Procedure for Blood Plasma Neutralization with ESP™

1. Heat-inactivate plasma enzymes by heating undiluted plasma to 60˚C for 30 minutes.
   - It is also possible to inactivate plasma enzymes with acidification. The suggested protocol for acidification is to add 10% final volume of 1-2 M hydrochloric acid to the sample and allowing it to incubate at room temperature for 5 minutes.
   - If using the acidification protocol remember to account for the 10% sample dilution effect in final calculations.
2. In a sterile, endotoxin-free borosilicate glass tube mix 270 µl of ESP™ Buffer #1 with 30 µl enzyme-inactivated plasma sample. Vortex for 10 seconds.
   - ESP™ Buffer #1 is specially designed to provide optimal buffer conditions (e.g. pH, divalent cation concentrations) for digestion.
2. Add 30 µl ESP™ Protease Solution and vortex for 10 seconds.
   - A control sample containing 30 µl of ESP™ Buffer #1 instead of ESP™ Protease Solution can be included with each set of reactions to determine endotoxin content without digestion.
4. Cover tube with Parafilm®.
5. Incubate sample in a shaking 37°C water bath.
   - Typical plasma samples show maximum endotoxin recovery after 30-60 minutes of treatment. Some samples may require longer incubation.
   - To verify complete sample digestion, polyacrylamide gel electrophoresis can be performed on digested samples.
6. Mix 450 µl Buffer #2 with 50 µl of digested sample.
   - ESP™ Buffer #2 is specially designed to provide optimal buffer conditions (e.g. pH, salt and divalent cation concentrations) for endotoxin detection.
7. Test samples for endotoxin using an endotoxin detection assay according to manufacturer's specifications.
   - Samples treated with ESP™ should be tested both with and without a positive product control (PPC) according to industry standards.
   - All standards, blanks and controls should be prepared using ESP™ Assay Control Buffer to eliminate any variations that may exist due to differences in buffer conditions.

Procedure for ESP™ Preparation and Storage

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

BioDtech, Inc. was organized in 2003 to develop and market products for detection, removal and neutralization of bacterial toxins.

Endotoxin Detection Products:

ESP™	>30 reactions	ESP-9001.01
EndoPrep™	>30 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™	10 ml bulk	EBR-3010.02

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ESP™ Instruction Booklet - Rev 3 11-18-14

ESP™

Endotoxin Sample Preparation Kit

Catalog No: ESP-9001

www.biodtechinc.com

Product Description

ESP™ is a sample treatment system to remove the interfering effects of blood plasma components on endotoxin and allow accurate detection and quantitation. The system consists of ESP™ Buffer #1, ESP™ Buffer #2, ESP™ Protease Solution and ESP™ Assay control buffer. Following the provided protocol, the system components are most compatible with recombinant Factor C endotoxin detection assays.

U.S. Patent Pending

Advantages

- Increases detection accuracy.
- Removes interfering effects of blood plasma components on endotoxin and endotoxin detection assays.
- Compatible with LAL and recombinant Factor C assays.
- Requires less than 60 minutes for most samples.
- Easy to use.

Blood Components Known to Alter Endotoxin Detection

- Serine Proteases
- Amidases
- Thrombin
- Urokinase
- Ribonuclease A
- Urokinase
- Bilirubin
- Plasmin
- Hemoglobin
- Immunoglobulins
- Esterases
- Apolipoprotein AI
- Lysozyme
- Serum Amyloid A
- Soluble CD-14
- Lactoferrin
- Platelets
- Bactericidal/ Permeability-Increasing Protein
- High-Density Lipoproteins
- Low-Density Lipoproteins
- Lipopolysaccharide-Binding Protein
- Cholesterol Ester Transport Protein

Comparison of ESP™ with Other Methods

To test **ESP™**, 10 control citrated human plasma samples (5 male, 5 female) were spiked with a known amount of endotoxin, treated with the full **ESP™** protocol and tested in triplicate using the Lonza Pyro-Gene® assay according to manufacturer's specifications. Each treated sample was also tested with a Positive Product Control (PPC) according to manufacturer's specifications. The spike recovery indicates the effectiveness of **ESP™** in detecting endotoxin in human plasma samples. The PPC recovery indicates the amenability of the final product for detection. To measure recovery, the results were compared to control samples that included water instead of plasma. The entire sample set was also treated with two additional protocols for comparison: (1) heat-inactivated with dilution in water and (2) digestion with **ESP™** but without heat-inactivation. The results are given in Table 1.

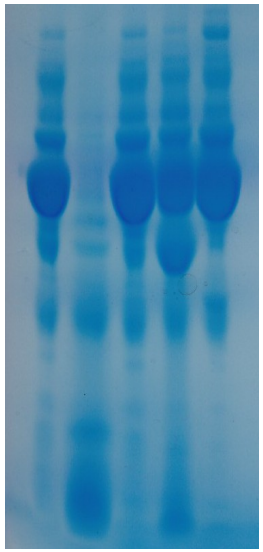

Figure 1. Plasma Digestion with Various Protocols. Normal citrated human plasma was treated with the indicated protocol (in the text), diluted 1:10 in water and examined by PAGE in non-reducing conditions. The gel was silver stained for visualization.

A protocol of heat-inactivation and dilution, which is prevalent in the literature, allows detection of less than 5% of the spiked endotoxin and a PPC recovery indicating over 80% inaccuracy. Alternatively, when the samples are digested without heat-inactivation the spike recovery is far too high, a result of active serine proteases that interfere with assay enzymes. This false-activation results in near-saturation of the assay and artificially low PPC recovery results that indicate inaccuracy of over 60%. When these two technologies are combined and the specially designed **ESP™** buffers are used, spike recovery is over 75% with an accuracy approaching 90%.

