Procedure for Protein/Peptide Neutralization with EndoPrep™

1. Prepare sample by diluting to a concentration of 0.1 to 1.0 mg/ml with BDTI™ Digestion Buffer.
    - BDTI™ Digestion Buffer should account for at least 90% of the sample volume for proper protease activity.
    - If dilution is not possible, use diluted hydrochloric acid to lower the pH of the sample below 4.5.
2. Remove an aliquot of 270 µl and transfer to a sterile, endotoxin-free borosilicate glass tube.
3. Add 30 µl of BDTI™ Protease Solution and mix by vortexing for 10 seconds.
    - A control sample containing 30 µl of BDTI™ Digestion Buffer instead of BDTI™ Protease Solution should be included with each set of reactions to determine baseline endotoxin content without digestion.
4. Cover tube with Parafilm®.
5. Incubate tube in a 37°C water bath.
    - Most proteins and peptides show maximum endotoxin activity after 60 minutes of treatment. Some samples may require longer incubation.
    - For examples of digestion times, refer to the BioDtech, Inc. EndoPrep™ Application Notes.
    - To verify complete sample digestion, polyacrylamide gel electrophoresis should be performed on digested samples.
6. After digestion, dilute samples 1:100 in endotoxin-free water.
    - Dilution of only 1:10 is possible but should include proper validation that enzyme or digestion products do not interfere with the endotoxin detection assay at this concentration.
7. Test with LAL or recombinant Factor C assay.
    - Samples treated with EndoPrep™ should be tested both with and without a positive product control (PPC).
    - Samples of BDTI™ Digestion Buffer and BDTI™ Protease Solution at equivalent concentrations should be tested alongside all samples as control.

Procedure for EndoPrep™ Preparation and Storage

1. Upon receipt, store EndoPrep™ kit at 4°C.
    - Before preparation of BDTI™ Protease Solution, the EndoPrep™ kit is stable for 2 years when properly stored.
2. Before use, add 1 ml BDTI™ Digestion Buffer to BDTI™ Protease Solution bottle.
3. Mix sample vigorously with vortexing for 5 minutes.
4. Assure full solubilization by visual inspection.
5. BDTI™ Protease Solution should be stored at 4°C.
    - After preparation of BDTI™ Protease Solution, the EndoPrep™ kit is stable for 3 months when properly stored.

Soluble Biotech, Inc. offers superior products for detection, removal and neutralization of bacterial toxins.

Endotoxin Detection Products:		
EndoPrep™	20 reactions	EDP-4001.01
Endotoxin Removal Products:		
EndoBind-R™	1 ml column	EBR-3001.01
EndoBind-R™	5 ml column	EBR-3005.01
EndoBind-R™	Bulk resin	Inquire

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Advantages

- Increases detection accuracy.
- Removes inhibitory effect of peptides and proteins on endotoxin.
- Works with LAL and recombinant Factor C assays.
- Requires less than 60 minutes for most samples.
- Easy to use.

Product Description

EndoPrep™ is a sample treatment system to remove the inhibitory effects of proteins and peptides on endotoxin and allow accurate detection and quantitation. The system consists of BDTI™ Digestion Buffer and BDTI™ Protease Solution. Following the provided protocol, the system components are compatible with both classical LAL and recombinant Factor C endotoxin detection assays.

U.S. Patent Pending

Soluble BIOTECH™ A Predictive Oncology Company

EndoPrep™

Protein/Peptide
Sample Treatment

Catalog No: EDP-4001

www.solublebiotech.com

EndoPrep™ and Endotoxin

The majority of lipid in the outer membrane of gram-negative bacteria consists of lipopolysaccharide (LPS), also called endotoxin. On average, a single *E. coli* cell contains 2,000,000 LPS molecules. Sub-nanogram levels of endotoxin can trigger immune responses and alter the phenotype and function of many cells including monocytes, neutrophils, dendritic cells, hepatocytes, vascular and respiratory epithelium, and arterial smooth muscle cells. Because of this activity, the FDA has established strict guidelines for allowable endotoxin content in injectible drugs. In addition, the presence of endotoxin affects cell culture and animal model studies. Given the above, accurate endotoxin detection and quantitation in protein/peptide samples is crucial. However, it is well documented that many proteins/peptides, especially cationic ones, bind to endotoxin and mask its activity. **EndoPrep™** is a sample treatment system which neutralizes the inhibitory effects of proteins/peptides on endotoxin and results in more accurate detection and quantiation.

