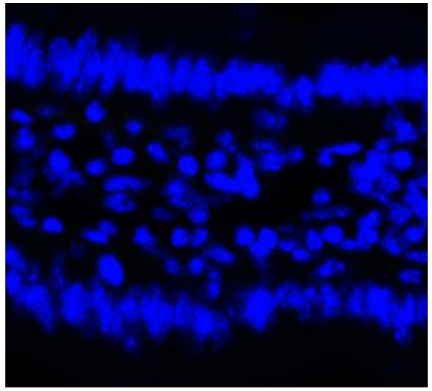
Antibody binding on Western blots to BoNT/A and BoNT/B toxin complex component proteins. 900 kDa BoNT/A complex (even numbered lanes), and BoNT/B complex (odd numbered lanes) separated on 10 % SDS-PAGE in the presence of 10 mM DTT, transferred to nitrocellulose membranes and probed with mAbs NAP80-5 (lanes 3-4), NAP80-7 (lanes 5-6), NAP80-33 (lanes 7-8), NAP83-27 (lanes 9-10), NAP83-71 (lanes 11-12), and NAP83-97 (lanes 13-14). Lanes 1-2 shows the serotype A and B compex components stained with SilverXpress Silver Stain, and lanes15-16 show biotinylated BoNT/A and BoNT/B toxin complexes, probed with 125 ng/ml ZyMax Streptavidin-HRP Conjugate in TBST (Zymed)

Detection of NAP HA70 by Capture ELISA

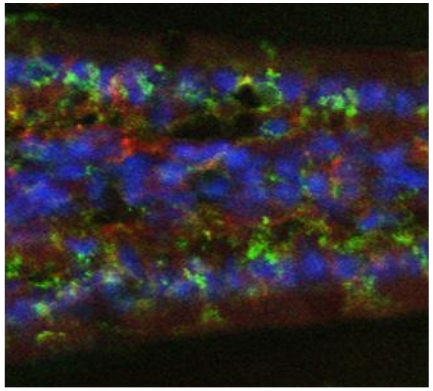


Passage of BoNT/A Complex Through the Mouse Small Intestine Over Time

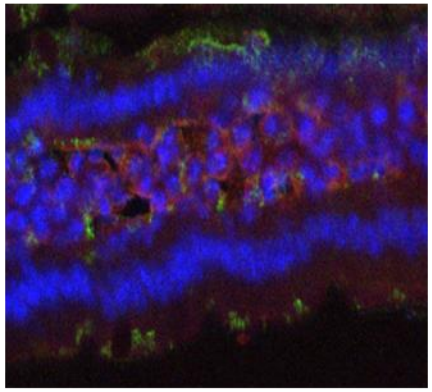
No Toxin



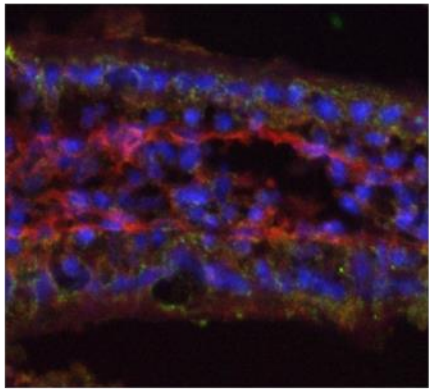
6 h



2 h



8 h



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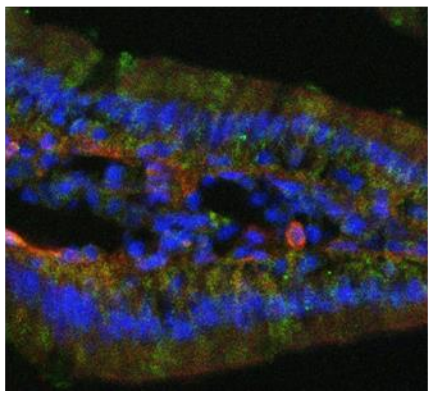

