Lucigen®
Simplifying Genomics

ClearColi® BL21(DE3) Electrocompetent Cells

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MA145 Rev. E

Table of Contents

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Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is imperative that the reagents supplied by the user are of the highest quality. Please follow the instructions carefully and contact our technical service representatives if additional information is necessary. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.

Lucigen Technical Support:

Email: techserv@lucigen.com

Phone: (888) 575-9695

Product Guarantee: Lucigen guarantees that this product will perform as specified for one year from the date of shipment. Please avoid using reagents for greater than one year from receipt.

Limited Use License

If you are a commercial entity, your ClearColi kit purchase comes with a 12-month Limited Use License from Research Corporation Technologies.

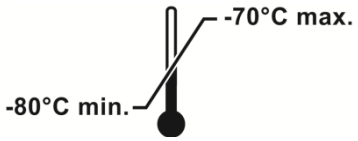
Please refer to Legal Information (p. 12) or this URL for the terms of use:
http://clearcoli.com/licensing/limited-use-license-2/

If you disagree with the terms of use, you have 10 days to contact Lucigen Corp. for permission to return unused cells.

Contact Lucigen at: custserv@lucigen.com

Components & Storage Conditions

All ClearColi® Competent Cells require storage at –80° C.



ClearColi BL21(DE3) Electrocompetent Cells

STRAIN	Efficiency (cfu/µg pUC19)	Transformations	Catalog #	Storage
ClearColi BL21(DE3) Electrocompetent Cells (White cap)	>1 x 10^9 cfu/ug	12 (6 x 50 µl) 24 (12 x 50 µl)	60810-1 60810-2	**-80°C**
Expression Recovery Medium (lactose minus)*	--	12 (1 x 12 ml) 24 (2 x 12 ml)	--	**-20 to -80°C**
Supercoiled pUC19 DNA (10 pg/µL)	--	1 x 20 µl	--	**-20 to -80°C**

As a control for transformation, ClearColi Electrocompetent Cells are provided with supercoiled pUC19 DNA at a concentration of 10 pg/µl. Use 1 µl for transformation.

* Additional Expression Recovery Medium (lactose minus) can be ordered separately as catalog # 80030-1, 96 ml (8 x 12 ml).

Expiration Dating

ClearColi competent cells are guaranteed for performance for 1 year after receipt of the product. Do not use this product past the expiration date. If you experience technical performance issues or have general questions, please contact Lucigen at techserv@lucigen.com

ClearColi Electrocompetent Cells

Introduction to ClearColi Technology

ClearColi® BL21(DE3) cells are the first commercially available competent cells with a modified LPS (Lipid IV_A - see Fig. 1) that does not trigger the endotoxic response in human cells. ClearColi cells lack outer membrane agonists for hTLR4/MD-2 activation; therefore, activation of hTLR4/MD-2 signaling by ClearColi® is several orders of magnitude lower as compared with *E. coli* wild-type cells. Heterologous proteins prepared from ClearColi® are virtually free of endotoxic activity. After minimal purification from ClearColi cells, proteins or plasmids (which may contain Lipid IV_A) can be used in most applications without eliciting an endotoxic response in human cells (see Fig. 2).

In ClearColi cells, two of the secondary acyl chains of the normally hexa-acylated LPS have been deleted, eliminating a key determinant of endotoxicity in eukaryotic cells. The six acyl chains of the LPS are the trigger which is recognized by the Toll-like receptor 4 (TLR4) in complex with myeloid differentiation factor 2 (MD-2), causing activation of NF-kB 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 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 resulting lipid IV_A from any downstream product.