Endotoxin Detection in an Inhibitory Peptide Sample Using EndoPrep™

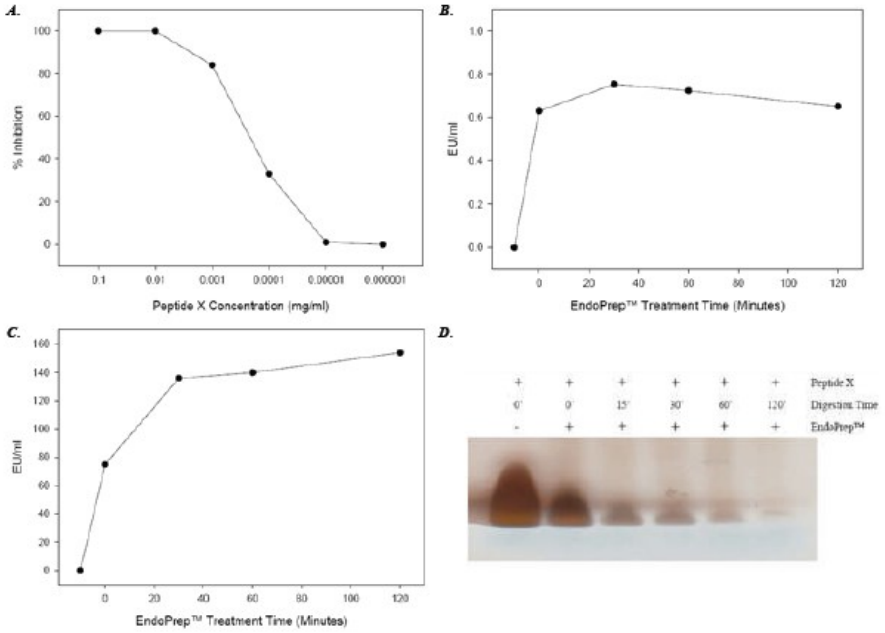

Figure 1. Treatment of Peptide X with EndoPrep™. (*A*) The PPC Inhibition Assay shows extent of peptide dilution required to overcome inhibitory effect. (*B*) Recovery of a 1 EU/ml PPC in Samples of Peptide X before and after treatment with EndoPrep™. (*C*) Recovery of defined endotoxin contamination in a sample of Peptide X. (*D*) PAGE data showing Peptide X degradation with EndoPrep™ treatment.

Peptide X (proprietary) is a mixture of short cationic peptides shown to be extremely effective in a therapeutic setting. However, the cationic nature of Peptide X causes it to bind endotoxin and mask its activity from LAL and recombinant Factor C assays. The extent of this inhibition is shown in Figure 1*A* which details the requirement of a 2,000,000-fold dilution (to 10 ng/ml) for full endotoxin spike recovery. A sample of 0.1 mg/ml Peptide X was prepared in BDTI™ Digestion Buffer containing 250 EU/ml exogenous endotoxin contamination. This sample was then incubated for various times with the BDTI™ Protease Solution and endotoxin content was measured with the Lonza PyroGene® assay. Figure 1*B* shows the results of 1 EU/ml PPC recovery experiments for all digestion samples. Without digestion, no endotoxin was detected. After **EndoPrep™** treatment, nearly 80% of the PPC was recovered, exceeding the standard 50-200% recovery requirements. Recovery of exogenous endotoxin contamination showed similar results (Figure 1*C*). Without treatment, none of the 250 EU/ml were detected. With treatment, over 150 EU/ml were detected. Figure 1*D* shows that Peptide X degradation from **EndoPrep™** treatment corresponds to the removal of endotoxin masking.

