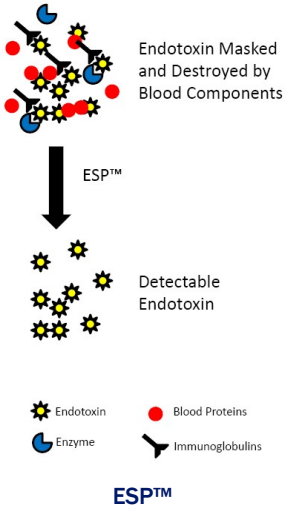
Predictive Oncology®

Endotoxin Sample Preparation (ESP™) Kit

Accurate Endotoxin Detection in Human Plasma With ESP™



Accurate endotoxin detection in plasma is impossible with current technologies. Endotoxin is a highly negative and hydrophobic molecule, causing it to bind to many factors in the blood. In addition, numerous blood components bind, activate and inactivate assay enzymes. Here we describe the Endotoxin Sample Preparation (ESP™) kit which can be used to treat citrated human plasma and allow for accurate endotoxin quantitation in under 60 minutes.

- Increases detection accuracy
- Removes inhibitory effect of peptides and proteins on endotoxin
- Removes enzymatic activity of blood enzymes
- Requires less than 60 minutes for most samples
- Easy to use

Introduction

Endotoxins are lipopolysaccharides associated with gram-negative bacterial membranes that act as potent immunostimulatory molecules. They have been implicated in a number of pathophysiological conditions and diseases. The current accepted assay used by the pharmaceutical and medical device industry for endotoxin detection is based on the clotting of horseshoe crab blood. This clotting scheme is comprised of a series of serine protease enzymes, initiated by Factor C, which are sequentially activated when endotoxin is present [1-3]. The most common form is the classic *Limulus* amoebocyte lysate (LAL) assay which uses lysate directly from horseshoe crab blood. More recently, a recombinant form of Factor C (rFC) has been developed allowing more defined assay conditions and circumventing the problem of relying on a wild species. Both LAL and rFC assays are dependent on serine protease activity and have been developed for endotoxin quantitation via gelation, turbidity, or fluorescence in either end-point or kinetic versions. However, using these assays for detection of endotoxin in biological samples is limited because they are affected by components in the blood.

The observation that factors in clinical samples inactivated the pyrogenic properties of endotoxin was first noticed in 1954 [4]. The prevailing explanation over the next decade was that the samples contained endotoxin-degrading enzymes. A set of experiments published in 1966 again showed that endotoxin incubated with human plasma lowered pyrogenic effects in the rabbit fever test. However, by using a protease digestion procedure followed by ethanol precipitation researchers were able to restore pyrogenic activity and reverse inhibitory effects [5]. This showed that the majority of endotoxin inhibition was due to complex formation with molecules in the blood. In the intervening years, specific blood components have been discovered which bind and inactivate endotoxin, alter aggregate formation, interfere with the enzymatic LAL and rFC assays, or even destroy endotoxin. Serine proteases involved in the blood coagulation cascade can activate LAL. Amidases, plasmin, thrombin and urokinase can cleave chromogenic substrates in certain LAL assays. Bilirubin can bind and inactivate these same substrates as well as endotoxin [6]. Esterases can directly cleave and inactivate endotoxin [7]. High- and low-density lipoproteins and apolipoprotein A1 bind endotoxin and can decrease activity by 40% [8]. Other studies have shown that lipoproteins in normal blood can inactivate 100 endotoxin units per milliliter [9]. Cationic proteins such as lysozyme, ribonuclease A, IgG and hemoglobin are known to make electrostatic interactions with endotoxin and impair detection by LAL assays [10]. Other proteins such as lipopolysaccharide-biding protein (LBP), serum amyloid A (SAA), bactericidal/permeability-increasing protein (BPI), soluble CD-14 and cholesterol ester transport protein have been shown to behave similarly [11-13]. Cytokine expression has shown that lactoferrin can diminish the physiological response to endotoxin [14]. Lastly, studies have shown that up to 92% of endotoxin in clinical samples may be bound to platelets in a process facilitated by Lipid A-associated proteins [15-16]. This is similar to a report showing that plasma components have the ability to neutralize 95% of endotoxin activity [17]. Difficulties of endotoxin detection in blood-derived samples are widespread in the literature. One study using cytokine ELISA as control showed 500-fold variations in LAL results [18]. Similar studies showed problems detecting endotoxin in bacteremia patients [19-22]. Other researchers have reported difficulties measuring endotoxin in clinical samples with about 30% recovery in serum and less than 60% in plasma [23-24]

