UDC 616.98:579.852.13]-07
DOI: 10.14739/2310-1210.2026.3.344174

Advancing botulism diagnostics: integrating molecularly specific in vitro platforms and high-sensitivity analytical technologies as alternatives to the mouse bioassay

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Accurate detection of botulinum neurotoxin (BoNT) requires assessing both its proteolytic SNARE-cleaving activity and its ability to inhibit synaptic transmission. While the mouse bioassay remains the traditional diagnostic standard, its prolonged turnaround time, limited reproducibility, and ethical constraints underscore the urgent need for advanced in vitro methodologies. Recent advances in analytical chemistry, stem-cell-derived neuronal platforms, and multi-omics profiling enable the development of sensitive systems that replicate BoNT's molecular and functional mechanisms with greater precision than conventional in vivo models.

Aim. To evaluate current methodologies for the diagnosis and functional analysis of botulinum neurotoxin and, synthesizing these findings, to propose a conceptual algorithmic model for comprehensive neurotoxin detection independent of *in vivo* methods.

Materials and methods. A literature review was conducted utilizing the PubMed, Scopus, and Web of Science databases. Studies detailing molecular, cellular, multi-omics, and computational approaches to BoNT detection were included. The extracted data were synthesized across molecular, cellular, and systems-level domains to construct an integrated diagnostic model.

Results. Modern diagnostic strategies increasingly surpass the mouse bioassay in terms of sensitivity, specificity, and mechanistic insight. SNARE-specific proteolytic profiling, notably Endopep-MS, enables precise detection of BoNT serotypes and their functional states by quantifying cleavage kinetics across a diverse array of substrates. Complementary human iPSC-derived neuronal platforms allow for direct functional assessment, revealing electrophysiological suppression, altered calcium signaling, and reporter-confirmed SNARE cleavage within living cells. Multi-omics analyses, including single-cell transcriptomics, epigenomics, and metabolomics, capture early stress signatures, serotype-specific responses, and determinants of neuronal susceptibility. Additional resolution is provided by receptor-binding kinetics and intracellular trafficking studies, which elucidate how serotype-dependent variations dictate overall toxicity. The integration of these molecular, cellular, and systems-level insights establishes the foundation for the ULTIMA-BoNT framework, a unified platform designed for high-precision BoNT detection.

Conclusions. The convergence of proteolytic assays, physiologically relevant neuronal models, and multi-omics analytics presents a robust, reproducible, and ethically sustainable alternative to the mouse bioassay. This integrative approach not only provides profound mechanistic insights but also supports the predictive analysis of emerging BoNT variants.

Keywords:
botulinum toxins, botulism, diagnostic techniques and procedures, mass spectrometry, endopeptidases, SNARE proteins, in vitro techniques.

Zaporozhye Medical Journal. 2026;28(3):267-273

Просування діагностики ботулізму: інтеграція молекулярно специфічних in vitro платформ та високочутливих аналітичних технологій як альтернативи мишачому біоаналізу

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Точне виявлення ботулінічного нейротоксину (BoNT) потребує оцінювання і його протеолітичної активності, що розщеплює SNARE-білки, і здатності пригнічувати синаптичну передачу. Хоча біоаналіз на мишах залишається традиційним діагностичним стандартом, його тривалість, обмежена відтворюваність та етичні обмеження підтверджують необхідність розроблення сучасних *in vitro* методів. Останні досягнення аналітичної хімії, нейронних платформ на основі стовбурових клітин і мультиомічного профілювання дають змогу створювати чутливі системи, що відтворюють молекулярні та функціональні механізми BoNT із більшою точністю, ніж традиційні *in vivo* моделі.

Мета роботи – оцінити сучасні підходи до виявлення та функціональної характеристики активного BoNT і запропонувати концептуальну *in vitro* діагностичну рамку як альтернативу біоаналізу на мишах.