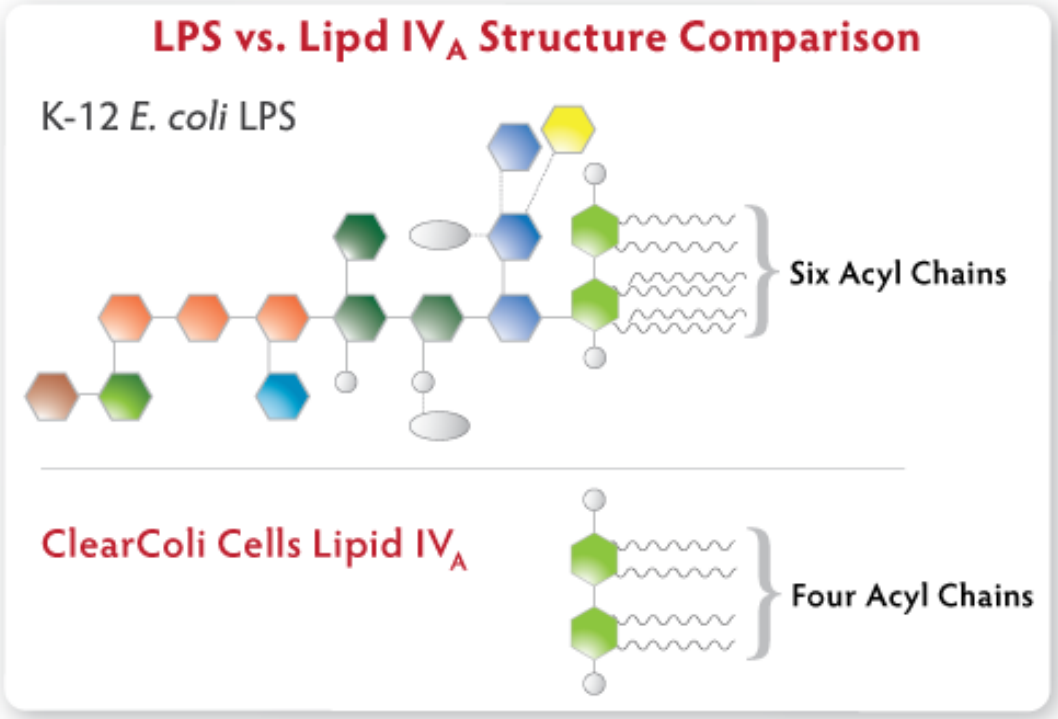


Figure 1. The LPS of normal *E. coli* cells compared to the genetically modified Lipid IV_A from ClearColi cells. In ClearColi, the oligosaccharide chain is deleted, and two of the six acyl chains are removed to disable the endotoxin signal.