Heat inactivation can remove some of the enzymatic activity in plasma but proteins still remain that bind endotoxin or act as substrate inhibitors. Also, heat-treatment alone has several inherent issues. Heating causes morphological changes in fibrinogen [6], lipoproteins [7] and platelets [16] which can alter the interactions of endotoxin and cause both false positives and altered binding. Heat-treated samples have variability in excess of 100% [7]. Usually heat-inactivation is accompanied by sample dilution. However, this is problematic due to the binding nature of endotoxin. Endotoxins have a net negative charge at physiological pH due to two phosphate groups on the disaccharide. In addition, endotoxins contain long hydrophobic fatty acids chains. Therefore, any molecule with a positive charge or containing a hydrophobic region may

bind to endotoxin. Often this binding is too strong to be diluted out or removed with heat or extraction. Because of these difficulties the more suitable method is inactivation and enzymatic degradation of the interfering molecules.

To solve these issues Soluble Biotech, Inc. has developed the **Endotoxin Sample Preparation (ESP™) kit.** **ESP™** is a plasma sample treatment kit that combines heat-inactivation, pH shift, and enzymatic degradation to remove the interfering factors in plasma. The protease cocktail in **ESP™** contains no serine proteases that would interfere with the LAL and rFC enzyme cascades, requires no special divalent cation conditions and typically produces testable plasma samples in less than 60 minutes. **ESP™** treatment requires only minimal dilution and is therefore suitable for detecting low levels of endotoxin.

Materials and Methods

Supplies. Endotoxin detection and quantitation was performed with the PyroGene® assay from Lonza (Walkersville, MD) according to manufacturer's specifications with and without 1 EU/ml positive product controls (PPC) to validate assay reliability. The assay has a range of detection from 0.01 to 10 EU/ml. Hemoglobin (bovine erythrocytes) was purchased from Calbiochem (La Jolla, CA). Endotoxin was purchased from List Biological Laboratories, Inc. (Campbell, CA) in the form of *Escherichia coli O55:B5* lipopolysaccharide or prepared in the lab by heat lysis of *E. coli N99* or *Salmonella enterica* Typhimurium LT2 strains. Rabbit plasma was obtained from project rabbits maintained by Capralogics (Harwick, MA). Human plasma was obtained from control patients by Innovative Research (Novi, MI) and Bioreclamation (Westbury, NY).

ESP™ Protocol. The ESP™ kit consists of ESP™ Buffer #1, ESP™ Buffer #2, ESP™ Protease Solution, and ESP™ Assay Control Buffer. All experiments were performed with the following protocol. The plasma sample was heated in a 60˚C water bath for 30 minutes. Next, 30 µl of the sample was mixed with 270 µl ESP™ Buffer #1 and 30 µl of ESP™ Protease Solution and incubated in a shaking 37˚C water bath for 30-180 minutes. After digestion, 50 µl was mixed with 450 µl ESP™ Buffer #2 and tested with the Lonza PyroGene® assay according to manufacturers specification. To maintain consistent buffer conditions the standards, blanks, and controls were prepared in ESP™ Assay Control Buffer. Any deviations from this protocol are indicated in the text.