Матеріали і методи. Здійснили огляд наукової літератури, що індексується у наукометричних базах PubMed, Scopus і Web of Science. Залучено дослідження, де описано молекулярні, клітинні, мультиомічні та комп'ютерні підходи до виявлення BoNT. Дані синтезовано на молекулярному, клітинному та системному рівнях для побудови інтегрованої діагностичної моделі.

Результати. Сучасні діагностичні стратегії дедалі більше перевищують біоаналіз на мишах за чутливістю, специфічністю та механістичним розумінням. Протеолітичне профілювання, специфічне до SNARE, особливо метод Endopep-MS, дає змогу точно визначати серотипи BoNT та їхній функціональний стан шляхом кількісного визначення кінетики розщеплення на різних субстратах. Крім того, нейронні платформи, отримані з людських iPSC, дають змогу здійснити безпосереднє функціональне оцінювання, виявляючи електрофізіологічне пригнічення, зміни кальцієвої сигналізації та підтверджене

Ключові слова:
ботулінічні токсини, ботулізм, діагностичні методи і процедури, мас-спектрометрія, ендопептидази, білки SNARE, методи in vitro.

Запорізький медичний журнал. 2026. Т. 28, № 3(156). С. 267-273

репортерами розщеплення SNARE у живих клітинах. Мультиомічний аналіз, включаючи транскриптоміку окремих клітин, епігеноміку та метаболоміку, фіксує ранні сигнатури стресу, серотипово-специфічні реакції та фактори чутливості нейронів. Додаткову точність забезпечують дослідження кінетики зв'язування з рецепторами та внутрішньоклітинного трафіку, що показали, як серотипово-залежні відмінності впливають на загальну токсичність. Інтеграція цих молекулярних, клітинних і системних даних формує основу платформи ULTIMA-BoNT – єдиної системи для високоточного виявлення BoNT.

Висновки. Поєднання протеолітичних тестів, фізіологічно релевантних нейронних моделей і мультиомічної аналітики є надійною, відтворюваною та етично стійкою альтернативою біоаналізу на мишах, пропонуючи механістичне розуміння та підтримуючи прогнозну оцінку нових варіантів BoNT.

Diagnosing botulism remains a formidable clinical and analytical challenge, as it necessitates the simultaneous detection of the highly specific proteolytic activity of botulinum neurotoxin (BoNT) and the empirical assessment of its capacity to block synaptic transmission [1,2]. Although the mouse bioassay has historically served as the definitive "gold standard", its clinical and translational relevance is progressively diminishing. This decline is driven by its prolonged turnaround time, inherent limitations in inter-laboratory reproducibility, and increasingly stringent ethical constraints regarding animal testing [1,2,3].

Modern analytical technologies enable the deployment of molecularly specific *in vitro* platforms capable of reproducing the key mechanisms of BoNT action, achieving diagnostic accuracy that rivals or exceeds that of traditional *in vivo* models [4,5]. The relevance of this study is driven by the need for a rapid, sensitive, and ethically acceptable platform for detecting active BoNT in various sample types. The combination of proteolytic, cell-functional, and molecular-genetic approaches enables not only toxin identification but also the elucidation of its underlying mechanisms of action.

The proposed paradigm encompasses three levels: the molecular level evaluated via SNARE-specific proteolysis combined with mass spectrometry to generate a proteolytic activity fingerprint (PAF); the cellular level assessed through the functional dynamics of human neuronal networks, yielding a functional potency vector (FPV); and the system-analytical level achieved through the integration of receptor binding kinetics, single-cell multi-omics (forming a systems response vector, SRV), and predictive machine-learning algorithms.

A primary innovation of this study is the development of a comprehensive integrative system that models BoNT toxicity *in vitro* while simultaneously addressing fundamental inquiries in toxicogenomics and neurobiology: identification of functional toxin subtypes, the delineation of substrate-specificity determinants, the characterization of cellular defense programs via scRNA-seq and scATAC-seq, and the predictive modeling of mutational impacts. This approach establishes a foundation for highly accurate diagnostics of active BoNT without the use of animals, effectively transforming routine detection into a robust tool for mechanistic analysis and the predictive characterization of emergent isolates.