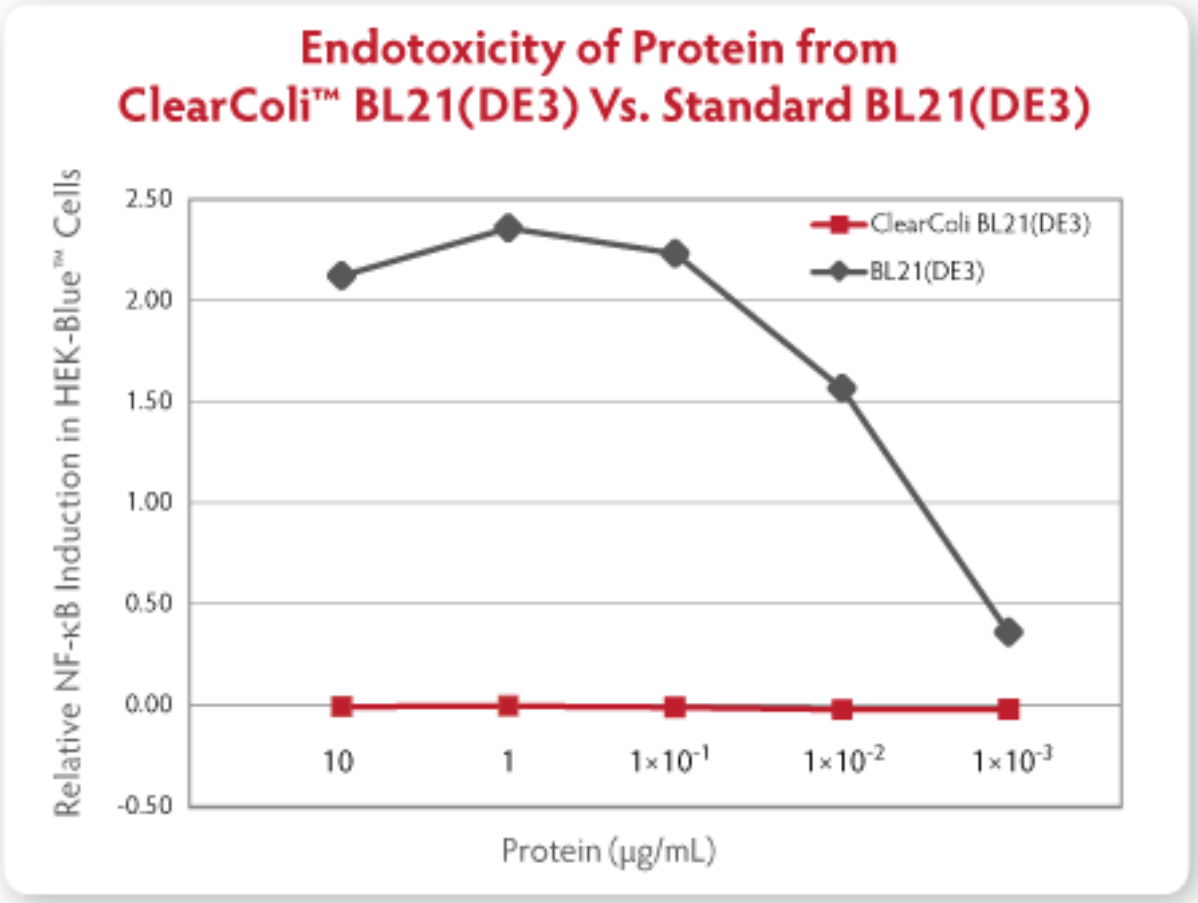


Figure. 2. Comparison of endotoxic response from protein derived from ClearColi BL21(DE3) and traditional BL21(DE3) competent cells. ApoA1 protein was expressed from a T7-promoter based plasmid in both cell types, followed by a simple Ni-column purification step and without any subsequent endotoxin removal steps. The purified proteins were then tested for Toll-Like Receptor (TLR) stimulation by assessing NF-κB activation in HEK293 cells expressing human TLR4. Protein derived from ClearColi BL21(DE3) cells demonstrated no activation at concentrations 5 orders of magnitude greater than the protein from traditional BL21(DE3) cells.

ClearColi competent cells have a genetically modified lipopolysaccharide (LPS) that disables the trigger for endotoxic response in mammalian cells. This was accomplished by incorporating seven genetic deletions that modify LPS to Lipid IV_A, while one additional compensating mutation (*msbA148*) enables the cells to maintain viability in the presence of the LPS precursor lipid IV_A. ClearColi BL21(DE3) cells are BL21(DE3) derived cells with several key mutations which result in significantly reduced endotoxicity. The specific set of mutations made to ClearColi cells is as follows:

msbA148 ΔgutQ ΔkdsD ΔlpxL ΔlpxM ΔpagP ΔlpxP ΔeptA

Growth/Colony Characteristics of ClearColi BL21(DE3) Cells:

ClearColi BL21(DE3) cells grow at approximately 50% of the rate of normal BL21(DE3) cells. Users should expect to see very small colonies for the first 24 hours after plating transformants. Lucigen recommends incubating plates for 32-40 hours before picking colonies

for future experiments (see Transformation Protocol and Sample Induction Protocol sections of this manual for more information). Longer growth times are necessary to reach desired cell densities prior to inducing protein expression.

In addition, users may observe some variation in colony size when plating ClearColi cells. A small portion (<2%) of colonies may be larger than the general population. These larger colonies have similar protein expression levels and endotoxin levels as the average size colonies.