Polyacrylamide Gel Electrophoresis (PAGE). PAGE analysis was performed to monitor correlation of endotoxin detection with protein degradation. For PAGE analysis, 20 µl of the undiluted digestion sample was added to a mixture containing 45 µl endotoxin-free water, 10 µl 5 mM DTT, and 25 µl CBS Scientific (Del Mar, CA) ClearPAGE™ 4x Sample Buffer (additional water replaced DTT for non-reducing electrophoresis). This sample was heated in a 70°C water bath for 10 minutes and 17 µl was loaded into a CBS Scientific ClearPAGE 10-20% TEO-CI SDS Gel submerged in CBS Scientific ClearPAGE 1x Tris-Tricine-SDS Run Buffer (Reducing or Non-Reducing) and electrophoresed at 200 Volts for 45 minutes with a current gradient from 60 to 30 mA. All gels were silver stained using Sigma (St. Louis, MO) ProteoSilver™ Silver Stain Kit according to manufacturer's specifications.

Results

Enzymatic Digestion of Blood Proteins

We have previously shown that endotoxin can be bound and masked by common proteins like hemoglobin [25]. Hemoglobin makes up 32-36% of whole blood in healthy patients and demonstrates the difficulties in detecting endotoxin in blood products. Ultrafiltration, density centrifugation, ethanol precipitation and non-denaturing PAGE experiments have demonstrated the affinity of hemoglobin for endotoxin [26-27]. By digesting samples of hemoglobin with our EndoPrep™ technology we have demonstrated the ability to remove interfering blood components for endotoxin detection. In the example below, the purchased hemoglobin contained approximately 330 EU/mg without treatment. Treatment with EndoPrep™ for 30 minutes degraded nearly all of the hemoglobin dimer and tetramer populations (Fig 1*B*) and increased endotoxin detection to 560 EU/mg (Fig 1*A*). This pattern continued over the 120 minute digestion time course with maximal recovery of 850 EU/mg. These experiments, along with others involving blood proteins such as albumin, immunoglobulins and transferrin [25], demonstrate the potential of using a digestion protocol in conjunction with other various blood treatments to accurately detect endotoxin in human blood plasma.

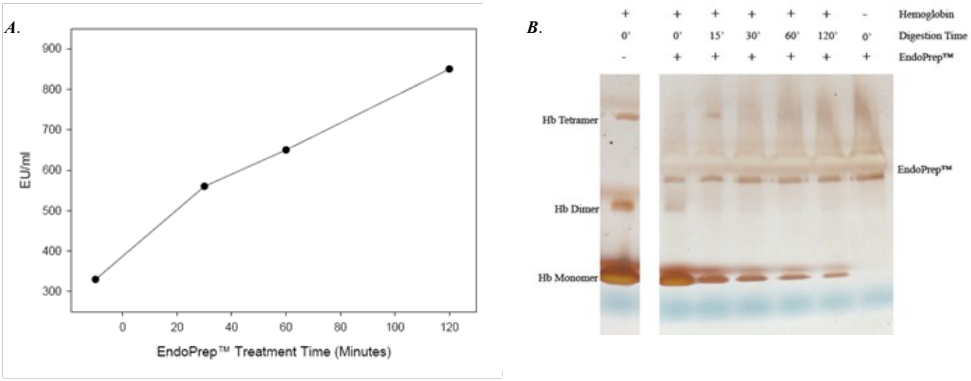


Figure 1. Treatment of Hemoglobin with EndoPrep™. (*A*) Recovery of endogenous endotoxin contamination in a sample of hemoglobin from bovine erythrocytes. (*B*) PAGE data showing hemoglobin degradation with EndoPrep™ treatment. The gel was run under non-reducing conditions resulting in three distinct hemoglobin populations. Locations of each hemoglobin population and components of the EndoPrep™ protease are indicated.

Enzymatic Digestion of Blood Plasma Samples

After establishing the ability to digest and remove some of the most prevalent blood components that interfere with endotoxin detection, the next step was to demonstrate that digestion was possible on samples of blood plasma. The initial step was to use EndoPrep™ technology to digest control blood from rabbits. Samples of plasma were diluted in the EndoPrep™ digestion buffer and digested according to product protocol [25]. Since EndoPrep™ is only active in acidic conditions, and physiological pH is about 7.4, it was assumed that the less dilute samples may have problems with complete digestion due to incompatible buffer conditions. However, the higher dilutions should have a more suitable pH and allow digestion. The results show that dilutions up to 1:10 showed little difference between the treated and untreated samples. This may be partially due to protein overload. At dilutions of 1:25 and 1:50 there is a distinct difference in protein content in the treated samples. There is a digestion band just above the EndoPrep™ band that is clearly visible in the 1:10 and 1:25 dilutions. This may indicate a fragment of IgG and demonstrate digestion. If so, this is not seen in the lower dilutions and indicates that these samples have incomplete digestion due to high pH. Supporting this line of thought, the pH in the samples diluted 1:5 or less have a pH above 6.0 while the samples diluted 1:10 or more have a pH in the 4.5-5.0 range.