To further validate these results, samples of citrated plasma were treated with various portions of the **ESP™** protocol and tested with PAGE analysis (Figure 1). Lane #1 contains untreated plasma. Lane #2 contains plasma treated with the full **ESP™** protocol with a 60 minute digestion step. Lanes #3 and 4 contain plasma that was treated with the **ESP™** protocol but using common laboratory buffers instead of **ESP™** Buffers #1 and #2. Lane #5 contains plasma that was heat-inactivated but undigested. From these results it is clear that the full **ESP™** protocol effectively removes the vast majority of proteins from plasma that interfere with endotoxin detection assays or bind and mask endotoxin. Treatments that omit the **ESP™** buffers or digestion step show only negligible differences compared to untreated plasma.

Treatment	% Spike Recovery	% PPC Recovery
Heat-Inactivation/Dilution	4.9 ± 6.2%	182.7 ± 30.5%
Digestion/Dilution	353.8 ± 292.7%	38.4 ± 54.9%
ESP™ Treatment	77.2 ± 26.7%	89.3 ± 12.8%

Table 1. Summary of Results. Summary of the effects of ESP™ treatment on citrated human plasma. The results are based on 10 independent samples tested in triplicate. "% Spike Recovery" was determined by comparing the plasma results to control experiments performed with endotoxin-free water. "% PPC Recovery" was determined according to manufacturer's specifications.

For a detailed explanation of the **ESP™** protocol and suggestions for sample preparation refer to the **BioDtech, Inc. ESP™** Application Notes.

Preparation of Plasma Prior to ESP™ Treatment

A lag time between sample collection and treatment can allow enzymatic destruction of endotoxin or the binding of endotoxin to proteins which will decrease the ability to detect total endotoxin. To minimize this, samples should be heat-inactivated or acidified as soon as possible.

The optimal treatment is to heat-inactivate blood immediately after collection and then proceed to plasma separation and then to step 2 of the protocol. However, treatment of whole blood at the time of collection is often not possible. In these situations, the plasma samples should be heat-inactivated immediately upon receipt/thawing. To demonstrate the extent of inactivation an aliquot of endotoxin was added to citrated plasma and allowed to incubate at room temperature for the indicated amount of time. As comparison, a plasma sample that was heat-inactivated according to the protocol was treated identically. After incubation all samples were treated with the normal **ESP™** protocol. The control was considered as 100% of recoverable endotoxin and the samples given as percentage of control. After one minute active endotoxin was decreased to 45% of the control (Figure 2). Continued incubation in the plasma further decreased the amount of recoverable endotoxin to 31% after 120 minutes. Though this extent of inactivation may not be typical of all patient samples or endotoxin species, it demonstrates the importance of rapid sample treatment.

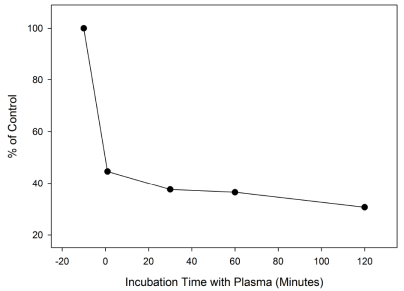

Figure 2. Inactivation of Endotoxin in Plasma. A known amount of endotoxin was added to aliquots of citrated plasma and allowed to incubate at room temperature for the indicated time. After incubation the samples were treated with ESP™ and detectable endotoxin was determined. Results are given as percent of a control sample which was heat-inactivated prior to endotoxin addition.

The other option is acidification. However, adding acid to whole blood will result in hemolysis, therefore this method should only be used on plasma. To demonstrate the ability of acidification to prevent endotoxin loss, aliquots of hydrochloric acid (HCl) representing 10% of the final volume were added to samples of citrated plasma and allowed to equilibrate. Next, the pH of the plasma was measured and a known amount of endotoxin was added. The samples were incubated at room temperature for 10 minutes and then treated with the **ESP™** protocol. All samples were compared to a control consisting of the same amount of endotoxin prepared in water. Samples receiving water instead of HCl measured as pH 8 and resulted in the recovery of 11.0% of the endotoxin (Figure 3). Addition of 0.1 and 0.3 M HCl decreased the pH to the 6-7 range and actually resulted in slightly lower endotoxin recovery. These samples also had a tendency to desolubilize and probably indicate the isoelectric point of a major plasma protein. Further acidification with 0.6 to 2.0 M HCl resulted in decreasing pH accompanied by increasing endotoxin recovery. The sample receiving 2 M HCl measured 93.6% of the total endotoxin.

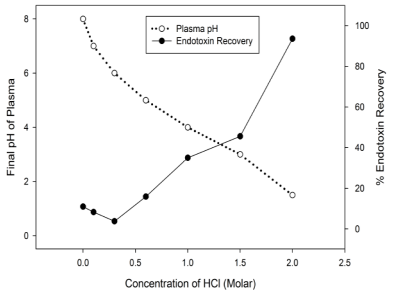

Figure 3. Overcoming Inactivation of Endotoxin in Plasma with Acidification. Samples of citrated plasma were acidified with 10% final volume of the indicated molarity of hydrochloric acid and a known amount of endotoxin was added to each. All samples were treated with ESP™ and total endotoxin recovery and final plasma pH were plotted as a function of acid molarity.

For a detailed explanation of the **ESP™** protocol and suggestions for sample preparation refer to the **BioDtech, Inc. ESP™** Application Notes.