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 endotoxin 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 ultrafiltration, 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.

Endotoxin/LPS Introduction

In E. coli, there are ~2 x 10^9 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 cytokines, cell growth, reduced DNA transfection efficiency, problematic differentiation, cell death, and compromised experimental results. In humans, LPS activates the immune system, resulting in endotoxin shock or even death.

Endotoxin Removal Challenges

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

- **Method**
  - **Dilution/Adsorption**
  - **Activated carbon**
  - **Antigen-exchange immunoaffinity**
  - **Histamine- and histamine-|nduced Sephasse**
  - **Poly(lysine)- |nduced Sephasse**

- **LPS vs. Lipid IVß Structure Comparison**

![Fig 2. Structure comparison of normal LPS from K-12 E. coli vs. polysaccharide deficient lipid IVß from ClearColi cells.](Image)

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-Clont® EXPRESS BL21(DE3) competent cells. Cells containing a T7 expression plasmid harboring a gene encoding the human antiplatelet protein ApoA1 (ApoA1) were grown in LB Miller medium at 37°C. When cultures reached OD600 of 0.8 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.](Image)

Endotoxicity of Final Proteins

ApoA1 protein was expressed from a T7-promoter based plasmid in normal BL21(DE3) and ClearColi 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.](Image)

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 with no 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 IVß
- ClearColi™ purified proteins do not activate relevant assays
- No stimulation of LAL4 cascade
- Significant time and cost savings – lower purification requirements prior to cell based screening
- Eliminate LPS carrier 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.

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'-monophosphoryl lipid A backbone of LPS. LAL activity is minimally influenced by acylation pattern of LPS, but the need for endotoxin removal methods. The LAL assay also recognizes a wider spectrum of LPS/lipid A variants than the central cellular endotoxin sensor system of the human immune 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 thresholds 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 IVß from ClearColi, it is suggested that researchers consider alternative methods of endotoxin measurement.

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

Fig 2. 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%.

ClearColi™ Competent Cells are subject to US Patent 8,303,964 and other US and foreign pending patents. For information on the ClearColi licensing program, please visit www.clearcoli.com