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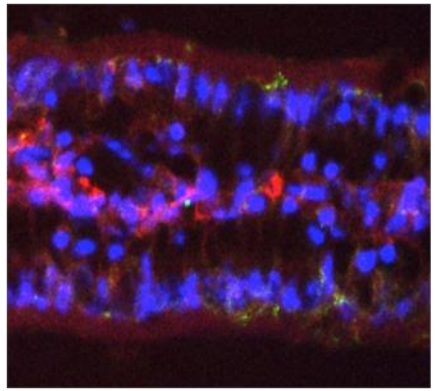
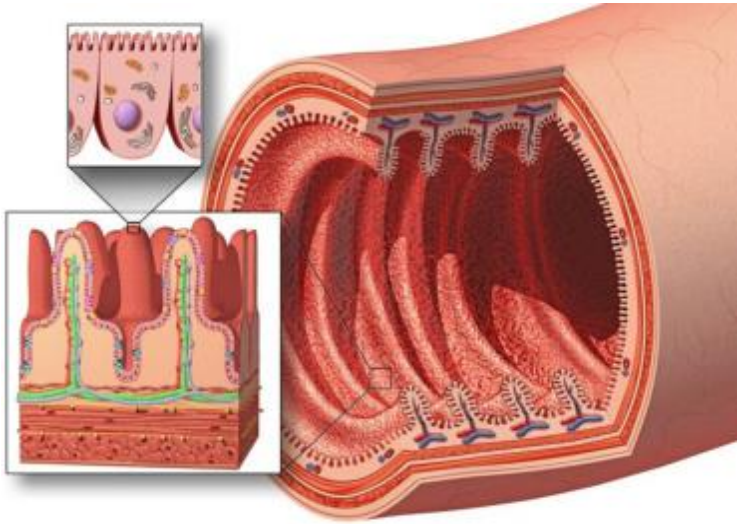
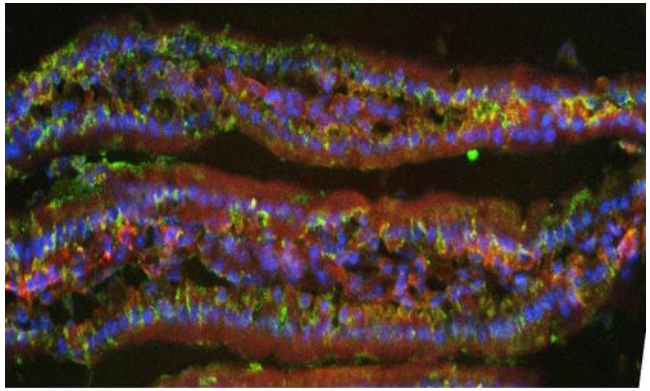