IMPORTANT Information About Medium Formulation

ClearColi cells have a specially engineered membrane composition and require a modified medium formulation compared to most *E. coli* strains. Consider the following:

- ClearColi cells are osmosensitive and require **1% NaCl** in their growth medium. We strongly recommend you use high salt **LB-Miller Medium** to achieve optimal growth. **LB-Miller differs from LB-Lennox**. See p.11 for the LB-Miller recipe.
- **We do not recommend growing ClearColi cells in LB-Lennox or Super broth medium. These media have resulted in slow and suboptimal cell growth.**
- **Do not** include Mg^{2+} and Ca^{2+} in your medium. They have been shown to inhibit growth of ClearColi cells.
- ClearColi cells tend to aggregate when grown in liquid culture. We suggest that you vortex the cell solution before measuring OD_{600}.

Endotoxin and ClearColi Cells

LAL Assay Testing Results

Limulus amebocyte lysate (LAL) assay testing is an FDA-approved method for detection of endotoxins and is the most common assay used. However, the LAL assay is an inappropriate method to discriminate between endotoxically active hexa-acylated LPS and endotoxically inactive tetra-acylated lipid IV_A. The structural requirements for activation of the LAL cascade by endotoxins differ from those for stimulation of the human immune cell system. While the acylation pattern of LPS/lipid A is a key determinant for stimulation of human immune cells, activation of the LAL cascade only marginally, if at all, depends on the number of acyl chains. Instead, reactivity in the LAL assay requires the 4´-monophosphoryl-diglucosamine backbone structure, which is present in both hexa-acylated LPS and tetra-acylated lipid IV_A of *E. coli*. As such, false positive results are due to the lack of specificity of the assay. The LAL assay recognizes a wider spectrum of LPS/lipid A variants than the central cellular endotoxin sensor system of the human immune cell system.

A simple Ni-column purification step for proteins produced from ClearColi cells significantly reduces LAL response levels. For example, Lucigen demonstrated a 99% reduction in LAL response comparing ApoA1 expressed using ClearColi cells versus *E. cloni* EXPRESS BL21(DE3) Electrocompetent Cells.

Cell Line	LAL Results (EU/mg)	Percent Reduction
ClearColi® Electrocompetent Cells	450	
E. cloni EXPRESS BL21(DE3) Electrocompetent Cells	53800	99.1%

Residual EU measurements are due to the non-specific nature of the assay unless extraneous LPS contamination from other sources is present. Alternative endotoxicity assays, such as those using HEK-Blue cells (Invivogen) suggest that even in the presence of EU levels above normal thresholds targeted by researchers, the actual stimulating effects from ClearColi-derived proteins are non-existent.

Due to the non-specific nature of the LAL assay giving false positive endotoxic results with lipid IV_A from ClearColi, it is suggested that researchers consider alternative physiologically relevant methods. These methods include measurement of TLR stimulation as assessed by NF-ƙB activation in HEK293 cells or Human Macrophage assays.

Lack of Endotoxin in ClearColi Cells for Mammalian Cell Applications

Lipid IV_A from ClearColi cells is incapable of inducing an endotoxic response in human immune cells. Seven separate deletions ensure that ClearColi cells cannot revert to normal LPS production. With proper controls, plasmids and proteins can be produced from ClearColi cells without need for downstream endotoxin removal steps. However, LPS contamination may be prevalent in your laboratory, and care must be taken to minimize LPS sources other than your cell strain.

While lipid IV_A is known as an endotoxin antagonist in human LPS-responsive cells, it has to be taken into account that the tetra-acylated LPS precursor may act as an endotoxic activator in other mammalian hosts such as mouse, Chinese hamster or equine cells, which reflects animal species-specific recognition and stimulatory activity of lipid IV_A due to species-specific differences in the structures of TLR4 and MD-2.

Avoiding Endotoxin Contamination from Other Sources

Although ClearColi cells will not produce endotoxin, it is still possible to contaminate your end product with endotoxins from other sources. Good laboratory sterile technique can adequately control extraneous LPS contamination. Lucigen recommends the following precautions.

