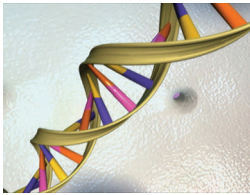




# EndoBind-R™



## Endotoxin Removal from DNA using EndoBind-R™

*Recombinant DNA methodologies have become a powerful tool in many fields of research and in gene therapy. However, DNA purified from gram-negative bacteria is contaminated with very high levels of a pyrogen termed lipopolysaccharide (or endotoxin). To optimize transfection efficiency and reduce adverse reactions to pyrogens, endotoxin must be removed. Here we describe the use of a peptide with high affinity and specificity for endotoxin in column chromatography to produce DNA samples with high recovery and low endotoxin.*

- High DNA recovery
- High specificity for endotoxin
- Endotoxin removal from low levels of contamination
- Rapid endotoxin removal
- Resistant to a wide range of buffer conditions
- Easy to use

### Introduction

The removal of endotoxin from DNA preparations for cell culture or therapeutic applications is a priority. The majority of lipid in the outer membrane of gram-negative bacteria consists of lipopolysaccharide (LPS), also called endotoxin. 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. For years, the *Limulus* amoebocyte lysate (LAL) test has been the standard for detecting even trace amounts of endotoxin. This test was developed from observations that horseshoe crab amoebocytes aggregate and degranulate in response to LPS as a defense mechanism against gram-negative bacteria [1,2]. This degranulation releases a series of enzymes that include Factor C, the initial activator of a serine protease cascade [3, 4]. In the LAL assay, Factor C detects picogram levels of LPS and initiates a clotting reaction. In recent years, the assay has been modified for detection with fluorescence, colorimetry, and turbidity, which made it more quantitative and less open to interpretation. Recently, a 34 amino acid LPS-binding Sushi domain was identified in Factor C. Expression and characterization of this linear peptide showed high binding to ( $K_d$   $10^{-6}$ - $10^{-8}$ ) and neutralization of ( $ENC_{50}$  2.25  $\mu$ M) LPS [5]. BioDtech's **EndoBind-R™** is a DADPA-agarose-conjugated Sushi peptide affinity chromatography column. It has been used to remove endotoxin from water, buffers, and cell culture media. It



BioDtech's EndoBind-R™

has also been used to remove endotoxin from protein solutions with minimal product loss. This included a wide variety of proteins including immunoglobulins [6].

The use of recombinant DNA for procedures such as transfection, gene therapy, microinjection, and transplantation has driven incredible scientific discovery in recent years. A significant drawback of this technology is that donor DNA typically comes from a bacterial origin and endotoxin copurifies with DNA in standard isolation techniques. Reports have shown that the level of contamination can be as high as 500 µg/ml (5,000,000 EU/ml) [7] and as little as 100 pg/ml (1 EU/ml) can cause toxicity [8]. Endotoxin contamination causes cytokine cascade activation which leads to altered cell structure and changes in metabolic function. Treatment with detergents, such as Triton X-114 and n-octyl-β-D-thioglucopyranoside (OSPG), can significantly reduce this but not to non-toxic levels [7, 9]. These procedures also cause the sample to be contaminated with detergent which must subsequently be removed. Affinity chromatography using Polymyxin B, which has an affinity for endotoxin, can reduce endotoxin levels in DNA to the range of 10 EU/ml (51 EU/mg) [10]. However, the product was greatly reduced and still contained significant endotoxin levels. In addition to activating the immune system, LPS contamination of DNA preparations results in low transfection rates and interferes with restriction digestion, cloning, PCR, and sequencing reactions [11, 12].

It has previously been shown that a wide variety of proteins can be purified using **EndoBind-R™** by optimizing the buffer conditions to account for the isoelectric point of the protein. Purifying DNA with the same technology presents a more difficult task given the similarities of endotoxin and DNA. These similarities have hindered DNA purification in the past. Endotoxins can form large micelles making it difficult to purify DNA with size exclusion columns. Along this line, these micelles often contain plasmid molecules. Both endotoxin and DNA have net negative charges due to high phosphate content, making it impossible to separate them using ion-exchange technology. In addition, both molecules have similar density making cesium chloride centrifugation protocols useless. Lastly, endotoxins are much more heat and pH stable than most biological samples preventing any inactivation protocols.

In this report we investigate endotoxin removal from DNA samples using **EndoBind-R™**. The initial binding between the Sushi peptide and LPS is due to electrostatic interactions between the positive residues near the N-terminus of the peptide and the negatively charged phosphoryl head groups of LPS. After this initial binding, hydrophobic interactions between the C-terminal end of the peptide and the acyl chains of LPS strengthen the binding [13]. Because of the nature of binding, buffer conditions may play an important role in product recovery and endotoxin removal. First, the minimum salt concentration required for removal of the DNA from the gel matrix was determined. Next, using this concentration of salt, the effect of buffer pH on DNA recovery and endotoxin removal was investigated. Finally, these conditions were applied to the purification of both small linear DNA fragments and a common cloning vector. The results show near complete DNA recovery with significant endotoxin removal.