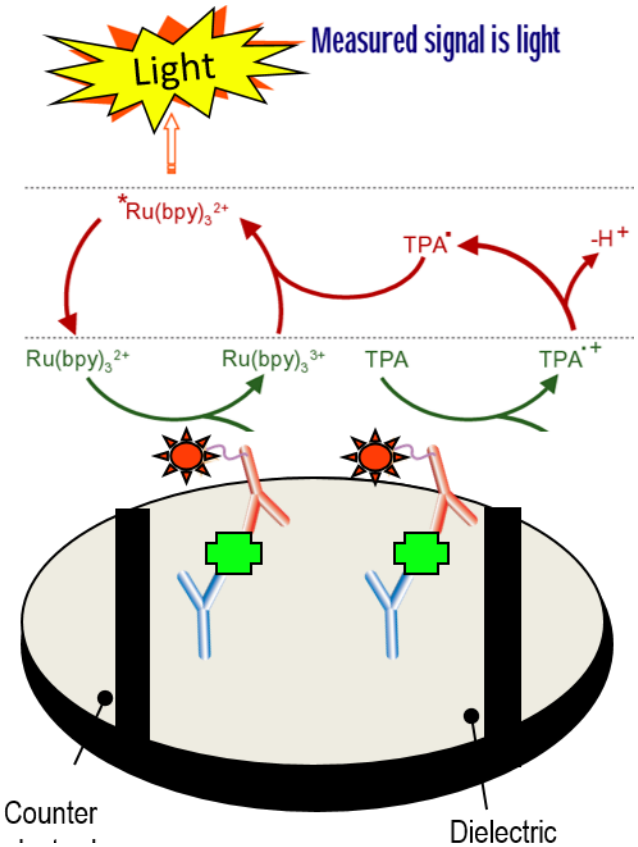


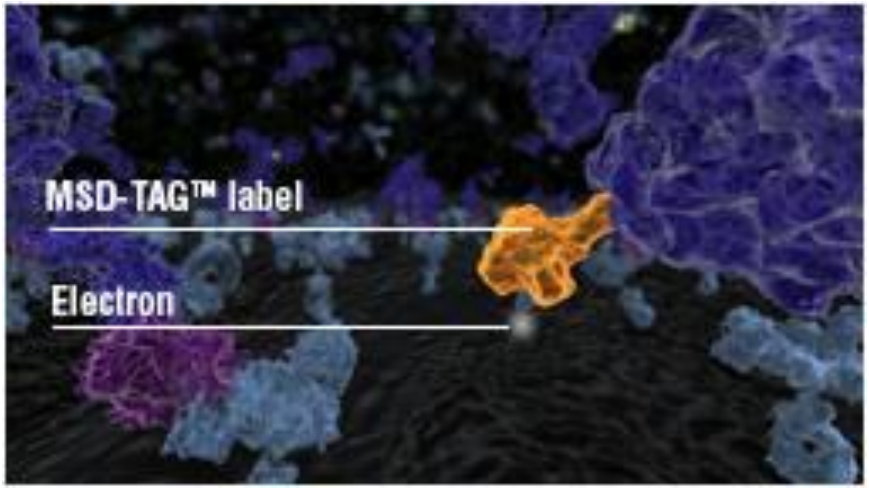


Green:=toxin Red:=HA70

Lam et al., Cellular Microbiology, 2015

Meso Scale Discovery (MSD) Electrochemiluminescense (ECL) assay





ELISA like assay

The detection process is initiated at electrodes located at the bottom of microplates.

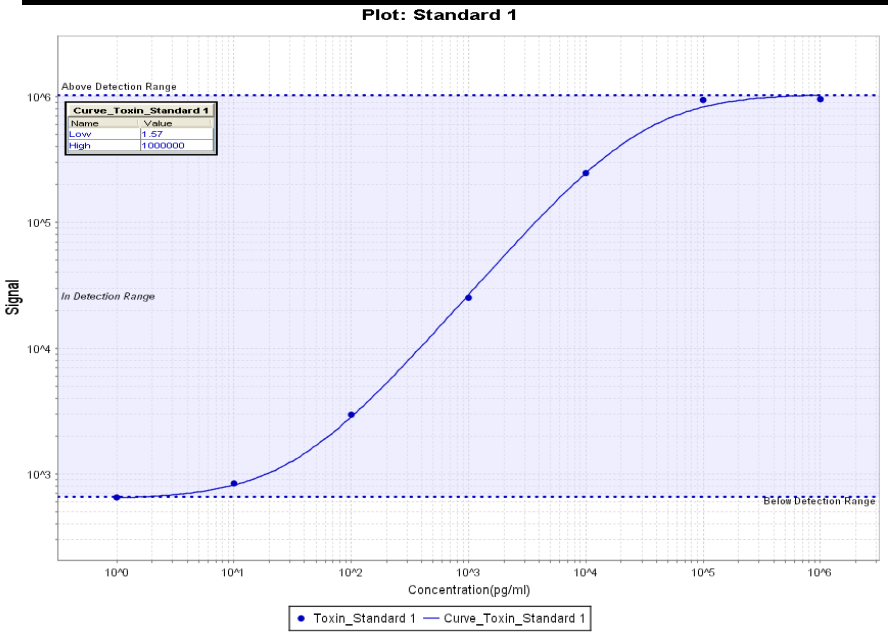
Electrochemiluminescent labels emit light when electrochemically stimulated