For a more detailed explanation of **EndoPrep™** protocol refer to the **BioDtech, Inc. EndoPrep™** Application Notes. This document outlines the use of **EndoPrep™** in treating peptides and proteins, including bovine serum albumin, immunoglobulin and hemoglobin.

Endotoxin Detection in Hemoglobin Solution using EndoPrep™

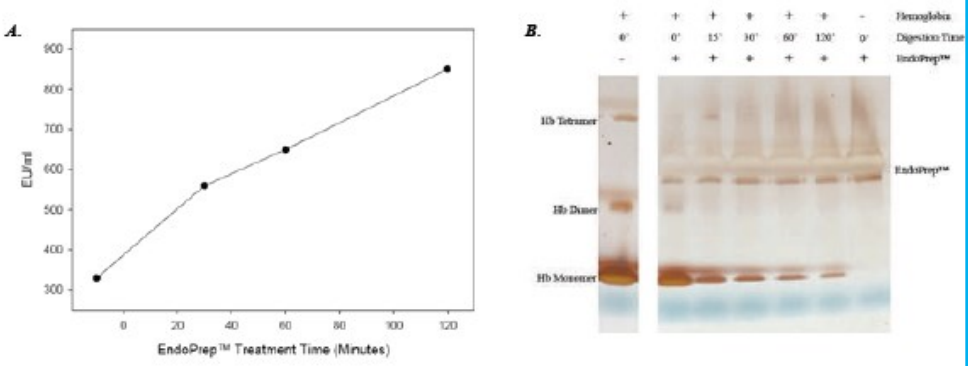

Figure 2. Treatment of Hemoglobin with EndoPrep™. (A) Recovery of endogenous endotoxin contamination in a sample of hemoglobin from bovine erythrocytes. (B) PAGE data showing hemoglobin degradation with EndoPrep™ treatment. The gel was run under reducing conditions resulting in three distinct hemoglobin populations. Locations of each hemoglobin population and the EndoPrep™ protease are indicated.

The example with Peptide X shows the use of **EndoPrep™** in a sample that completely masks endotoxin activity. However, the **EndoPrep™** system can also be used to increase the accuracy of endotoxin detection in protein samples that do not exhibit such dramatic effects. Hemoglobin is an example of a protein that is known to bind endotoxin and mask its activity. To increase the accuracy of endotoxin quantitation in a sample of hemoglobin, a 1 mg/ml sample was prepared in BDTI™ Digestion Buffer. Previous experiments with this untreated hemoglobin stock showed that it contained about 300 EU/mg of endogenous endotoxin contamination. BDTI™ Protease Solution was added to the hemoglobin sample and incubated at indicated times. Figure 2*A* shows that untreated hemoglobin measured at slightly more than 300 EU/mg, as previously reported. However, with increasing lengths of **EndoPrep™** treatment, the amount of detectable endotoxin increased to about 650 EU/mg after one hour of treatment and 850 EU/mg after two hours of treatment. This indicates a 150% increase in detection and could be significant for biological samples. Figure 2*B* shows that hemoglobin digestion correlates to endotoxin liberation. The PAGE experiments were performed in reducing conditions resulting in monomer, dimer, and tetramer hemoglobin populations. With **EndoPrep™** treatment, the dimer and tetramer populations are quickly degraded followed by continuous degradation of the monomer population over the entire two hour time course.

For a more detailed explanation of **EndoPrep™** protocol refer to the **BioDtech, Inc. EndoPrep™** Application Notes. This document outlines the use of **EndoPrep™** in treating peptides and proteins, including bovine serum albumin, immunoglobulin and hemoglobin.