505 S. Rosa Road, Suite 105
Madison, WI 53719
1-608-441-8174
Email: info@biosentinelpharma.com

BioSentinel Part No: L1019, Release Date: October 16, 2014

BoTest® KO Non-specific Protease Detection Kit Protocol

BoTest KO Non-specific Protease Assay Kit 200 assays Cat. A1039

1.0 INTRODUCTION

The BoTest KO Reporter was designed to serve as a control for the BoTest and BoTest Matrix botulinum neurotoxin (BoNT) Detection Assays. The BoTest KO Reporter is insensitive to up to 3 nM BoNT but can be cleaved by other non-specific proteases (i.e. proteases other than BoNT) that are commonly found in complex samples such a culture supernatants or food. The BoTest KO Reporter, therefore, can be used to control for false positives or to optimize assay conditions for samples that contain non-specific protease activity.

The BoTest A/E and B/D/F/G Assays reply upon the recombinant BoTest Reporters to measure BoNT endopeptidase activity. The BoTest reporters are also susceptible to BoNT-independent cleavage by non-specific protease contamination, which can lead to false positives or an overestimation of sample potency. The BoTest KO Reporter differs from the BoTest A/E Reporter by three point mutations that render the reporter insensitive to BoNT cleavage while retaining sensitivity to non-specific protease degradation.

2.0 DESCRIPTION

2.1 Materials Supplied

BoTest KO Non-specific Protease Detection Kit

Description	Composition	A1039	
		Size	Part #
BoTest KO Reporter	20 μM in 50 mM HEPES-NaOH, 10 mM NaCl, 15% Glycerol	250 μl	A1038
10x BoTest Reaction Buffer	500 mM HEPES-NaOH, pH 7.1, 50 mM NaCl, 1% Tween-20, 100 μM $ZnCl_2$	2 x 1.25 ml	A1002

2.2 Additional Required Materials

- Fluorescence microplate reader with 434 nm excitation, 470 nm emission, and 526 emission filters
- Black, flat-bottom microtiter plates with covers (Nunc Part Number 237105 recommended)
- Incubator set to 30 °C or 37 °C (optional)
- EDTA-free protease inhibitor (optional, Roche Number 04693132001 recommended)

3.0 STORAGE

Description	Storage Temp.	Notes
BoTest KO Reporter	-80 °C	Upon thawing, aliquot into single use amounts to avoid repeated freeze-thaw cycles. Stable for a minimum of five days at 4 ^{0}C upon thawing.
10x BoTest Reaction Buffer	-20 or -80 °C	Stable for a minimum of five days at 4 ^{0}C upon thawing.

4.0 SAFETY PRECAUTIONS

All reagents in this kit are considered non-hazardous according to 29 CFR 1910.1200. Normal precautions exercised in handling laboratory reagents should be followed.

5.0 GENERAL ASSAY CONSIDERATIONS

5.1. Suggested Use

BoTest KO is used in parallel with the BoTest and BoTest Matrix Assays to monitor test samples for non-specific protease activity. The BoTest KO Reporter is especially useful as a BoTest Matrix Assay control when testing samples that may contain high concentrations of non-specific proteases (*e.g.* culture supernatant, food, or environmental samples). In cases where protease contamination is a concern, one or more of the test samples should be assayed both with its serotype-specific BoTest Reporter and the BoTest KO Reporter. Testing a sample dilution series with both the specific (BoTest A/E or BoTest B/D/F/G) or non-specific (BoTest KO) reporters will determine the sample dilution at which no significant non-specific protease activity remains, while testing only the highest concentration sample can monitor for the absolute presence of a non-specific protease contamination in the assay. See Section **8.0 Example Data** for examples of BoTest KO use.