Aim

To evaluate current methodologies for the diagnosis and functional analysis of botulinum neurotoxin and, synthesizing these findings, to propose a conceptual algorithmic model for comprehensive neurotoxin detection independent of *in vivo* methods.

Materials and methods

A systematic literature review was conducted utilizing the Scopus, PubMed, and Web of Science databases. The search was performed using combinations of key terms ("botulinum neurotoxin", "diagnosis", "proteolytic activity", "functional assay", "*in vitro*", "neuronal networks", "multi-omics", "machine learning"), allowing coverage of molecular, cellular, and systems-level approaches to BoNT analysis. Inclusion criteria comprised original research articles and reviews describing functional or molecular methods for BoNT detection, *in vitro* and *in vivo* models, proteolytic, cellular, and multi-omics approaches, and the use of machine-learning algorithms.

The review included original research articles and comprehensive reviews published between 2000 and 2025 that described functional or molecular methods for BoNT detection, *in vitro* and *in vivo* comparative models, proteolytic and cellular assays, multi-omics applications, or the integration of machine-learning algorithms. Exclusion criteria encompassed studies lacking primary data, technical reports without rigorous validation, non-peer-reviewed preprints, and non-English publications.

Study selection proceeded in two phases: an initial screening of titles and abstracts, followed by an in-depth full-text evaluation. Extracted data were systematically categorized into three hierarchical domains (molecular, cellular, and systems-analytical) facilitating the integration of findings regarding proteolytic kinetics, neuronal network functional shifts, and multi-omics profiling. The selection and data extraction processes were performed independently by two reviewers; any discrepancies were resolved via consensus or consultation with a third independent expert. The structured data subsequently served as the foundation for identifying diagnostic trends and conceptualizing the proposed analytical framework.

Results

Current in vitro and in vivo models for BoNT analysis. Contemporary BoNT diagnostics rely on an evolving matrix of complementary methodologies that are systematically replacing the traditional mouse bioassay [6,7]. The most informative *in vitro* approaches are synthetic SNARE peptides coupled with mass spectrometry and cellular reporter models that reproduce the natural action of BoNT and provide high specificity without ethical constraints [8,9,10,11,12]. Concurrently, immunochemical methods serve as rapid screening tools, while PCR and advanced sequencing technologies are essential for precise *bont* gene typing [13,14,15,16,17]. Despite the development of alternatives, the mouse bioassay retains reference status due to its ability to confirm the full spectrum of toxic action, stable sensitivity, and regulatory consolidation [5,7]. Molecular methods are auxiliary, as they do not determine toxin activity [18,19,20]. Based on the obtained data during the analysis of modern *in vitro* models for botulotoxin diagnostics, we have revealed a possible pattern that collectively may form a multilevel, accurate, and reproducible diagnostic system capable of establishing the basis for updated standards in botulism testing.

The conceptual ULTIMA-BoNT platform in modern botulotoxin diagnostics. In the rapidly advancing field of botulotoxin diagnostics, the search for methodologies capable of combining high sensitivity, rapid result acquisition, and multilevel analytics is becoming particularly relevant. Unfortunately, the available alternatives remain analytically fragmented, capturing isolated facets of BoNT biology, such as isolated proteolytic cleavage or singular cellular responses, rather than providing a comprehensive functional profile [17–22]. This highlights the critical need for a unified diagnostic platform that would ensure the integration of heterogeneous phenotypic data and allow the formation of a holistic diagnostic-scientific algorithm.