These results suggest that it is possible to remove the majority of protein in plasma with dilution and digestion.

Even though the experiments with EndoPrep™ were very successful in digesting blood components, these samples were still not amenable for endotoxin testing using the traditional LAL or rFC assays. To achieve this we developed a specialized plasma digestion system which incorporates enzymatic digestion, heat-inactivation, pH shift and divalent cations. We have named this technology the **Predictive Oncology Endotoxin Sample Preparation (ESP™) Kit** and describe it below.

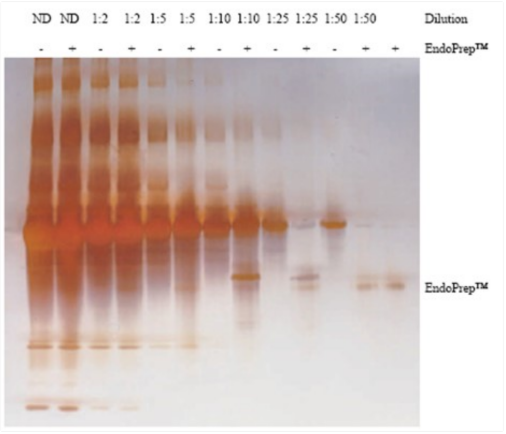


Figure 2. Plasma Digestion with EndoPrep™. Normal rabbit plasma was diluted by the indicated amounts in digestion buffer and treated with EndoPrep™ digestion for 60 minutes at 37°C. Samples were then diluted 1:10 in water and examine by PAGE in non-reducing conditions. The gel was silver stained for visualization. A band representing a component of the EndoPrep™ technology is indicated to the right of the gel. ND, no dilution.

Endotoxin Sample Preparation (ESP™) Kit

Summarily, **ESP™** works as follows. A sample of citrated plasma is heat-inactivated at 60-65˚C for 30 minutes. Next, the plasma is diluted 1:10 in the special low pH **ESP™** Buffer #1. This buffer acidifies the plasma, causing inactivation of neutral and alkaline enzymes and preparing it for digestion with the **ESP™** Protease Solution. **ESP™** Buffer #1 also contains divalent cations to chelate interfering anticoagulants. After a 30-180 minute digestion at 37˚C, the sample is then prepared in **ESP™** Buffer #2 for detection with an rFC assay. **ESP™** Buffer #2 is specially formulated to not interfere with LAL-based assays and to adjust the sample pH to an optimum level. Lastly, **ESP™** Assay Control Buffer is provided to prepare all samples, blanks, and controls. Since endotoxin detection assays are sensitive to differences in buffer type, cation concentration and pH, using this control buffer will ensure the most accurate results.

To test **ESP™**, ten (10) control citrated human plasma samples (five (5) male, five (5) female) were spiked with a known amount of endotoxin, treated with the full **ESP™** protocol and tested in triplicate using the Lonza PyroGene® assay according to manufacturer's specifications. In addition, each treated sample was tested with a Positive Product Control (PPC), also 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 but were otherwise identical. The entire sample set was also treated with two additional protocols for comparison: first, a set was heat-inactivated and diluted but without using the **ESP™** buffers. The other protocol involved the **ESP™** enzymatic digestion step but without heat-inactivation. The results are given in Table 1.

A protocol of heat-inactivation and dilution, which is prevalent in the literature, produces 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%.

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 experiment performed with endotoxin-free water. "% PPC Recovery" was determined according to manufacturer's specifications.