5.2. Non-specific Protease Activity Reduction

The inclusion of ***EDTA-free*** protease inhibitors (Roche Number 04693132001**)** in all BoTest or BoTest Matrix Assay buffers can reduce non-specific protease activity, if observed. (**Note:** a small increase in the emission ratio of intact, uncleaved reporter is commonly seen with protease inhibitor addition.) In addition, alternate blocking buffers and additional Matrix bead washes also aid in reducing the carryover of non-specific proteases throughout the BoTest Matrix Assay. See Section **8.0 Example Data** for an example of using protease inhibitors to reduce non-specific protease activity in *Clostridium* culture supernatants.

5.3. Special Consideration when using BoTest KO with BoTest B/D/F/G

The BoTest KO reporter is based on BoTest A/E and has a lower intact, uncleaved emission ratio value than BoTest B/D/F/G. The emission ratio values obtained with BoTest KO-tested samples should only be compared to a BoTest KO-tested control sample that is known to lack any non-specific proteases such as a sample contain only BoTest Reaction Buffer made with laboratory-grade water.

5.4. Equipment and Buffer Considerations

Please see the BoTest and BoTest Matrix BoNT Detection Kit Protocols for additional instrumentation, equipment, and buffer considerations.

6.0 ADDITIONAL INFORMATION

Protocols, publications, and application guides can be found at www.biosentinelpharma.com.

7.0 BASIC ASSAY PROTOCOL

1. BoTest KO Reporter is diluted and used as described for the BoTest A/E and BoTest B/D/F/G Reporters.
    a. Follow the specific protocol for the **BoTest** (Protocol L1002) or **BoTest Matrix** (Protocol L1007, L1011, or L1013) Assay to be executed.
    b. Include additional test sample and control wells for BoTest KO testing. At a minimum, to detect the presence of non-specific protease activity, BoTest KO should be run with a buffer control (no test sample) and the highest concentration sample that will be tested with the other BoTest reporters.

2. Based on the number of wells to be run, prepare a **KO Reporter Master Stock** containing 1.25 µl BoTest KO reporter and 3.75 µl **1X** BoTest Reaction Buffer per well.
    a. See specific BoTest or BoTest Matrix BoNT Detection Kit protocols for direction on making the 1X BoTest Reaction Buffer.

b. The total volume of the Reporter Master Stock can be increased 10-25% to account for pipetting error and loss during dispensing.

3. Add 5 µl of KO Reporter Master Stock to each well to be tested with the BoTest KO Reporter at the same time that the BoTest A/E or BoTest B/D/F/G Reporters are added to their respective wells.
4. See specific BoTest or BoTest Matrix BoNT Detection Kit protocols for guidance on incubation time and temperatures, reading the assay, and converting raw fluorescence emission RFU values into emission ratio values.
5. For determining the presence of non-specific proteases, compare the BoTest KO Reporter emission ratio value of the control sample (buffer, no test sample) to the BoTest KO Reporter emission ratio value of the test sample. If the emission ratio value of the test sample is >10% lower than the control value, non-specific proteases are likely present in the test sample.

