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Endotoxin-Free Protein Production - ClearColi™ Technology

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Abstract

The Gram-negative bacterium *E. coli* is the workhorse of molecular biology, regularly used as the first choice host for DNA cloning, small-scale protein expression, and large-scale protein production for FDA approved biologics. One of the major limitations in using *E. coli* relates to the lipopolysaccharide (LPS) component of the outer membrane. LPS is an endotoxin that is a potent activator of many immune cells through the Toll-like receptor-4 (TLR4) and can directly trigger endotoxic shock (septic shock), resulting in severe medical problems and death. Such a toxic component requires extensive, and expensive, removal during protein purification. In addition to medical applications, LPS is unwanted in basic research that involves human cells and tissue as toxicity experiments are compromised due to the presence of endotoxins. Current methods for endotoxin removal are varied, including ultra-filtration, activated carbon, surfactants, anion exchange chromatography, and immobilized sepharose. Each of these strategies involves negative effects: significant yield loss, high cost, loss of bioactivity of the protein, or bioactivity of the additives used for endotoxin cleanup.

Eliminating LPS from the *E. coli* outer membrane is a technically more desirable pathway to clean recombinant proteins, which can lead to new *E. coli* strains that are useful as research tools and potentially as a protein production platform for therapeutic proteins. Lucigen, in combination with Research Corporation Technologies, has developed a new line of *E. coli* competent cells called ClearColi™. These cells have been genetically modified to remove the immune response triggers associated with LPS while still retaining viability and protein expression capabilities. By eliminating the offending LPS, purification of recombinant proteins for downstream cell-based assays is reduced to a simple nickel column process. Immune response data from multiple methods for endotoxin detection will be presented, demonstrating that proteins expressed from this novel cell line cause virtually no inflammatory cytokine production, eliminating the need for endotoxin removal treatments.

Endotoxin/LPS Introduction

In *E. coli,* there are ~2 x 10^6 LPS molecules per cell, accounting for 30% of total outer membrane gross weight (see Fig 1). In mammalian cell culture, LPS contamination triggers secretion of pro-inflammatory cytokines, poor cell growth, reduced DNA transfection efficiency, problematic differentiation, cell death, and compromised experimental results. In humans, LPS activates the immune system, resulting in endotoxic shock or even death.

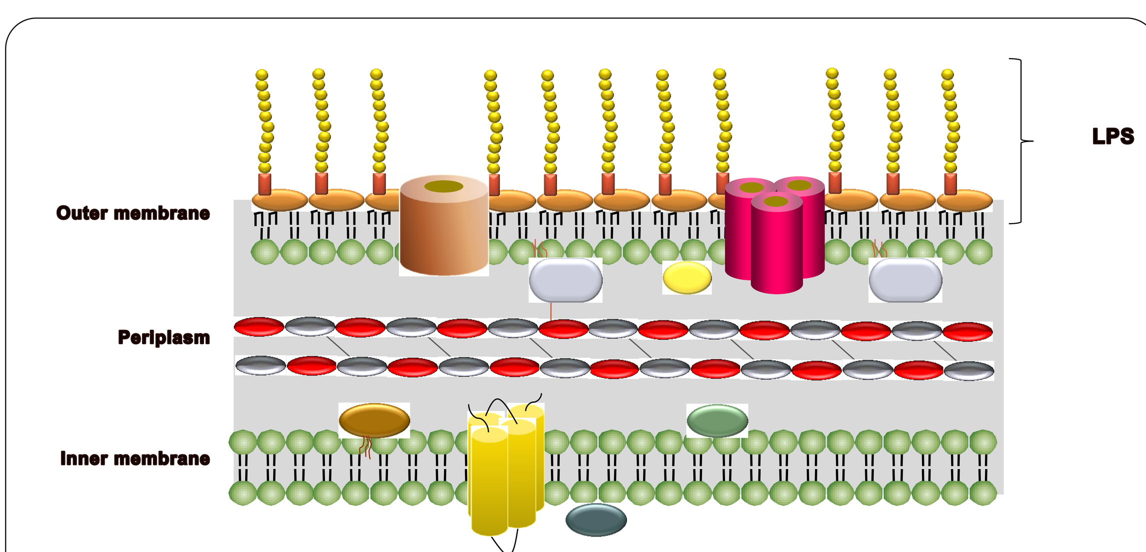

Fig 1. Diagram of *E. coli* membrane with LPS extending outside the cell.