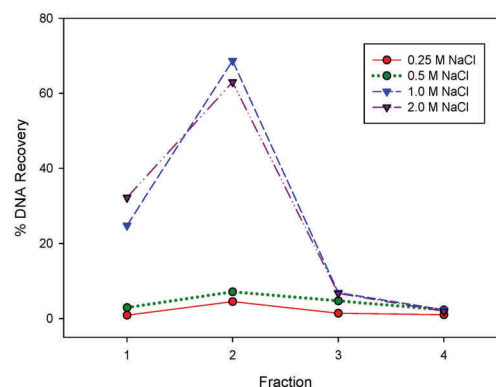
## Materials and Methods

DNA elution was performed at room temperature using BioDtech's **EndoBind-R™** with a column volume of 1 ml. Solutions were added to the column in 1 ml aliquots and collected with gravity flow in endotoxin-free tubes. The column was equilibrated with buffer before each use and was cleaned with washes of 2 M sodium chloride and 0.2% sodium deoxycholate before and after each use and stored in 0.02% sodium azide at 4°C. Salt and pH optimization experiments were done by applying 1 ml of the sample load to the column and collecting the flow-through. Next, the column was washed with three volumes of corresponding buffer with variable salt concentrations. The DNA content of the load was compared to the washes and recovery was calculated. Endotoxin removal experiments were done by applying 1 ml of the sample to the column and collecting the flow-through. Next, the column was washed with 3 sequential 1 ml volumes of salt-containing buffer and collected. The DNA and endotoxin content of the load and each fraction were determined and DNA recovery and endotoxin removal were calculated.

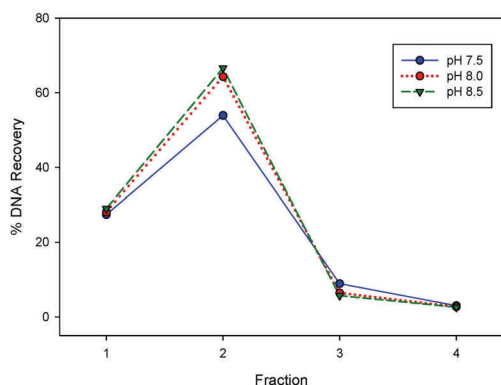
Endotoxin levels were determined with the PyroGene Recombinant Factor C Endotoxin Detection System (Lonza, Walkersville, MD) according to manufacturer's specifications. Samples were tested at multiple dilutions and the absence of inhibition/enhancement was verified with endotoxin spikes. DNA concentrations were determined by absorbance at OD<sub>260</sub> as measured by the Synergy II plate reader (BioTek, Winooski, VT). A 5 mg/ml stock of Salmon sperm DNA (Calbiochem, La Jolla, CA) was made in TE pH 8.0. pUC19 was isolated from DH5α cells using the Promega (Madison, WI) PureYield Plasmid Midiprep System. The plasmid was eluted with TE pH 8.0 to make a 30 µg/ml stock. All samples, buffers, and chemicals were prepared using Pyrogen-Free Water (available from BioDtech).

## Results

*The Effect of Sodium Chloride Concentration on DNA Recovery.* A previous report [10] using Polymyxin B affinity chromatography, which has a much lower affinity to endotoxin than does the Sushi peptide, showed modest success in purifying DNA samples from endotoxin using a TE (10 mM Tris, 1 mM EDTA) buffer at pH 8.0 with 0.25 M sodium chloride. This result, and the fact that a majority of DNA applications use a TE buffer at or near this pH, prompted the use of these criteria for initial experiments with **EndoBind-R™**. To characterize DNA purification, salmon sperm DNA was used. This preparation of DNA consisted of linear DNA fragments with a median size of 100 base pairs. From a 5 mg/ml stock solution, a 50 µg/ml solution was made which gave an OD<sub>260</sub> absorbance value of about 0.8. This concentration allowed DNA tracking using absorbance. At sodium chloride concentrations less than 1 M little to no DNA recovery was achieved (Figure 1). However, at 1 M and above the cumulative recovery was over 99% of the load. The flow-through typically contained 20-30% of the initial load and the second fraction contained the peak value of 60-70%. The third and fourth fractions



**Figure 1. The Effect of Salt Concentration on DNA Recovery.** The effect of sodium chloride concentration on salmon sperm DNA recovery was determined using 50 µg/ml samples in TE pH 8.0. The salt range tested was from 0.25 to 2.0 M.