To further validate these results, samples of citrated plasma were treated with various portions of the **ESP™** protocol and tested with PAGE analysis (Figure 3). 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.

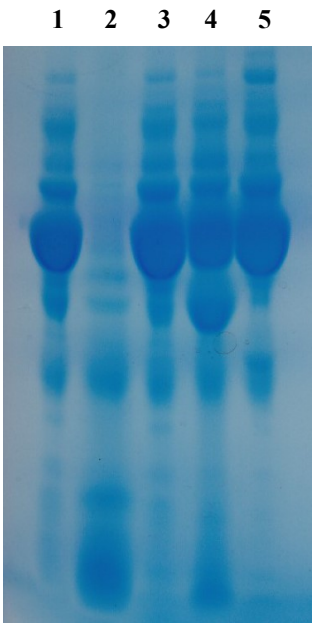


Figure 3. Plasma Digestion with Various Protocols. Normal citrated human plasma was treated with the indicated protocol (in the text), diluted 1:10 in water and examined by PAGE in non-reducing conditions. The gel was silver stained for visualization.

***ESP™** Treatment Does Not Significantly Alter Endotoxin Activity*

To establish that **ESP™** treatment does not affect the potency of endotoxin, and therefore result in artificially high recovery, samples from a stock endotoxin solution were treated with the **ESP™** protocol and the activity was compared. A sample of 100 EU/ml endotoxin was prepared in **ESP™** Buffer #1. Aliquots of **ESP™** Protease Solution were added to a final concentration of 10% volume and allowed to digest at 37°C for up to 120 minutes. Two series of experiments were included. One contained the concentration of **ESP™** Protease Solution included in the kit (3.5 units/ml). A second series was tested using 10-fold higher amounts of enzyme (35 units/ml). All samples were then diluted in **ESP™** Buffer #2 and tested using the Lonza PyroGene® assay. Mock digestions that were not incubated are included. The results were standardized and expressed as percent of standard. Figure 4 shows that regardless of the amount of protease solution added or digestion time, there was little change in endotoxin detection. All three reactions had about a 5% increase, which is significantly less than the increases over untreated samples. Actually,

this small increase demonstrates the utility of the proposed system. The stock solution from List Biologics (Campbell, CA) was prepared using the Westphal & Jann [28] method which leaves small amounts of contaminating protein. Treatment of the stock with **ESP™** removes this protein which "unmasks" the endotoxin and gives a more accurate measurement.

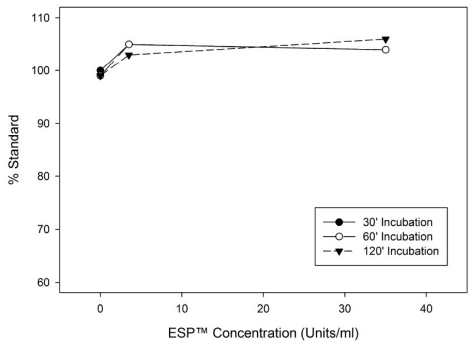


Figure 4. Purified LPS digestion with ESP™. A 100 EU/ml stock solution of LPS in ESP™ Buffer #1 was incubated with 0, 3.5, and 35 units/ml of ESP™ Protease Solution (3.5 units/ml is the normal protocol amount). Incubation was allowed to proceed for 30, 60, and 120 minutes. After digestion, samples were diluted in ESP™ Buffer #2 and tested with the Lonza PyroGene® assay. The data in fluorescent units was normalized to a standard and is expressed as percent of standard.

*Pre-Treatment of Plasma Prior to **ESP™***

Though **ESP™** is very effective at removing most interfering components in plasma, we have demonstrated that these factors can enzymatically inactivate or irreversibly bind endotoxin during the time between sample collection and **ESP™** treatment. Therefore, for optimal quantitation measures should be taken to inactivate plasma components as soon as possible. This can be achieved through heat-inactivation or acidificaion.

Heat-Inactivation

As discussed, heat-inactivation is important to remove interfering factors in plasma. However, 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. One option to prevent this is to heat-inactivate blood immediately at the time of collection. In this scenario, the protocol should be altered so that the whole blood is collected, heat-inactivated, plasma separated and then diluted in ESP™ Buffer #1. The normal protocol would be followed from here.