In this context, the concept of the Unified Layered Toxicity Investigation with Multi-phenotype Analytics of Botulinum Neurotoxin (ULTIMA-BoNT) *(Fig. 1)*, or "Ultima", is proposed, representing a multilevel architecture for multiphenotype analysis of botulotoxin. This conceptual platform is designed as a stepwise layering: Stage 0 – Sample reception and unified preparation; Stage 1 – Quantitative multi-substrate proteolysis profiling; Stage 2 – Replicative functional assay; Stage 3 – Single-cell multi-omics response; Stage 4 – Receptor binding and intracellular trafficking; Stage 5 – Genetic analysis; Stage 6 – Integrative Bayesian and machine learning (ML) analytics. Through this hierarchical architecture, ULTIMA-BoNT transcends conventional applied testing, providing a new conceptual framework from registering proteolytic activity to identifying systemic cellular response programs. It yields both highly accurate diagnostic determinations and profound insights into the evolutionary and neurobiological properties of the toxin.

Stage 0 – sample receipt and preparation (unified control). The starting point of the algorithm is a rigorously standardized procedure for receiving and initial preparation of biological or food material, aimed at maximizing analytical integrity and controlling reproducibility between independent laboratory cycles. For liquid samples, the recommended volume is at least 1–5 mL, whereas for food or microbial matrices it is 1–5 g, which allows maintaining sufficient mass/volume for multiparametric studies while minimizing the loss of informational content, which in turn was based on the established protocols adapted from S. R. Kalb et al. [23]. The subsequent stage involves preliminary purification of the sample to remove macromolecular debris and bacterial contaminants that may cause artifacts during proteolytic and functional testing. Membrane filtration of 0.22 μm is applied, which reduces bacterial contamination and ensures a controlled composition of low-molecular-weight components (adapted from S. Pellett et al. [24]). The resulting filtrate is aliquoted for parallel processing across the Endopep-MS, carbon-based photo-absorber (CBPA) microelectrode array (MEA), and multi-omics modules to minimize cross-contamination, consistent with workflows

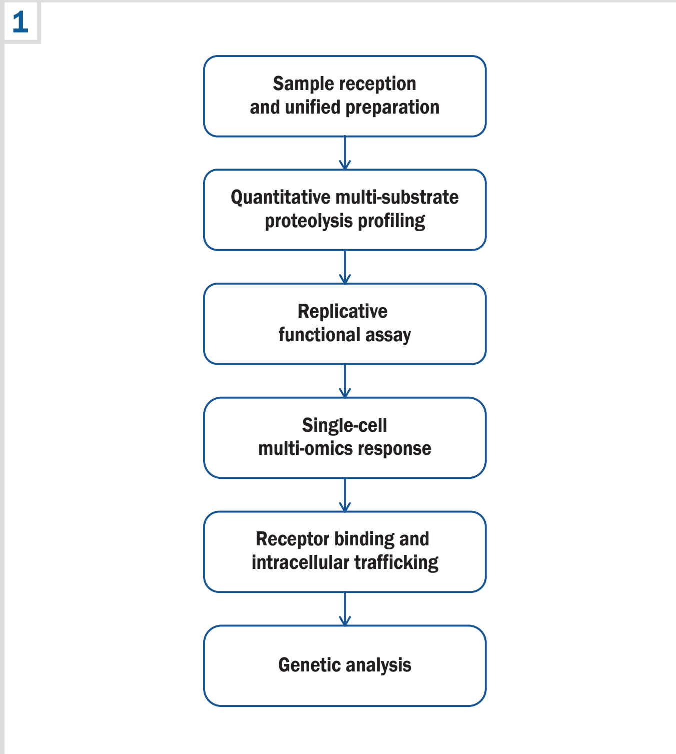

Fig. 1. ULTIMA-BoNT Steps.

described by O. Rosen et al. [25]. A dual internal standard system is implemented at this stage: an isotope-labeled BoNT fragment for mass-spectrometric calibration and a phospholipid nanoparticle control to monitor matrix interference in downstream cellular assays.