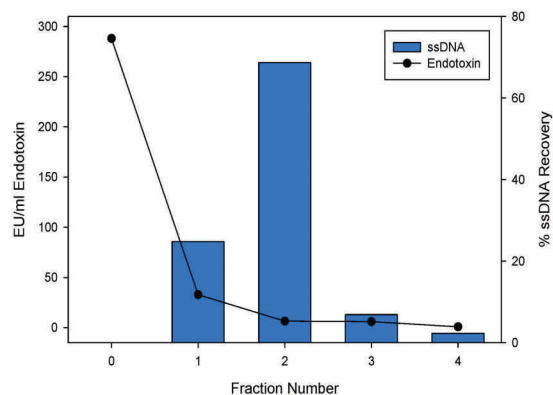
**Figure 2. The Effect of Buffer pH on DNA Recovery.** The effect of buffer pH on salmon sperm DNA recovery was determined using 50 µg/ml samples in TE containing 1 M sodium chloride. The pH range tested was from 7.5 to 8.5.

7.5 sample being just slightly less efficient at DNA recovery (Figure 2). In all cases, the first fraction contained 25-30% of the load and the second contained the peak of 55-70%. Fractions 3 and 4 contained the remainder. The concept that buffer pH is not as crucial in DNA purification as it is in protein purification is not surprising considering that both endotoxin and DNA molecules carry extensive negative charges due to their phosphate content. In addition to DNA recovery, endotoxin removal was virtually identical at all three pH levels (Data Not Shown).

**Endotoxin Removal from Salmon Sperm DNA.** After establishing conditions to recover DNA from EndoBind-R™, the ability to remove endotoxin was examined. As before, this was first tested with salmon sperm DNA. A 50 µg/ml sample of DNA was prepared in TE pH 8.0 with 1 M sodium chloride and 25 ng/ml (250 EU/ml) *E. coli* O55:B5 endotoxin. The DNA was tested for endogenous endotoxin contamination and was found to contain negligible amounts. A 1 ml aliquot of this load (fraction 0) was placed onto the column and the flow-through (fraction 1) was collected. Next, the column was rinsed with three 1 ml washes of TE pH 8.0 containing 1 M sodium chloride (fractions 2-4). The DNA content and endotoxin levels of each fraction were determined and compared to the load. Nearly all of the DNA was recovered in the four fractions in a similar pattern as before (Figure 3). In addition, the endotoxin level was reduced significantly in all fractions. The initial load contained 288 EU/ml and was reduced to 32 EU/ml in the flow-through. The peak DNA fraction contained nearly 70% of the product and retained only 6 EU/ml endotoxin. This represents removal of 98% of the endotoxin.

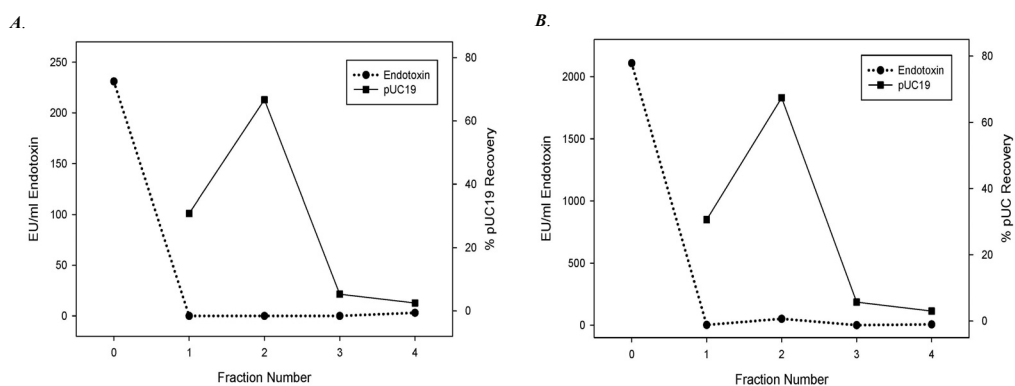
usually contained less than 10% combined. These results dictated that 1 M salt would be necessary to sufficiently remove DNA from the column.

**The Effect of Buffer pH on DNA Recovery.** Given the importance of electrostatic interactions in the initial binding of the Sushi peptide to endotoxin, buffer pH may contribute to optimal purification. Previously, protein recovery was shown to be affected by buffer pH in a pI-dependent manner. Samples of 50 µg/ml salmon sperm DNA were prepared in TE at pH's of 7.5, 8.0, and 8.5 containing 1 M sodium chloride. In three independent experiments, the samples were applied to EndoBind-R™ and washed with TE at the same pH also containing 1 M sodium chloride. The DNA content of all the fractions was measured and compared against the initial load. The pH 8.0 and 8.5 samples gave almost identical results with the pH



**Figure 3. Endotoxin Removal from Salmon Sperm DNA.** Salmon sperm DNA samples at 50 µg/ml containing 25 ng/ml endotoxin were prepared in TE pH 8.0 containing 1 M sodium chloride and applied to EndoBind-R™. After collecting the flow-through, the remaining DNA was recovered in three subsequent washes. DNA recovery was determined by absorbance and endotoxin levels were determined by PyroGene (Lonza) assay.