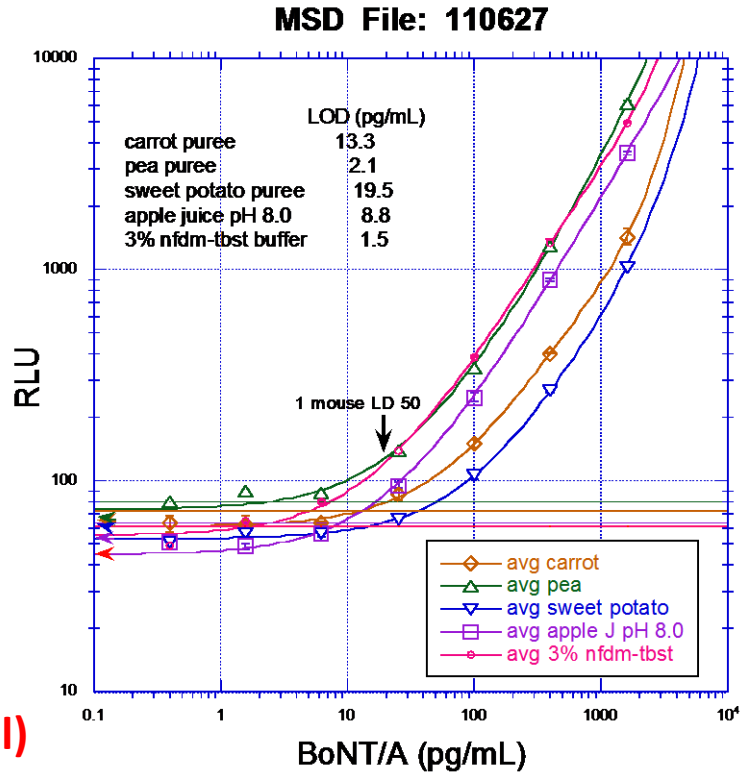
Only labels near the electrode are excited and detected, enabling non-washed assays

Sector Imager 2400

MSD Data Output

	1	2	3	4	5	6	7	8	9	10	11	12
A	956004	964955	955946	902401	957625	939171	433769	270475	176738	79628	860336	523661
B	941479	945102	926408	780927	960364	958094	229296	209654	146949	70549	823134	470945
C	246885	241862	212538	164669	658604	641151	30981	29254	26366	17643	106560	104594
D	25165	25491	23033	17362	83630	76453	3153	3236	2778	1912	10698	11002
E	2957	2847	2622	1962	8604	7000	415	414	346	259	1624	1556
F	839	629	585	353	1612	1347	157	134	127	111	763	654
G	649	347	327	184	1080	712	133	113	93	87	710	624
H	610	339	264	186	1065	581	139	116	102	93	695	577





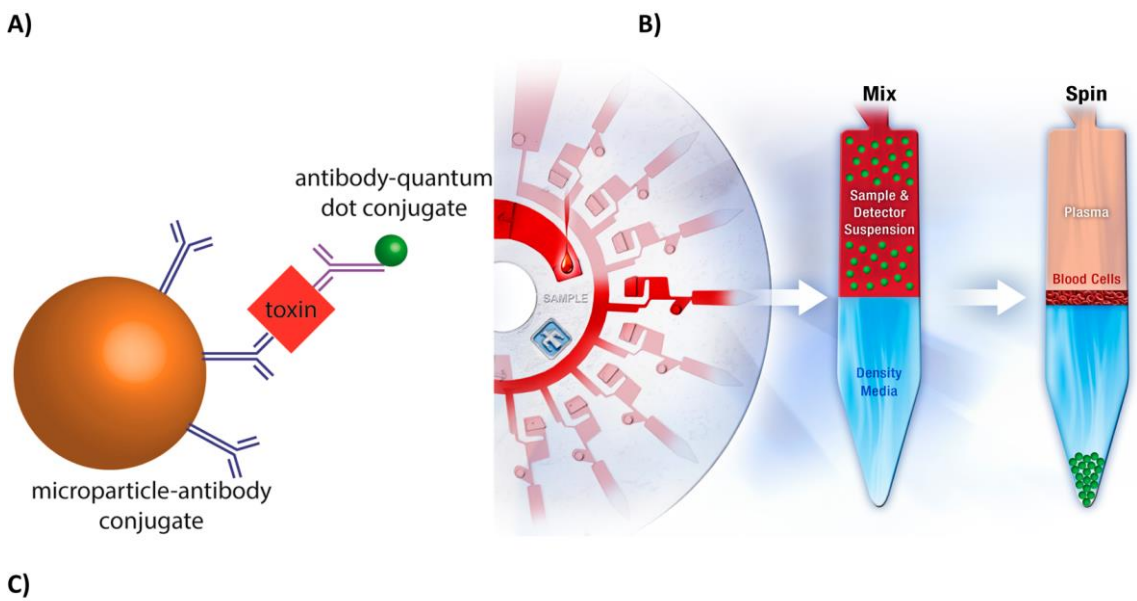
Linear range of 3-4 logs (10 pg/ml – 10,000 pg/ml)

Detecting Biotoxins using Sandia's SpinDx Platform

Sandia: Anup Singh, Greg Sommer, Ulrich Schaff, Chung-Yan Koh,
Matt Piccini, Ron Renzi
USDA: Larry Stanker, Luisa Cheng
U Mass, Dartmouth: Bal Ram Singh, Easwaran Ravichandran

