# Advances in assays and analytical approaches for botulinum-toxin detection

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**We review methods to detect botulinum toxin, the most poisonous substance known. Current assays are being developed with two main objectives in mind:**

**(1) to obtain sufficiently low limits of detection to replace the mouse bioassay with an *in vitro* assay; and,**

**(2) to develop for screening purposes rapid assays that are as sensitive as possible while requiring an hour or less to process the sample and to obtain the result.**

**We emphasize the diverse analytical approaches and devices that have been developed over the past decade. Also, to provide background and context, we briefly review representative older immunoassays.**



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## 1. Introduction

Botulinum neurotoxin (BoNT) is the most toxic of the microbial toxins, and, indeed, is among the most poisonous substances known to man [1–8]. It is produced by the Gram-positive bacterium species *Clostridium botulinum*, *C. barati*, and *C. butyrium*. This toxin is estimated to be 1000 times more toxic than ricin, 15,000 times more toxic than VX nerve gas, and 100,000 times more toxic than sarin, by weight. The BoNT LD50 is approximately 1 ng/kg in humans [8]. Alternatively, by extrapolation from primate studies, lethal amounts of crystalline BoNT serotype A have been estimated to be 0.09–0.15 μg by injection, 0.70–0.90 μg by inhalation, or 70 μg orally for a 70 kg human [6].

Natural cases of BoNT intoxication can occur through three primary mechanisms:

- consumption of contaminated food (food-borne botulism);
- infection of an open wound (wound botulism); and,
- infections in the intestines of infants (infant botulism) [6,8].

In addition, inhalational botulism is a fourth intoxication mechanism that can result from intentional release of aerosols in acts of warfare or terrorism. The Centers for Disease Control (CDC) in the United States has listed BoNT as one of the six highest-risk threat agents for bioterrorism (''Class A agents'') [1,4,5].

BoNT acts by binding to the neuronal cell membrane and entering the neuron by endocytosis. Once inside, the active zinc endoprotease of the toxin structure cleaves a specific protein, ultimately preventing the release of acetylcholine and leading to muscular paralysis [6–9]. In lethal cases, death typically results from respiratory paralysis and paralysis of the airways; the patient cannot breathe. Patients who survive face intensive care, mechanical ventilation, and extended long-term health effects. The effects on the individual are less severe if early, rapid detection enables appropriate medical treatment [6,8].

The gold-standard method for BoNT detection is the mouse bioassay, which can detect BoNT to <10 pg/mL [7,9–12]. The quantity that is lethal is 1 mouse lethal dose (MLD), which is approximately 10 pg. Given a 0.5 mL injection, the limit of detection (LOD) would be 2 MLD/mL in terms of

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concentration [9]. This assay requires intraperitoneal injection of samples into mice followed by observation for 2–4 days. To identify specific antigenically-distinct serotypes of BoNT (see below), additional neutralization steps are required. Thus, although sensitive, the assay is laborious and time-consuming, poses potential hazards to personnel during injection, and requires special animal facilities. *In vitro* assays to replace the mouse bioassay are highly desirable.

Two articles published in 2005 reviewed assays for BoNT especially as they relate to detection in foods [7,12]. A further review on *in vitro* assays was published in 2007 [9]. Historically, two primary approaches have been used to develop *in vitro* assays. Immunoassays use antibodies to recognize and to bind to epitopes on the surface of the three-dimensional structure of the toxin. Hence, these are structure-based assays, and are generally carried out as enzyme-linked immunosorbent assays (ELISAs) where the enzyme provides amplification. Alternatively, assays have been developed to detect the proteolytic activity of the toxin; these are functional assays; and, some functional assays also use structural recognition to capture and to concentrate the toxin prior to assaying the catalytic activity. Typically, either type of *in vitro* assay requires hours, rather than the days associated with the mouse bioassay.

In this review, we describe assay approaches developed for botulinum toxin, with emphasis on the most recent methods. Other reviews on immunoanalytical methods in this journal in recent years have focused on pesticides, aflatoxins, pollutants, and tumor markers as analytes [13–16] or electrochemistry or chemiluminescence as technical methodologies [17,18]. Focusing the present review on botulinum toxin addresses an analyte that is important for food safety, biosecurity, and clinical applications, while also presenting a cross-section of modern biodetection approaches that illustrate how they can be used to achieve very challenging LODs.

Two trends have emerged in botulinum-toxin detection:

- first, there are continuing efforts to develop sensitive *in vitro* assays to replace the mouse bioassay; and,
- second, there has been a recent emphasis on rapid assays.

Whereas the typical ELISA requires 4–6 h to complete, there is interest in assays of $\leqslant$1 h to enable the rapid screening of potentially contaminated samples, diagnosis of potentially exposed humans, and determination of serotype for serotype-specific therapies. Assays using tissues or living cells, and their responses to the toxin, are beyond the scope of this review, as is the detection of cells of the *Clostridium* species by immunoassay or nucleic-acid-based methods.

After describing the botulinum toxin in some detail in the next section, we describe structure-based immunoassays, followed by functional assays. Finally, in a section on rapid assays, we describe assays for the sensitive

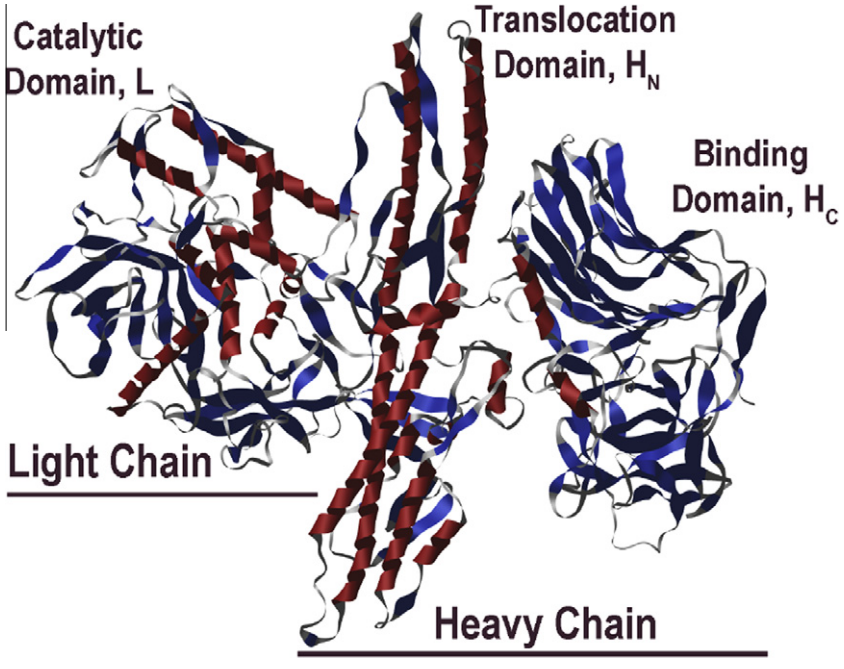


**Figure 1.** Three-dimensional structure of 150 kDa botulinum neurotoxin showing the catalytic domain that comprises the light chain; the translocation and receptor-binding domains comprise the heavy chain. The structure [4] was rendered using POV-Ray rendering software. This image was made with VMD (http://www.ks.uiuc.edu/Research/vmd/), developed with NIH support by the Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign, USA.