Endotoxic-Free Protein Production - CleanColi™ 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, mini-prep, 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 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.

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 CleanColi. 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 10^9 LPS molecules per cell, accounting for 30% of total outer membrane mass weight (see Fig 1). In mammalian cell culture, LPS contamination triggers secretion of pro-inflammatory cytokines, 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.

Endotoxin Removal Challenges

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

- **Unfractionated**: Only useful for small proteins
  - **Endotoxin removal may mask the membrane due to endotoxin/protein interaction**
  - **Inefficient for proteins which can be damaged by physical stress**

- **Activated carbon**: Absorbing activity for both endotoxin and protein
  - **Adsorption**
  - **Difficult to completely remove**
  - **Difficult to scale-up the process**

- **Antigen-exchange chromatography**: High adsorption of both endotoxin and acidic protein
  - **High adsorption to the resin**
  - **Difficult to completely remove**

- **Hepes- and histidine-based affinity chromatography**: Removing capacity dependent on the ionic strength and biological activity of the protein

- **Polyacrylamide and Sepharose**: B and protein
  - **Polyacrylamide is cytotoxic**
  - **Low bioactivity due to the interaction between polyacrylamide and protein**

Endotoxin-Free Protein Production

CleanColi™ Technology

CleanColi 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 (ΔlpxO ΔlpxD ΔlpxL ΔlpxM ΔlpxR ΔlpxP ΔlpxA). One additional compensating mutation (msbA149) enables viability in the presence of lipid IVa.

In CleanColi cells, the polysaccharide chains are eliminated and two secondary acyl chains of the normally hexaacylated 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 myxoid differentiation factor 2 (MD-2), causing activation of NF-κB and production of pro-inflammatory cytokines. The deletion of the two secondary acyl chains results in lipid IVa, which does not induce formation of the activated heterotetrameric TLR4/MD-2 complex and thus does not trigger the endotoxic response. In addition, the oligosaccharide chain is deleted, making it easier to remove the lipid IVa from any downstream product (see Fig 2).

Protein Expression Comparison

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

Toxicity of Protein from CleanColi™ BL21(DE3) vs. Standard BL21(DE3)

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

Non-Specificity of LAL Assay

LAL test 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, 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 may occur and will result due to the lack of specificity of the assay.

A simple Ni-column purification step for proteins produced from CleanColi 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® cells (see Fig 4) suggest that even in the presence of EU levels above thresholds normally targeted by researchers, the actual immunogenic effects from CleanColi-derived proteins are non-existent.

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

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 IVa**
- **CleanColi™ purified proteins do not activate relevant assays**
- **No stimulation of TLR4 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 CleanColi strains for plasmid production and phase display applications are also in development.

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

Fig 2. Structure comparison of normal LPS from K-12 E. coli vs. polysaccharide deficient lipid IVa from CleanColi cells.

Fig 3. Comparison of protein expression in CleanColi BL21(DE3) and Lucigen’s E. Coli® 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 OD600 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.

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

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