Stage 1 – quantitative multi-substrate proteolysis profile (deep endopep-MS). Accurate diagnostics demand not only the detection of BoNT but also the precise elucidation of its serotype and functional state. Single-substrate assays are insufficient because they do not reflect the diversity of SNARE motifs. An expanded library of 50–200 peptide substrates encompassing essential SNARE sequences and other BoNT-sensitive fragments provides high sensitivity required to detect minor inter-serotype variations [26,27,28]. Prepared samples are incubated with a panel of isotope-labeled peptides, followed by time-resolved LC-MS/MS analysis at standardized intervals (0, 15, 60, 240 minutes). The calculated reaction kinetics and catalytic efficiencies of each substrate yield a multidimensional PAF – a multidimensional profile of proteolysis dynamics. This PAF vastly enhances diagnostic resolution, allowing for definitive serotype differentiation based on distinct substrate specificities [2,26,27,28]. This approach also enables the detection of allosteric changes, co-factor effects, and even multi-toxin exposures. Finally, Deep Endopep-MS transforms the analysis into a high-precision tool defining the toxin's immediate functional state.

Stage 2 – replicative functional verification in iPSC neurons using MEA, Ca^{2+} imaging, and SNARE-reporter system. The biological relevance of the detected proteolytic activity is subsequently validated using living neuronal networks. Utilizing human induced pluripotent stem cell (iPSC)-derived motor neurons and neuromuscular co-cultures ensures physiological relevance and high assay reproducibility, building upon models established by J. D. Lamotte et al., C. Y. Lin et al., and O. F. Vila et al. [29,30,31]. Functional activity is quantified via multimodal endpoints: Microelectrode Array (MEA) analytics record critical electrophysiological disruptions (altered network synchronization and firing amplitude) directly resulting from SNARE blockade [8–31]. Concurrently, live Ca^{2+} imaging monitors the kinetics of calcium signaling, a direct correlate of neuronal excitability [32].

Enzymatic activity is tracked in real-time utilizing a targeted SNARE-reporter system, wherein specific fluorescence attenuation indicates active intracellular cleavage [8–23]. Short time-lapse series additionally capture morphological changes in neurites, loss of synaptic contacts, and early degenerative features. All indicators are integrated into the FPV – a multidimensional description of electrophysiological, calcium, biosensor, and morphological characteristics in temporal dynamics (0, 2, 6, 24, 48 h) [33,34,35]. The FPV robustly confirms the presence of functional toxin and discriminates between serotypes exhibiting homologous proteolytic profiles but divergent neuronal network impacts.

Stage 3 – single-cell multi-omics response (scRNA-seq + scATAC + metabolomics). The third stage of ULTIMA-BoNT moves from population-level assessments to the analysis of cellular heterogeneity. Single-cell multi-omics allows simultaneous profiling of the transcriptome, epigenome, and metabolome, revealing the full spectrum of cellular reactions [36]. Evaluations are anchored at 6 hours (capturing the early Integrated Stress Response (ISR) and Unfolded Protein Response (UPR)) and 24 hours (capturing consolidated epigenetic remodeling and allowing for the delineation of resistant versus apoptotic cellular trajectories) [26–36]. The analytics combines three platforms: scRNA-seq (subpopulations, differential expression, trajectories), scATAC-seq (open chromatin, epigenetic regulators), and targeted metabolomics (energetics, neurotransmitters, redox profile) [33,34,35,36,37,38]. Integration via MOFA+ and Seurat WNN forms the SRV, used to perform pathway enrichment of key pathways – stress-related, synaptic, endocytosis, autophagy, and apoptosis [33,34,35,36,37,38]. The system captures serotype-specific programs: BoNT/A activates compensatory vesicular pathways and alternative SNARE isoforms; BoNT/E induces rapid polycomb repression of synaptic genes; BoNT/C forms a UPR+ERAD signature [39,40,41]. Trajectories, in turn, should show that resistant neurons have high Rab11/35 and NAD^+/NADH, while sensitive ones exhibit an early glycolytic shift [26–41]. Correlating the early SRV with the FPV allows for the precise predictive modeling of ultimate cellular fate at 24–48 h based on the early profile (6 h) and the identification of pivotal neuroprotective regulators (ATF4, CREB, MEF2) [26–41].

