



Endotoxin-free protein production—ClearColi™ technology

A new *Escherichia coli* strain with a genetically modified lipopolysaccharide (LPS) molecule has been created that enables protein expression without the endotoxin. Proteins expressed and purified from ClearColi™ BL21(DE3) cells will not trigger LPS-related immune response in human cells, making them ideal for immunogenicity, toxicity and cytokine assays.

Introduction

ClearColi™ competent cell strains are derivatives of *E. coli*, which are regularly used as the first-choice host for DNA cloning and protein production, including in FDA-approved biologics. One of the major limitations in using traditional *E. coli* strains relates to the LPS component of the outer membrane. Such a toxic component requires extensive and expensive removal during protein purification. Additionally, the endotoxin can compromise many basic research experiments involving human cells and tissues. Current methods for endotoxin removal are varied and include ultrafiltration, activated carbon, surfactants, anion exchange chromatography and immobilized sepharose. The use of these strategies often results in significant yield reduction, increased cost or a loss of the bioactivity of the desired protein. Genetically eliminating LPS from the *E. coli* outer membrane is technically a more efficient means to prepare endotoxin-free recombinant proteins. This approach has led to new *E. coli* strains that are potentially useful as research tools and protein production platforms for therapeutic proteins.

Lucigen and Research Corporation Technologies have 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. Here we summarize immune response data from multiple methods for endotoxin detection, demonstrating that proteins expressed from this new cell line cause virtually no inflammatory cytokine production, eliminating the need for endotoxin removal treatments.

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ClearColi™ technology

In *E. coli*, there are about 2×10^6 LPS molecules per cell, accounting for 30% of the total outer membrane gross weight. In mammalian cell culture, LPS contamination leads to secretion of proinflammatory 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.

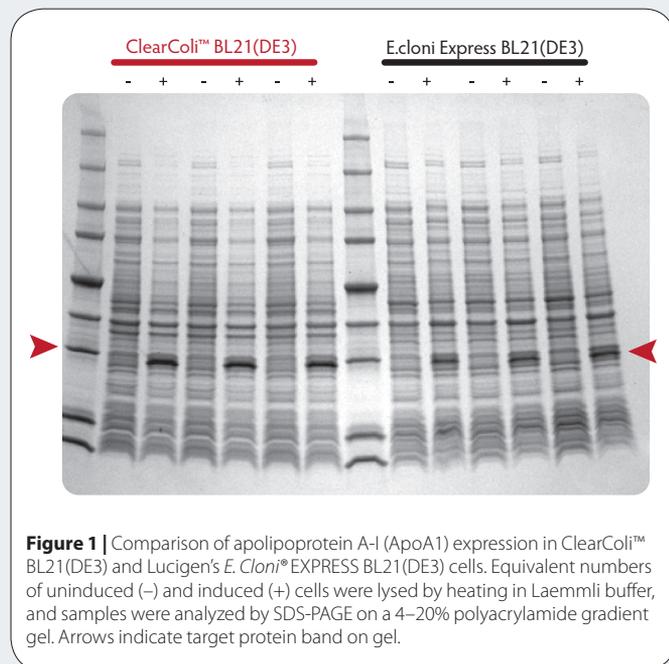
ClearColi™ competent cells have a genetically modified LPS that does not cause an endotoxic response in human cells. This has been accomplished by radically modifying the synthesis of LPS through the incorporation of seven genetic deletions ($\Delta gutQ$, $\Delta kdsD$, $\Delta lpxL$, $\Delta lpxM$, $\Delta pagP$, $\Delta lpxP$ and $\Delta eptA$), which remove all of the carbohydrate decorations usually attached to LPS. One additional compensating mutation (*msbA148*) enables the viability of ClearColi™ cells in the presence of lipid IV_A, a nonglycosylated precursor of LPS biosynthesis that lacks the two secondary acyl chains of the normally hexa-acylated LPS. The six acyl chains of *E. coli* LPS are the trigger, which is recognized by the human toll-like receptor 4 (hTLR4) and myeloid differentiation factor-2 (MD-2) complex, leading to activation of NF-κB and the production of proinflammatory cytokines. In contrast, lipid IV_A does not induce formation of the activated heterotetrameric hTLR4-MD-2 complex and thus does not trigger the endotoxic response. Additionally, the lack of the oligosaccharide chain in lipid IV_A allows for easy downstream removal from any product.