**Endotoxin Removal from Plasmid DNA.** After successful recovery and endotoxin removal from linear DNA fragments, purification of plasmid DNA was tested. This is the more applicable test since most procedures use recombinant DNA vectors for experimentation. The common cloning vector pUC19 was chosen for purification. It is a high-copy, 2686 base pair plasmid with a 54 base pair multiple cloning site and ampicillin resistance. A stock solution of pUC19 was prepared from a DH5 $\alpha$  strain as described (Materials and Methods). The ability of **EndoBind-R™** to remove endotoxin in DNA samples with both high- and low-level contamination was tested. The low-endotoxin sample contained 30  $\mu\text{g/ml}$  pUC19 in TE pH 8.0 with 1 M sodium chloride and 25 ng/ml (250 EU/ml) *E. coli* O55:B5 endotoxin. The high-endotoxin sample was identical but contained 10-fold more of the endotoxin stock (2500 EU/ml). As with the salmon sperm DNA, a 1 ml portion of each sample was applied to the column and the flow-through was collected (fraction 1). The column was then rinsed with three 1 ml washes of TE pH 8.0 with 1 M sodium chloride (fractions 2-4). The low-endotoxin load contained 231 EU/ml (fraction 0) and was reduced to below the level of detection (0.01 EU/ml) after treatment with **EndoBind-R™** (Figure 4A). This represents removal of over 99.99% of the endotoxin. The plasmid recovery was as efficient as linear DNA with about 30% in the flow-through (fraction 1) and nearly 70% in the peak fraction (fraction 2). Results with the high-endotoxin pUC19 were similarly impressive (Figure 4B). The initial endotoxin load of 2109 EU/ml (fraction 0) was reduced to less than 2 EU/ml in the flow-through (fraction 1) and to 50 EU/ml in the peak DNA fraction (fraction 2). This represents removal of nearly 98% of endotoxin in the peak fraction and over 99.9% in the flow-through. DNA recovery was nearly identical in both low- and high-endotoxin experiments.



**Figure 4. Endotoxin Removal from pUC19 DNA.** pUC19 plasmid samples at 30  $\mu\text{g/ml}$  containing either (A) 25 ng/ml or (B) 250 ng/ml endotoxin were prepared in TE pH 8.0 containing 1 M sodium chloride and applied to EndoBind-R™. After collecting the flow-through, the remaining DNA was recovered in three subsequent washes. DNA recovery was determined by absorbance and endotoxin levels were determined by PyroGene (Lonza) assay.

## Discussion

DNA purification with **BioDtech's EndoBind-R™** produced samples with high DNA recovery and removal of endotoxin to very low levels. Efficient endotoxin removal owes to the high affinity of the Sushi peptide on the column to the lipid A portion of LPS. This binding occurs via initial electrostatic interactions followed by hydrophobic interactions that reinforce the association [13]. Linear salmon sperm DNA in fragments of approximately 100 base pairs were used to characterize the conditions required for optimal product recovery. Salt content of the samples showed a critical importance with sodium chloride levels at or above 1 M required for full DNA removal (Figure 1). Buffer pH was less important with a pH level at or above 8.0 slightly better than one below (Figure 2). In combination, a TE buffer at pH 8.0 with 1 M sodium chloride provided nearly 100% product recovery with up to 70% in the peak fraction in all experiments. Endotoxin

removal at these conditions was also very efficient. Endotoxin contamination in small DNA fragments was reduced by 98% (Figure 3). Plasmid purification gave similar numbers with low-level contamination being reduced to levels below detection (0.01 EU/ml) (Figure 4A) and high-level contamination being reduced by approximately 98% (Figure 4B). These results demonstrate that **EndoBind-R™** can be used to efficiently remove endotoxin from DNA solutions with negligible product loss. Purification of a common cloning vector showed 98% endotoxin removal with loads in excess of 20,000 ng/ml and removal to below detection limits with 10-fold less starting endotoxin. Similar efficient endotoxin removal has been shown with protein solutions with a wide variety of physical characteristics [6]. DNA purification with **EndoBind-R™** will prove invaluable in technologies such as transfection, gene therapy, microinjection, and transplantation.

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

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*Endotoxin removal products:*

<i>EndoBind-R™</i>	<i>1 ml column</i>	<i>EBR-3001.01</i>
<i>EndoBind-R™</i>	<i>5 ml column</i>	<i>EBR-3005.01</i>
<i>EndoBind-R™</i>	<i>Bulk resin</i>	<i>Inquire</i>

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