Stage 4 – receptor-binding and intracellular dynamics. The fourth stage of the ULTIMA-BoNT platform is aimed at an in-depth quantitative investigation of the physicochemical mechanisms of botulinum toxin interaction with cellular receptors and the subsequent processes of intracellular transport. The main goal is to determine whether differences in toxic activity among various serotypes or isolates are driven by features of binding, complex stability, or efficiency of endocytosis and translocation.

Receptor-level kinetics are mapped using Surface Plasmon Resonance (SPR) and Biolayer Interferometry (BLI), which measure real-time interaction of the toxin with SV2A/B/C, synaptotagmin I/II receptors and cofactors (GT1b, GD1a, phospholipids) [41,42,43,44,45]. For each "toxin-receptor" pair, KD, kon and koff are determined, forming a Receptor Binding Signature (RBS) that reflects affinity and binding kinetics and allows capturing serotype-specific differences and the influence of mutations or environmental conditions. Additionally, cooperative binding (SV2 + GT1b) is assessed, which characterizes the synergy between protein and glycolipid receptors. After affinity determination, analysis of toxin endocytosis and translocation in live neurons is conducted using dual labeling (BoNT–AF488/pHrodo) and confocal / TIRF microscopy. Next, the rate of endocytosis, endosomal half-life, translocation efficiency, and the balance of endosomal/cytosolic fractions must be measured; pH-sensitive FRET sensors determine the moment of translocation. These parameters form the Intracellular Trafficking Vector (ITV) [41,42,43,44,45].

Integration of RBS and ITV produces the Receptor–Trafficking Profile (RTP), which combines receptor binding and intracellular transport, allowing one to distinguish affinity differences from defects in toxin delivery. RTP identifies mechanistic causes of toxicity variations and forms the basis for developing binding inhibitors or modulators of endosomal dynamics as potential antidotes.

Stage 5 – genetic support and structural analytics. The fifth level of the ULTIMA-BoNT platform provides systematic mapping of the genetic and structural-functional variability of botulinum toxin, creating an analytical bridge between nucleotide sequence, conformational dynamics, and phenotypic manifestations of toxicity. Its task is to move from descriptive genomics to mechanistic interpretation, determining how microgenetic variations in the bont cluster translate into changes in substrate specificity, stability of the SNARE–toxin complex, and functional potency at the cellular level [46,47,48]. At the first substage, hybrid NGS sequencing (Illumina + Nanopore / PacBio) is used for complete reconstruction of the bont locus, including flanking regions and mobile elements. The data must undergo gene annotation (bont, ntnh, orfX, ha), GC-profile analysis, phylogenetic reconstruction, and identification of SNPs, indels, and recombination events that may affect active sites [46,47,48].

The comparative locus profiling module builds graph models of interactions between mobile elements and toxigenic genes, determining evolutionary trajectories and possible homologous exchange events. The second substage includes structural-dynamic analysis: homology modeling (AlphaFold2 / ColabFold), cryo-EM if needed, and molecular dynamics (100–500 ns) to refine conformations. Evaluate RMSF of the active site, flexibility of the SNARE-recognition loop, binding free energy of SNARE peptides, and allosteric shifts. These parameters form the Structural Impact Vector (SIV) [46,47,48]. Integration of genomic, structural, and functional data forms a genotype–phenotype map of the toxin, and ML models (PLS, XGBoost) will allow establishing the "genotype – activity" relationship and predicting toxicity of new isolates.

Stage 6 – integrative analytics: Bayesian inference + interpretable machine learning. The final level of ULTIMA-BoNT involves the synthesis of all generated descriptors, PAF, FPV, SRV, RTP, and SIV, into a unified probabilistic space through hierarchical Bayesian modeling. Each level of toxic action is described by separate priors linked through a shared hyperprior structure that reflects the pathway from genetic variation to cellular response. Variational or MCMC inference enables computation of posterior (active BoNT) and identification of key parameter contributions (changes in kon/koff or reduction in MEA activity). In parallel, an interpretable ML module (XGBoost / ElasticNet) operates for serotype classification using SHAP decomposition to explain major features. The combination of these approaches forms the Composite Diagnostic Score (CDS) – a numerical metric with a confidence interval and an explanation of determinant factors. The final stage transforms multimodal data into a causally grounded diagnostic model capable not only of identifying active BoNT but also explaining which parameters led to the prediction.