8.0 EXAMPLE DATA

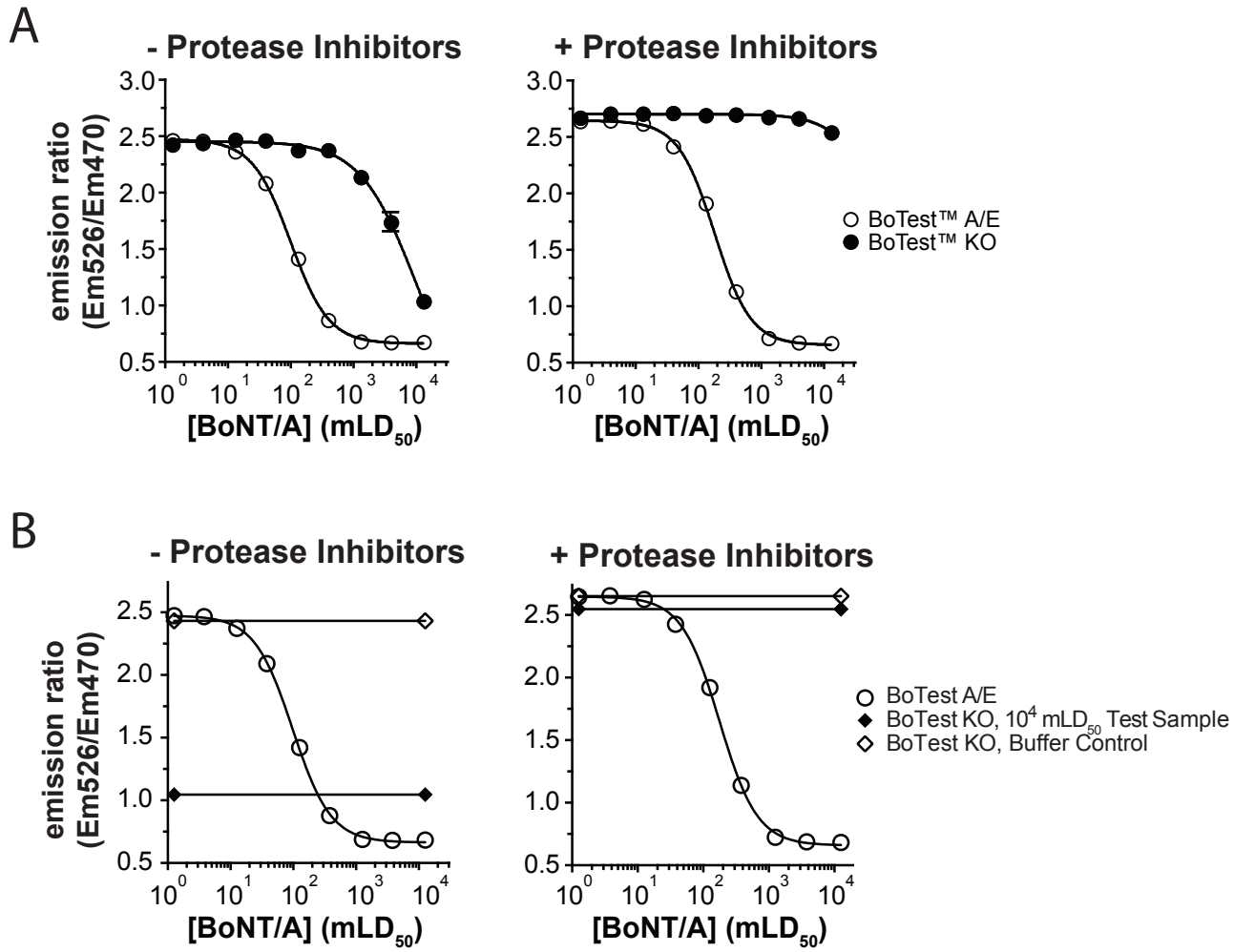

Figure 1. Use of BoTest KO as a control to monitor non-specific protease activity. *Clostridium* BoNT/A culture supernatant was serially diluted in PBS and assayed with the BoTest Matrix A BoNT Detection Kit (Cat. A1015) using both BoTest A/E and BoTest KO Reporters. Non-specific protease activity is monitored by assaying either each sample of the dilution series with BoTest KO (A panels) or the highest concentration test sample along with a buffer control containing no test sample (B panels). Non-specific protease activity is evident by a dose-dependent loss of the BoTest KO Reporter's emission ratio value (A panels) or by comparing the BoTest KO Reporter's high concentration test sample assay response to the response with the buffer control (B panels).

The inclusion of **EDTA-free** protease inhibitors can significantly reduce non-specific protease activity as demonstrated by comparison of the left data panels (no protease inhibitors) to the right panels (with protease inhibitors). The lack of BoTest KO Reporter responses in the presence of protease inhibitors demonstrates that activity measured with the BoTest A/E Reporter is specifically due to the presence of BoNT/A. Error bars represent the standard deviation of the mean.