FUNDING:
NIAID (PI: Anup Singh)
Contact: aksingh@sandia.gov

Detecting Biotoxins using SpinDx Platform



Additional Targets:

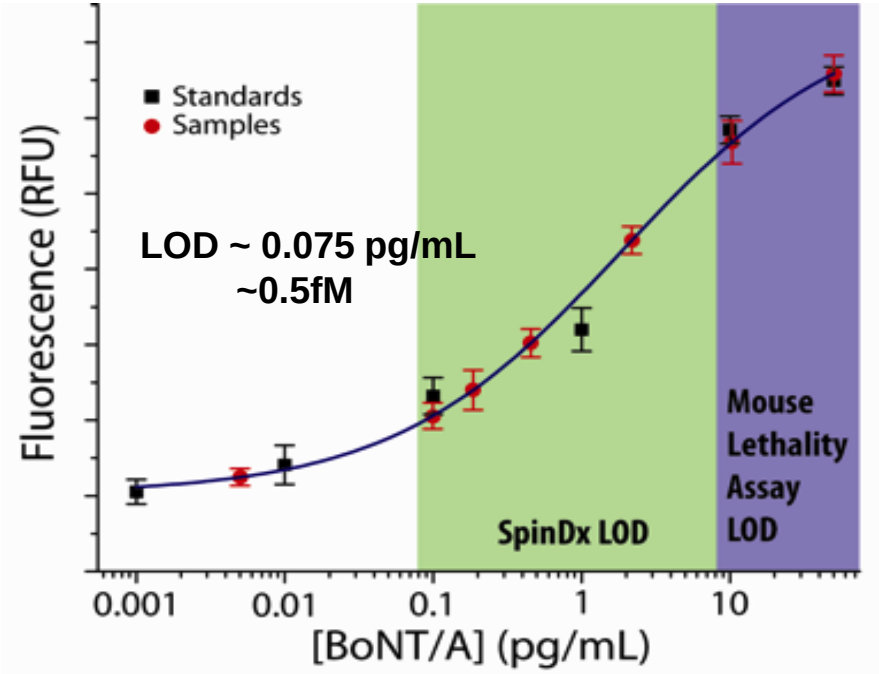
- Botulinum toxin serotypes
- Ricin
- Abrin
- Shiga toxin

Sandia National Laboratory: Anup Singh, Greg Sommer, Ulrich Schaff, Chung-Yan Koh, Matt Piccini, Ron Renzi

SpinDx BoNT/A assay performance vs. gold standard mouse bioassay and ELISA

Head-to-head comparison with mouse lethality assay and ELISA results from USDA-Albany (all experiments conducted on same day with same samples).

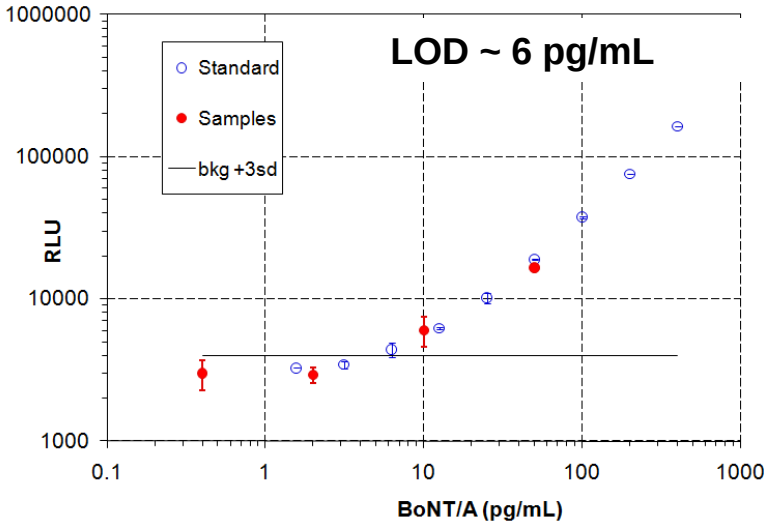
SpinDx



Mouse lethality assay

BoNT/A (pg/mL)	Estimated Mouse LD_{50}	Deaths (X/10)	Wasp Waist (X/10)
100	3.4	10/10	10/10
50	1.7	8/10	9/10
10	0.6	0/10	8/10
2	0.1	0/10	0/10

ELISA



Lateral Flow Immunoassay (LFIA)

LFIA Format

Sandwich ELISA

Antibodies

Capture mAb – BioJet on BioDot XYZ platform.