The practical workflow with time windows provides stepwise execution of a comprehensive analytical cycle aimed at rapid and multimodal diagnostics of biological samples *(Table 1)*. At stage T0 (0–1 h), sample reception, aliquoting, and addition of internal standards are performed, ensuring accuracy and reproducibility of subsequent analytical measurements. After primary material preparation, at T1 (0–6 h) Deep Endopep-MS analysis is performed, including incubation with corresponding enzymatic systems and subsequent mass spectrometric detection of specific peptide fragments. This stage is critical for rapid detection of toxic or biomarker components with high sensitivity. In parallel, during T2 (0–48 h), samples are incubated with differentiated iPSC neurons for subsequent electrophysiological (MEA) and imaging studies. Key observation time points at 6 and 24 hours enable assessment of both early functional alterations and delayed cellular responses. At stage T3 (6/24 h), cells are harvested for parallel scRNA-seq and scATAC-seq. This includes library preparation (1–2 days), followed by sequencing to achieve detailed transcriptomic and epigenomic characterization of cellular responses. Additionally, at T4 (1 day), receptor-binding assays are conducted to quantitatively evaluate interactions between candidate ligands and their corresponding cellular receptors, thereby complementing the molecular and cellular profiling of the system. The final stage, T5, comprises integrative analytics – the unification of multiomic, functional, and biochemical datasets into a unified interpretative model. This process continues over the next 24–48 hours after obtaining all experimental results.

Discussion

The synthesized evidence demonstrates that advanced in vitro technologies now possess the analytical precision and mechanistic resolution required to supersede the mouse bioassay. SNARE-targeted proteolytic profiling, particularly via Endopep-MS, affords exceptional serotype-specific quantification of catalytic activity, revealing molecular dynamics obscured in in vivo models. Research by K. Björnstad et al. highlights that Endopep-MS achieves limits of detection equivalent to or surpassing those of standard bioassays, while effectively distinguishing closely related BoNT mosaics in complex matrices [27].

Table 1. ULTIMA-BoNT analytical workflow: temporal framework

Stage	Timeframe	Procedure	Output
T0	0–1 h	Sample reception, aliquoting, internal standard addition	Quality-controlled aliquots
T1	0–6 h	Deep Endopep-MS: substrate incubation + LC-MS/MS analysis	PAF (Proteolytic Activity Fingerprint)
T2	0–48 h	iPSC neuron exposure; MEA + Ca^{2+} imaging + SNARE-reporter (readouts at 6 h, 24 h)	FPV (Functional Potency Vector)
T3	6/24 h – 3–4 d	Cell harvest for scRNA-seq + scATAC-seq; library prep (1–2 d) + sequencing	SRV (Systems Response Vector)
T4	1 d	Receptor binding assays (SPR/BLI) + trafficking imaging	RTP (Receptor–Trafficking Profile)
T5	Final 1–2 d	Bayesian integration + interpretable ML (XGBoost, SHAP)	CDS (Composite Diagnostic Score)

Complementary human iPSC-derived neuronal systems validate functional toxicity with high fidelity, revealing electrophysiological suppression, calcium dysregulation, and reporter-confirmed SNARE cleavage that directly reflect BoNT's mechanistic action. Lin C. Y. et al. used complementary human iPSC-derived neuromuscular junction models to confirm BoNT functional toxicity with high fidelity, showing robust suppression of neuronal firing, calcium dysregulation in myotubes, and reporter-verified SNARE cleavage that mirror its canonical mechanism of action [30]. The authors note that these 2D hNMJ cultures recapitulate stepwise synapse maturation in vitro and add that the same platform can be used to model neuromuscular disease-associated vulnerability, including impaired AChR clustering and contraction deficits under pathological conditions [30].