Endotoxin Removal Challenges

There are multiple different methods for endotoxin removal, all of which have disadvantages.

Method	Disadvantages
Ultrafiltration	• Only useful for small proteins • Endotoxin monomers may permeate the membrane due to endotoxin/protein interaction • Inefficient for proteins which can be damaged by physical forces
Activated carbon	• Adsorbing activity for both endotoxin and protein
Surfactants	• Expensive • May affect bioactivity of protein • Difficult to completely remove • Removal may lead to product loss
Anion-exchange chromatography	• High adsorbtion of both endotoxin and acidic protein • No selectivity to adsorb endotoxin
Histamine- and histidine-immobilized Sepharose	• Removing capacity dependent on the ionic strength • Biological activity of histamine
Polymyxin B-immobilized Sepharose	• Protein losses due to the ionic interaction between polymyxin B and protein • Polymixin B is physiologically active

ClearColi™ Technology

ClearColi competent cells have a genetically modified LPS that does not cause an endotoxic response in human cells. This has been accomplished by radically modifying the production of LPS through the incorporation of seven genetic deletions (Δ*gutQ* Δ*kdsD* Δ*lpxL* Δ*lpxM* Δ*pagP* Δ*lpxP* Δ*eptA*). One additional compensating mutation (*msbA148*) enables viability in the presence of lipid IV_A.

In ClearColi cells, the polysaccharide chains are eliminated and two secondary acyl chains of the normally hexa-acylated LPS have been deleted, which is a key determinant of endotoxicity in eukaryotic cells. The six acyl chains of the LPS are the trigger which is recognized by TLR4 in complex with myeloid differentiation factor 2 (MD-2), causing activation of NF-κB and production of proinflammatory cytokines. The deletion of the two secondary acyl chains results in lipid IV_A, which does not induce formation of the activated heterotetrameric hTLR4/MD-2 complex and thus does not trigger the endotoxic response. In addition, the oligosaccharide chain is deleted, making it easier to remove the resulting lipid IV_A from any downstream product (see Fig 2).