Protein expression and endotoxin measurements

Apolipoprotein A-I (ApoA1) protein was expressed from a T7 promoter-based plasmid in normal BL21(DE3) and ClearColi™ BL21(DE3) cells, followed by simple ion affinity chromatography column purification without any subsequent endotoxin removal steps. When grown to sufficient densities, ClearColi™ BL21(DE3) cells produce protein levels similar to those generated by an equal number of normal BL21(DE3) cells (Fig. 1). The purified proteins were then tested for endotoxin

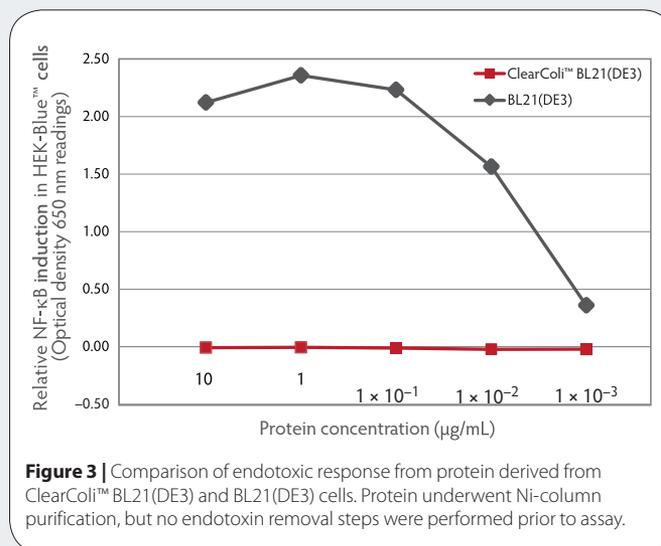
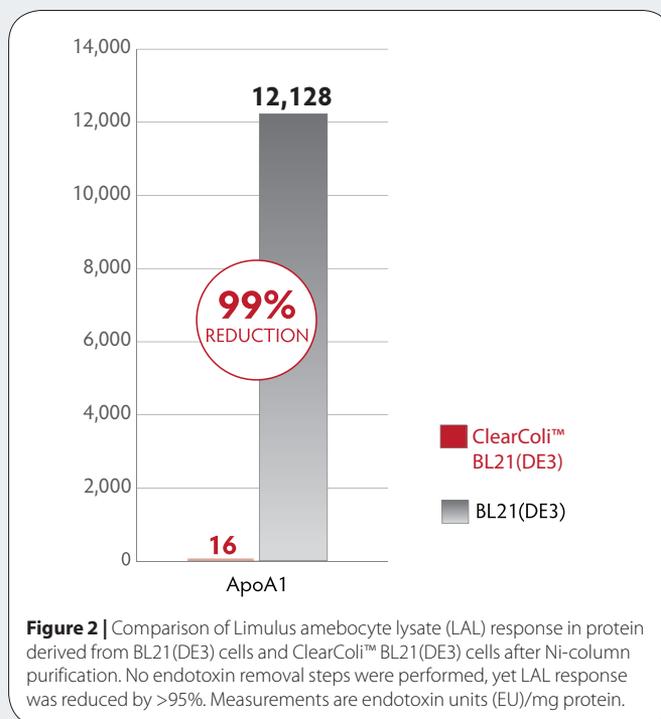
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levels as determined by the Limulus amoebocyte lysate (LAL) assay and for hTLR4-MD-2 stimulation by assessing NF- κ B activation in HEK-Blue™-4 cells (InvivoGen).



The LAL assay is the most common FDA-approved method for the detection of endotoxins. However, the assay cannot discriminate between variants of lipid A. The LAL assay is activated solely by the 4'-monophosphoryldiglycosamine backbone of LPS. LAL activity is minimally influenced by the acylation pattern of LPS, the key determinant of endotoxicity in eukaryotic cells. The LAL assay also recognizes a wider spectrum of LPS and lipid A variants than the central cellular endotoxin sensor of the human immune cell system. As such, false-positive results can, and do, occur because of the lack of specificity. Although lipid IV_A is known as an endotoxin antagonist in LPS-responsive human cells, the tetra-acylated LPS precursor may act as an endotoxic activator in other nonhuman mammalian hosts, which reflects the species-specific recognition and stimulatory activity of lipid IV_A caused by species-specific differences in the structures of TLR4 and MD-2.

Purification of the ClearColi™-expressed protein is required for optimal performance. As an example, simple Ni-column purification will reduce LAL response levels by 95% or more (Fig. 2). The residual endotoxin (EU) levels are likely due to the nonspecific nature of the LAL assay. Alternative endotoxin detection assays, such as those using HEK-Blue™-4 cells, suggest that even in the presence of endotoxin levels above thresholds normally targeted by researchers, the actual immunogenic effects from ClearColi™-derived proteins are nonexistent. In the HEK-Blue™ assay, protein derived from ClearColi™ BL21(DE3) cells demonstrated no NF- κ B activation at a concentration four orders of magnitude greater than that for protein derived from traditional BL21(DE3) cells (Fig. 3).



Conclusions

Lucigen has created cell lines capable of expressing proteins suitable for assays in human cells without the need for endotoxin removal. Protein yields are similar to those of standard strains, with only simple purification methods required in most cases to remove residual lipid IV_A. ClearColi™-derived purified proteins do not activate the hTLR4-MD-2 cascade, resulting in fewer false positives in cytokine assays and improved confidence in experimental results. The use of ClearColi™ competent cells will allow researchers to achieve significant time and cost savings by eliminating LPS contamination and the associated side effects at the source.

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