Single-cell multi-omics adds an additional layer of diagnostic depth, capturing serotype-dependent stress signatures, chromatin remodeling, and metabolic shifts that define neuronal susceptibility. Stuart T. et al. developed the anchors method for integrating single-cell data, which corrects for technical differences across experiments and enables combining multiple omics technologies [36]. The approach accurately matches shared cellular states, builds integrated atlases, and transfers annotations between datasets, even in the absence of certain cell populations. This allowed detailed characterization of neuronal subtypes, chromatin and protein signatures, and integration of spatial data. Their work demonstrates that multi-omics integration improves cell type classification and enables detection of rare or novel populations [36]. Together with receptor-binding kinetics and intracellular trafficking dynamics, these data elucidate how serotype-specific differences in entry pathways and translocation efficiency contribute to overall toxicity.

When synthesized with receptor trafficking data, the ULTIMA-BoNT framework provides a mechanistically transparent and highly informative diagnostic continuum, proving that a multi-phenotypic in vitro architecture can establish a new, ethical, and highly precise standard for botulism diagnostics.

Conclusions

1. The integration of molecularly specific *in vitro* platforms with advanced analytical and computational technologies provides a rigorously validated alternative to the traditional mouse bioassay in the diagnosis of botulism.

2. The combination of SNARE-proteolysis profiling, cellular reporter models, and molecular-genetic verification provides high sensitivity, specificity, and a significant reduction in analytical turnaround time.

3. The obtained data demonstrate the feasibility of creating a multilevel, ethically acceptable, and standardized system for detecting active botulinum toxin of various serotypes in clinical, food, and environmental samples.

Limitations of current in vitro approaches. Although contemporary in vitro technologies provide a mechanistically grounded alternative to traditional toxin detection, several constraints must be acknowledged to ensure a rigorous evaluation of the translational potential of the ULTIMA-BoNT platform. The standardization of highly complex neuronal, proteolytic, and multi-omics systems remains inherently challenging due to their sensitivity to methodological variability, while the implementation of such platforms requires substantial financial, infrastructural, and technical resources. Additionally, inter-laboratory differences in cell-line characteristics, analytical settings, and data interpretation may produce variability in readouts, including occasional false-negative signals or reduced informativeness of specific analytical modules under certain experimental conditions. Despite these limitations, the integrative architecture of ULTIMA-BoNT, encompassing multi-substrate proteolysis, physiologically relevant neuronal functionality, and systems-level molecular profiling, provides far superior mechanistic resolution and diagnostic fidelity compared with the traditional mouse bioassay. Consequently, even with the noted challenges, the platform represents a fundamentally more advanced, ethically sustainable, and scientifically robust framework that has the potential to redefine evidence-based standards in botulism diagnostics.

Prospects for further research. Future research will focus on advancing the capabilities of the ULTIMA-BoNT platform. Priority objectives include diversifying the SNARE peptide panel for Deep Endopep-MS, which is essential for enhancing serotype discrimination and identifying emerging toxin variants. Furthermore, the standardization of iPSC-derived neuronal models remains a critical endeavor to ensure the reproducibility of functional assays. Emerging avenues include integrating novel sensing technologies for the real-time monitoring of neurotransmission blockade. Moreover, high-resolution single-cell multi-omics will facilitate the elucidation of temporal cellular responses to BoNT and the identification of toxin resistance mechanisms. The application of machine-learning algorithms holds significant potential for predicting toxin variant properties and refining diagnostic precision. Ultimately, the standardization of methodologies and the development of standardized diagnostic kits are necessary to support the transition of ULTIMA-BoNT into routine laboratory practice.

Funding
The study was conducted without financial support.

Conflicts of interest: authors have no conflict of interest to declare.
Конфлікт інтересів: відсутній.

Надійшла до редакції / Received: 24.11.2025
Після доопрацювання / Revised: 03.02.2026
Прийнято до друку / Accepted: 11.02.2026

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