1. Antigen specific capture mAb at (**T**) test line
2. Anti-mouse capture mAb at (**C**) control line

Detection mAb – AirJet on BioDot XYZ platform.

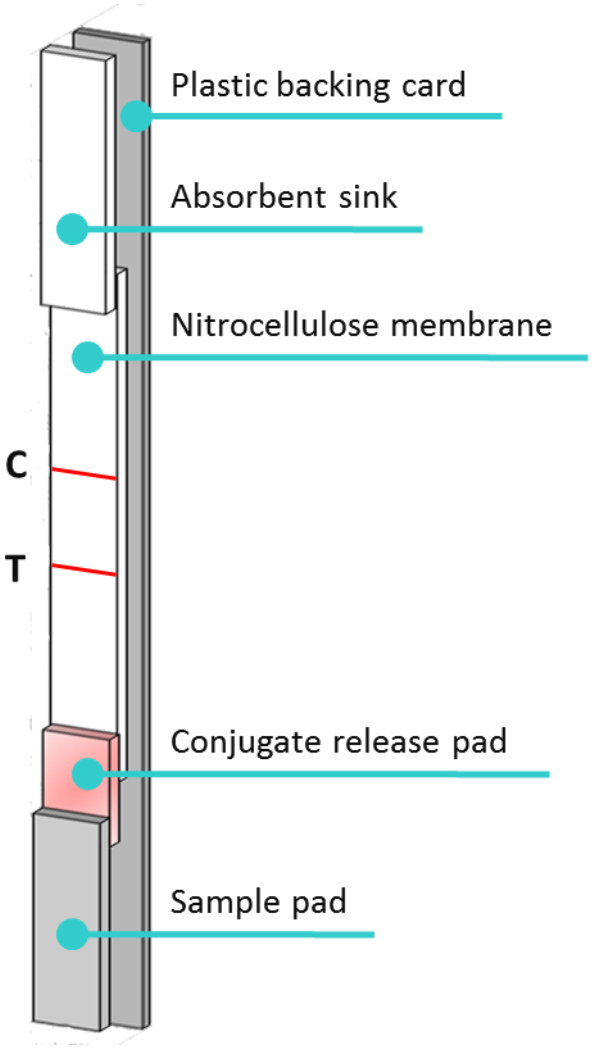
Antigen specific mAb labeled with a reporter molecule embedded in conjugate release pad