To demonstrate the extent of inactivation an aliquot of endotoxin was added to citrated plasma samples 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 only one minute in plasma active endotoxin was decreased to 45% of the control. Continued incubation in the plasma further decreased the amount of recoverable endotoxin to 31% after a 120 minute incubation. Though this extent of inactivation may not be typical of all patient samples or endotoxin species, it demonstrates the importance of rapid sample treatment.

pH Inactivation

The other option to prevent endotoxin inactivation is acidification. However, adding acid to whole blood will result in hemolysis, therefore this method should only be used on plasma. To demonstrate this, 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. 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.

These results highlight the heat- and acid-sensitive components in plasma that inactivate endotoxin. Extreme care that should be taken when collecting and preparing biological samples for endotoxin detection.

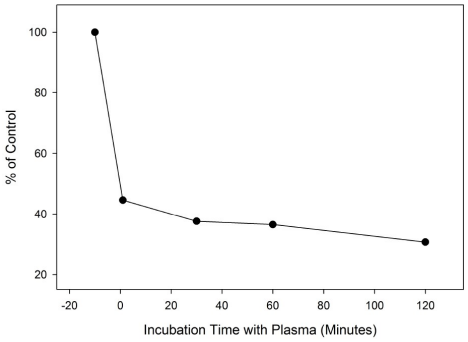


Figure 5. Inactivation of Endotoxin in Plasma. A known amount of endotoxin was added to aliquots of citrated plasma and allowed to incubate at room temperature for the indicated time. After incubation the samples were treated with ESP™ and detectable endotoxin was determined. Results are given as percent of a control sample which was heat-inactivated prior to endotoxin addition.

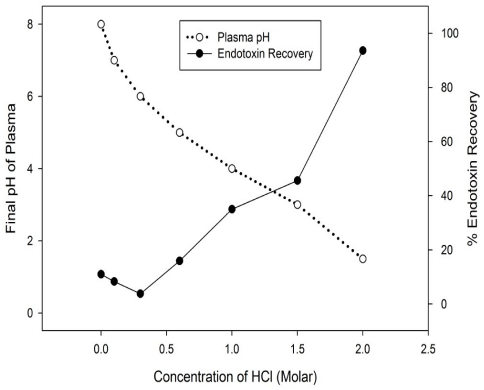


Figure 6. Overcoming Inactivation of Endotoxin in Plasma with Acidification. Samples of citrated plasma were acidified with 10% final volume of the indicated molarity of hydrochloric acid and a known amount of endotoxin was added to each. All samples were treated with ESP™ and total endotoxin recovery and final plasma pH were plotted as a function of acid molarity.

Discussion

Endotoxin detection in complex solutions can be problematic and inaccurate. The epitome of this is measuring endotoxin in blood products. Here, the problem of detection has been investigated and a novel solution has been provided with **ESP™**. In summary, blood plasma samples are prepared in a specialized low pH buffer, digested with an enzyme mixture designed to be compatible with LAL-based assays, and finally prepared in a second buffer for detection at neutral pH. The **ESP™** protocol typically requires less than 60 minutes and increases endotoxin detection 15-fold over common heat/dilution protocol. Endotoxin recovery using **ESP™** is usually over 75% of total endotoxin with PPC recovery exceeding 80%. These results make detection with **ESP™** the most accurate, sensitive and reliable reported.

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Endotoxin detection products:

EndoPrep™	*20 reactions*	*EDP-4001.01*
ESP™	*20 reactions*	*ESP-9001.01*

Endotoxin removal products:

EndoBind-R™	*1 ml column*	*EBR-3001.01*
EndoBind-R™	*5 ml column*	*EBR-3005.01*
EndoBind-R™	*10 ml bulk resin*	*EBR-3010.02*
EndoBind-R™ Single-Use		*EBR-3001.11*

Predictive Oncology also offers Endotoxin Detection and Removal Services. This includes routine sample treatment and testing as well as protocol development for difficult samples. Inquire for specifics.