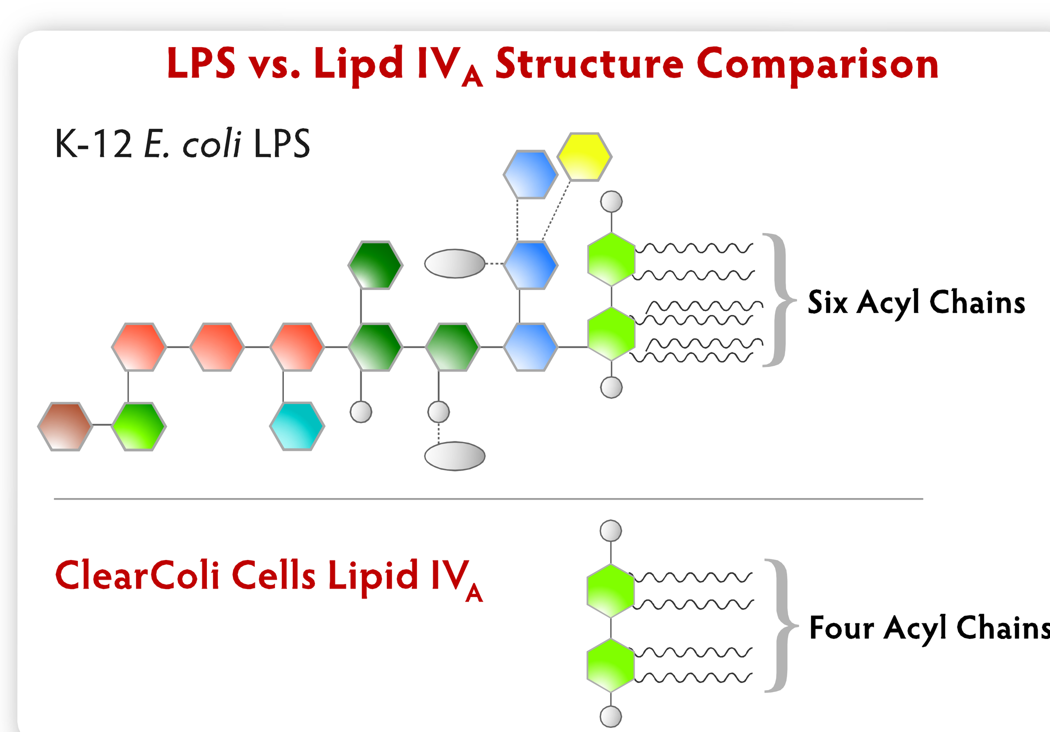

Fig 2. Structure comparison of normal LPS from K-12 *E. coli* vs. polysaccharide deficient lipid IV_A from ClearColi cells.

Protein Expression Comparison

When grown to sufficient densities, ClearColi BL21(DE3) cells produce similar protein levels as normal BL21(DE3) cells when comparing equal numbers of cells (see Fig 3).