Reporter

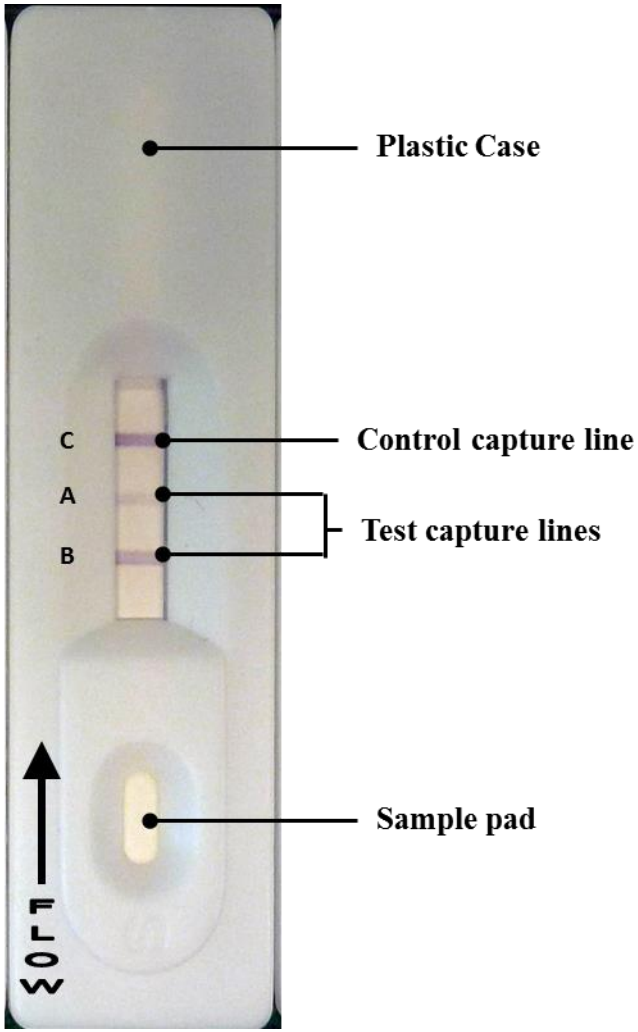
Gold Nanoparticles

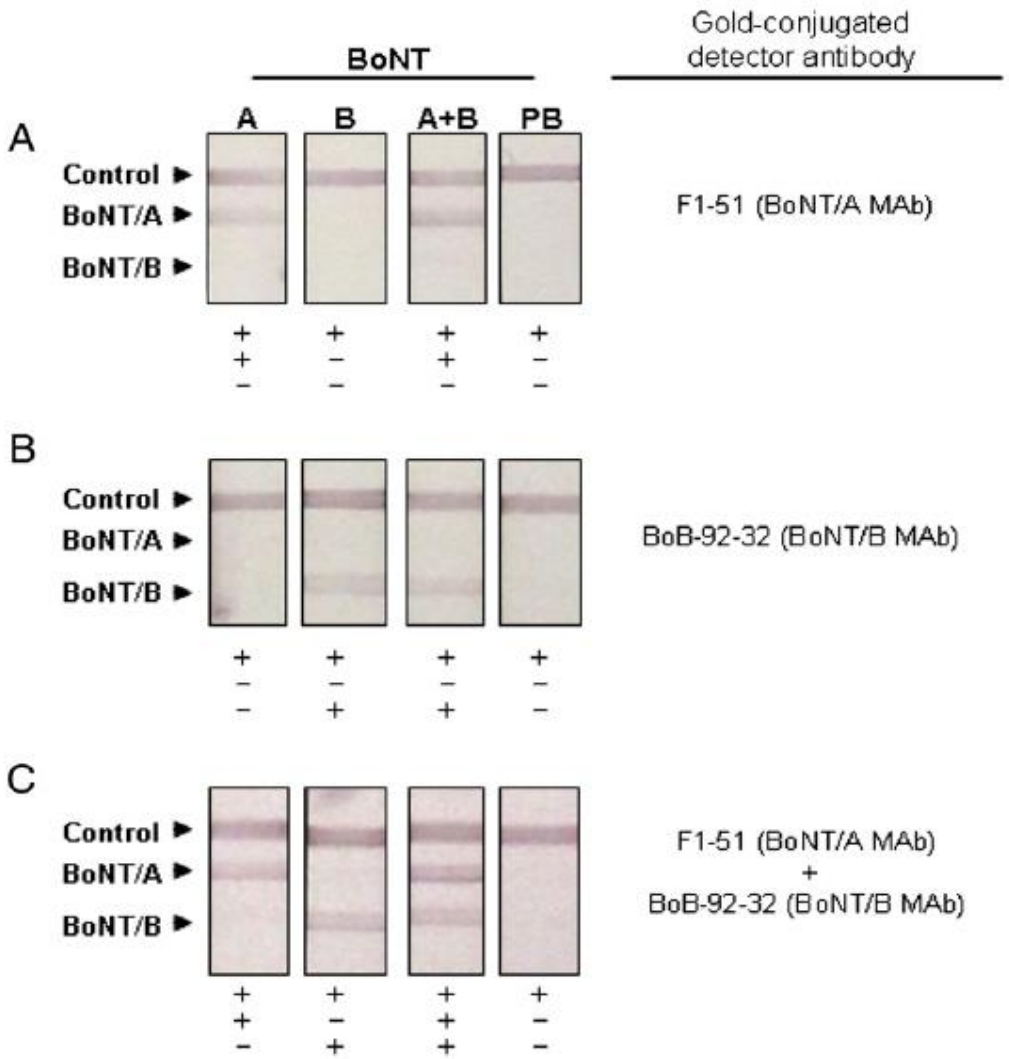
Detection

Visual Inspection



Detection of BoNT Serotypes by LFIA

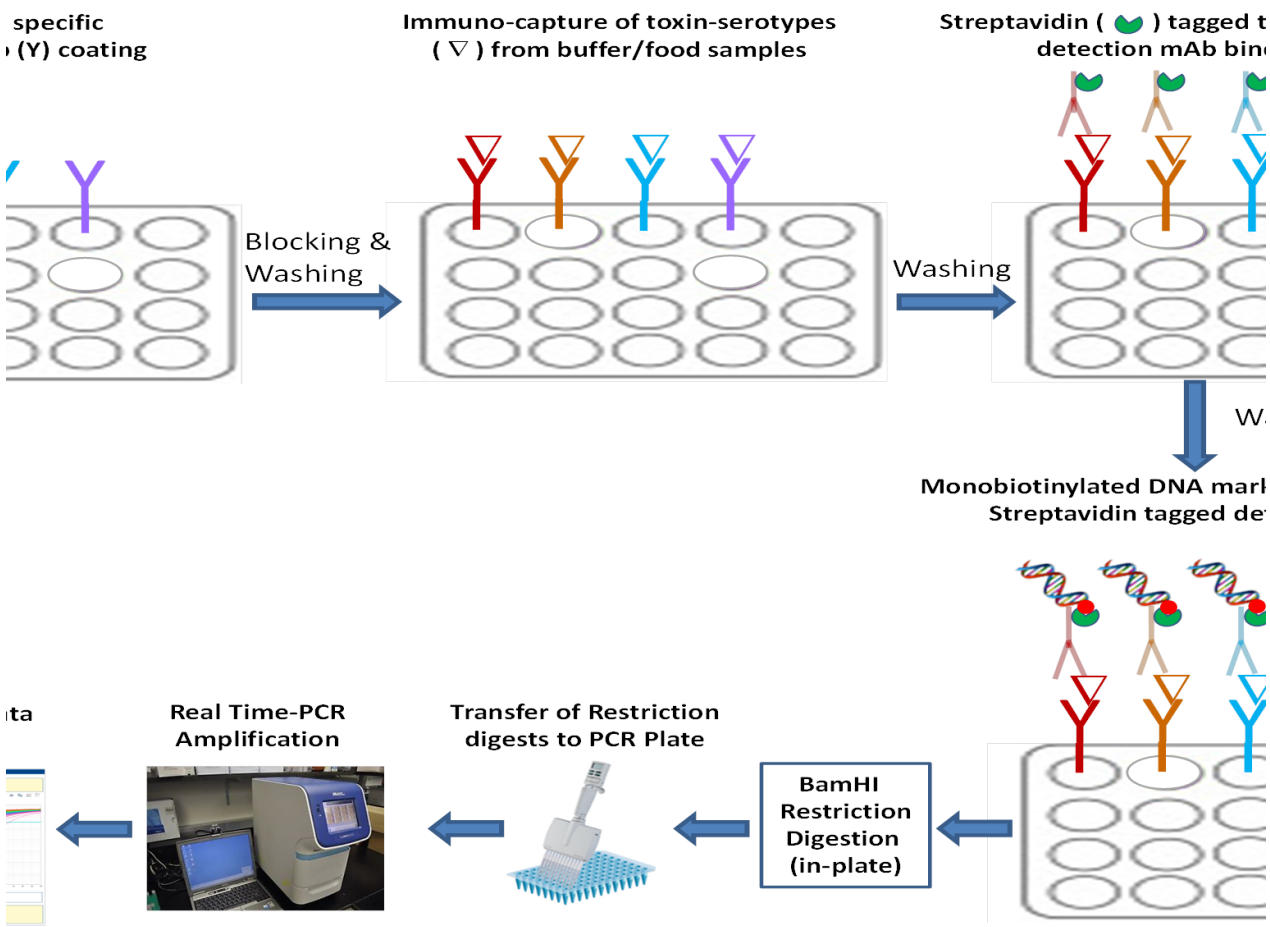




Ching et al., *J. Immunol. Meth.*, 2012

Immuno-PCR Assay for the Detection of Type A, B, E, and F Botulinum Neurotoxins in Food Matrices

Praseeda Mullasseril[1], Nagarajan Thirunavukkarasu[1], Gowri Manickam[1], Ajay Singh[2], Christine Pillai[1], Larry Stanker[3], David Hodge[4], Donald Zink[5], Eric Brown[1], Thomas Hammack[1] and Shashi Sharma[1],*



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US Department of Homeland Security
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Xoma Inc
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Allergan Inc

License Agreements

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Bioo Scientific

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