- Use disposable pipette tips and centrifuge tubes certified as sterile and non-pyrogenic
- Depyrogenate any glassware by heat treating at >250° for 1 hour prior to use
- Do not use purification columns or resins that have come in contact with *E. coli*
- Use reagents certified as low endotoxin or test reagents prior to use
- Use a water source that is regularly tested for endotoxin contamination
- Clean all laboratory surfaces with disinfectants

Measuring Endotoxin Prior to Downstream Applications

In applications where minimal endotoxin levels are critical, it is strongly recommended that all normal precautions are taken. Lucigen cannot guarantee a total absence of LPS due to the possibility of contamination from other sources. Safety and downstream applications are the sole responsibility of the user.

Endotoxin Removal Prior to Downstream Applications

The need for endotoxin removal steps will depend on the user's method of endotoxin measurement and application. As previously discussed, the lipid IV_A of ClearColi cells does not cause an endotoxic response in human cells; however, the use of LAL testing may result in a relatively low EU measurement. Normally, a simple plasmid purification or Ni-column protein purification will be sufficient to lower LAL levels below threshold. If lower levels are desired, additional cleanup steps should be taken.

Genotype Information

ClearColi BL21(DE3)
F⁻ *ompT hsdS$_B$ (r_B^- m_B^-) gal dcm lon* λ(DE3 [*lacI lac*UV5-T7 gene 1 *ind1 sam7 nin5*]) *msbA148* Δ*gutQ* Δ*kdsD* Δ*lpxL* Δ*lpxM* Δ*pagP* Δ*lpxP* Δ*eptA*

Preparation for Transformation

ClearColi Electrocompetent Cells are provided in 50 µl aliquots (DUOs), sufficient for two transformation reactions of 25 µl each. Transformation is carried out in a 0.1 cm gap cuvette. Optimal settings for electroporation are listed in the table below. Typical time constants are 3.5 to 4.5 msec.

Optimal Setting	Alternate Settings (~ 20-50% lower efficiencies)	Alternate Settings (~ 20-50% lower efficiencies)
1.0 mm cuvette 10 µF 600 Ohms 1800 Volts	1.0 mm cuvette 25 µF 200 Ohms 1400 – 1600 Volts	2.0 mm cuvette 25 µF 750 Ohms 2400 Volts

Suggested Electroporation Systems
Bio-Rad Micro Pulser #165-2100; Bio-Rad *E. coli* Pulser #165-2102; Bio-Rad Gene Pulser II #165-2105; BTX ECM630 Electroporation System

Suggested Electroporation Cuvettes
Successful results are obtained with cuvettes from BTX (Model 610), BioRad (Cat. #165-2089), or Eppendorf (Cat. # 4307-000-569). Users have reported difficulties using Invitrogen cuvettes (Cat.# 65-0030).

Optional transformation control reactions include electroporation with 1 µl (10 pg) of supercoiled pUC19 DNA.

To ensure successful transformation results, the following precautions must be taken:

- For best results, ligation reactions must be purified or heat killed at 70ºC for 15 minutes before transformation.
- The DNA sample to be used for electroporation must be dissolved in water or a buffer with low ionic strength, such as TE. The presence of salt in the electroporation sample leads to arcing at high voltage, resulting in the loss of the cells and DNA. *NOTE: Ligation reactions performed with Lucigen's CloneDirect® Ligation Buffer (included with Lucigen's Cloning or Ligation Kits) can be used immediately after heat inactivation, without purification of the ligation products.*

- Microcentrifuge tubes and electroporation cuvettes must be thoroughly pre-chilled on ice before use.
- The cells must be completely thawed **on ice** before use.
- For highest transformation efficiency, use the provided Expression Recovery Medium to resuspend the cells after electroporation. Use of TB or other media will result in lower transformation efficiencies.

Transformation Protocol

Before proceeding with transformation, read **IMPORTANT Information About Medium Formulation** on page 6.