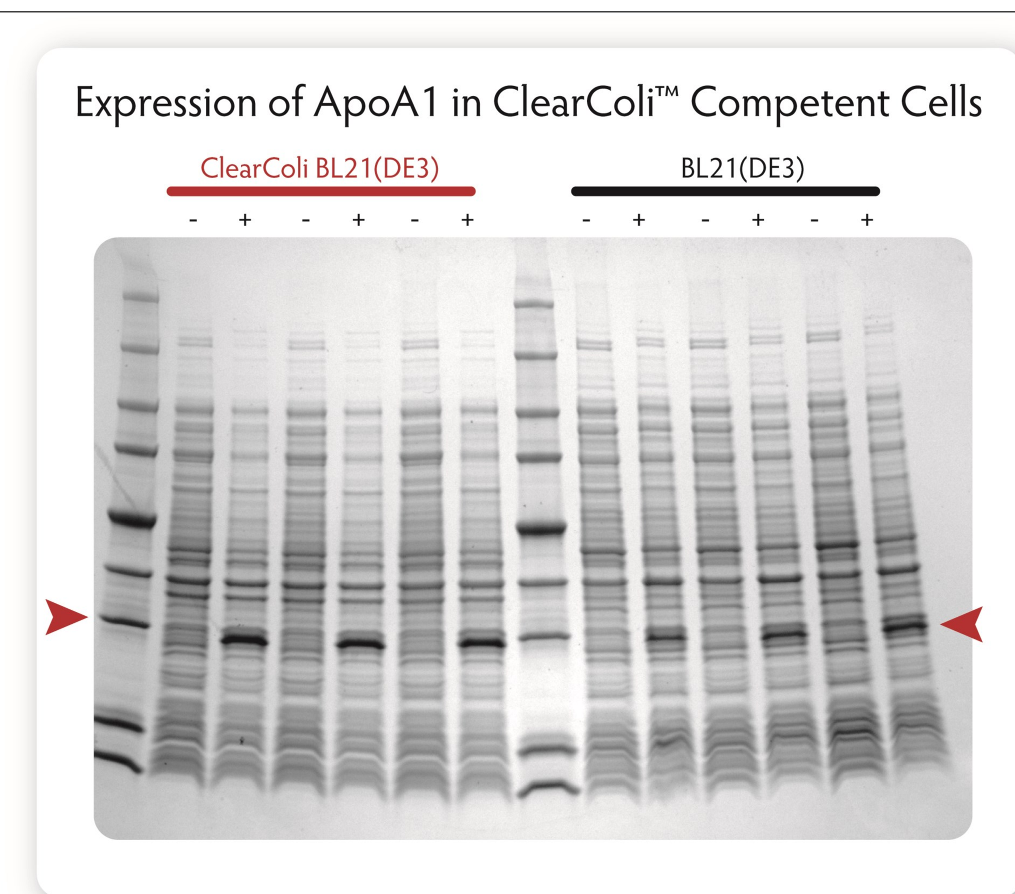

Fig 3. Comparison of protein expression in ClearColi BL21(DE3) and Lucigen's *E. Cloni*® EXPRESS BL21(DE3) competent cells. Cells containing a T7 expression plasmid harboring a gene encoding the human apolipoprotein A1 (ApoA1) were grown in LB Miller medium at 37°C. When cultures reached OD_{600} of 0.6 to 0.8, expression was induced by the addition of 0.4 mM IPTG and incubation was continued for 3 hours. Equivalent numbers of uninduced (-) and induced (+) cells were lysed by heating in Laemmli buffer and samples were analyzed by SDS-PAGE on a 4% - 20% polyacrylamide gradient gel.

Endotoxicity of Final Proteins

ApoA1 protein was expressed from a T7-promoter based plasmid in normal BL21(DE3) and CleatColi BL21(DE3) cells, followed by a simple IMAC-column purification step without any subsequent endotoxin removal steps. The purified proteins were then tested for TLR stimulation by assessing NF-κB activation in HEK-Blue™-4 cells expressing human TLR4. Protein derived from ClearColi BL21(DE3) cells demonstrated no activation at concentrations 4 orders of magnitude greater than the protein from traditional BL21(DE3) cells (see Fig 4).

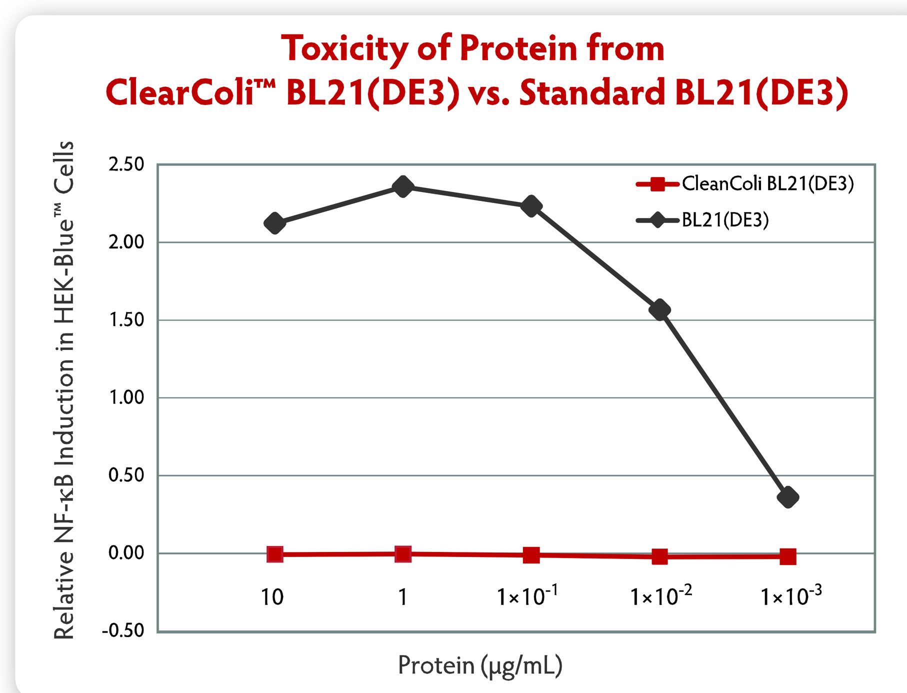

Fig 4. Comparison of endotoxic response from protein derived from ClearColi BL21(DE3) and traditional BL21(DE3) competent cells.

Non-Specificity of LAL Assay

Limulus amebocyte assay (LAL) testing is an FDA-approved method for detection of endotoxins and the most common assay used; however the LAL assay is activated solely by the 4´-monophosphoryldiglucosamine backbone of LPS. LAL activity is minimally influenced by acylation pattern of LPS, the key determinant of endotoxicity in eukaryotic cells. The LAL assay also recognizes a wider spectrum of LPS/lipid A variants than the central cellular endotoxin sensor system of the human immune cell system. As such, false positive results can and will result due to the lack of specificity of the assay.

A simple Ni-column purification step for proteins produced from ClearColi cells will reduce LAL response levels by 95% or greater (see Fig 5). However, the residual endotoxin unit (EU) measurements are due to the non-specific nature of the assay unless extraneous LPS contamination from other sources is present. Alternative toxicity assays, such as those using HEK-Blue™-4 cells (see Fig 4) suggest that even in the presence of EU levels above threshholds normally targeted by researchers, the actual immunogenic effects from ClearColi-derived proteins are non-existent.

Due to the non-specificity of the LAL assay when combined with lipid IV_A from ClearColi, it is suggested that researchers consider alternative methods of endotoxin measurement.

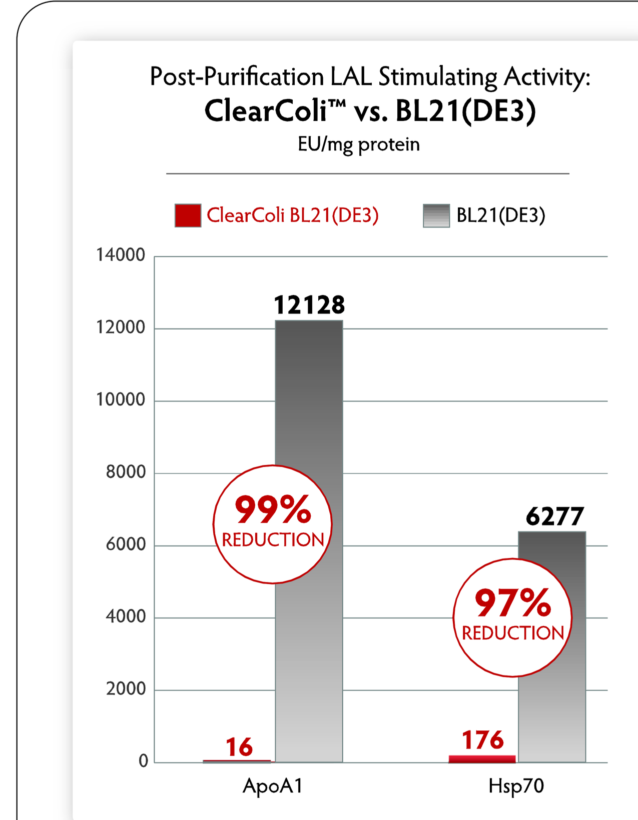

Fig 5. Comparison of LAL response in proteins derived from normal BL21(DE3) cells and ClearColi BL21(DE3) cells after Ni-column purification. No endotoxin removal steps were performed, yet LAL response is significantly reduced by >95%.

Conclusions

By genetically modifying the LPS of *E. coli* BL21(DE3) cells, we have created competent cells capable of expressing protein suitable for downstream toxicity assays in human cells without the need for endotoxin removal methods. Simple IMAC-column purification is sufficient in most cases.

- Protein purification yields are similar to standard strains
- Minimal purification needed to remove lipid IV_A
- ClearColi™ purified proteins do not activate relevant assays - no stimulation of hTLR4 cascade
- Significant time and cost savings – lower purification requirements prior to cell based screening
- Eliminate LPS carryover and associate side effects
- Reduce false positives in cytokine assays, improve confidence in your results

Additional ClearColi strains for plasmid production and phage display applications are also in development.

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COMPETENT CELLS

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