1. Have Expression Recovery Medium and 17 mm x 100 mm sterile culture tubes readily available at room temperature (one tube for each transformation reaction). Transformation efficiency may decrease with the use of SOC or other media.
2. Place electroporation cuvettes (0.1 cm gap) and microcentrifuge tubes on ice (one cuvette and one tube for each transformation reaction).
3. Remove ClearColi cells from the -80°C freezer and place on wet ice until they thaw **completely** (10-20 minutes).
4. When cells are thawed, mix them by tapping gently. Add 25 µl of ClearColi cells to the chilled microcentrifuge tube on ice.
5. Add 1 µl of DNA or heat-denatured ligation reaction to the 25 µl of cells on ice. (Failure to heat-inactivate the ligation reaction will prevent transformation.) Stir briefly with pipet tip; **do not** pipet up and down to mix, because this can introduce air bubbles and warm the cells. Use of more than 2 µl of ligation mix may cause electrical arcing during electroporation.
6. Carefully pipet 25 µl of the cell/DNA mixture into a chilled electroporation cuvette without introducing bubbles. Quickly flick the cuvette downward with your wrist to deposit the cells across the bottom of the well. Electroporate according to the conditions recommended above.
7. Within 10 seconds of the pulse, add 975 µl of Expression Recovery Medium to the cuvette and pipet up and down three times to resuspend the cells. Transfer the cells and Recovery Medium to a culture tube.
8. Place the tube in a shaking incubator at 200 - 250 rpm for 1 hour at 37°C.
9. Spread up to 100 µl of transformed cells on LB-Miller plates containing the appropriate antibiotic.
10. Incubate the plates **32-40 hours** at 37°C. Very small colonies may be visible at 24 hours.

    Note: **Do not store ClearColi strains on agar plates at 4°C for more than one week.** While cells remain viable, there is an increased lag time before growth. Transformed ClearColi strains can be stored at -80°C in LB Miller medium with the addition of 20% glycerol.
11. Transformed clones can be further grown in **LB-Miller** and the appropriate antibiotic (see p. 11 for recipe).

Protein Induction Protocol

1. Inoculate a single colony from a freshly streaked plate into 40 ml of **LB-Miller** medium containing the appropriate antibiotic for the plasmid and host strain. To minimize the amount of expression of the target protein prior to induction, add glucose to the growth medium at a concentration of 0.5% (w/v).
2. Incubate with shaking at 37°C overnight.
3. Inoculate 1L of LB-Miller medium (no glucose) containing the appropriate antibiotic with all 40 ml of the overnight culture prepared in step 2. Alternatively, measure the OD_{600} of your overnight culture and inoculate to a final OD_{600} of 0.1.
4. Incubate with shaking at 37°C until the OD_{600} reaches 0.6 - 0.8 (approximately 4-5 hours).
5. Add IPTG to a final concentration of 0.4 – 1 mM. (Prepare a 1 M solution of IPTG by dissolving 2.38 g of IPTG in water and adjust the final volume to 10 ml. Filter sterilize before use). To determine the optimal concentration of IPTG for maximum expression of the target protein, test a range of IPTG concentrations.
6. Incubate, shaking at 37°C for 3-4 hours. To determine the optimal time for induction of the target protein, it is recommended that a time course experiment be performed varying the induction from 2-16 hours. Note: Final OD_{600} of the ClearColi cells may be as low as 50% of that normally achieved with standard BL21(DE3) cells due to slower growth rates.
7. Place the culture on ice for 10 minutes. Harvest cells by centrifugation at 5,000 x g for 10 minutes at 4°C.
8. Remove the supernatant . Depending on your workflow and specific protein, you can proceed to:
   a. Store the pellet at -20°C.
   b. Store the pellet at -80°C.
   c. Immediately continue with processing (isolation and purification of protein).

Media Recipes

LB-Miller Culture Medium for Growth of Transformants

Per liter: 5 g yeast extract
10 g tryptone
10 g NaCl
Add all components to deionized water. Autoclave and cool to 55°C.

LB-Miller Plates

Per liter: 5 g yeast extract
10 g tryptone
10 g NaCl
15 g agar

Add deionized water to 1 liter. Autoclave and cool to 55°C before adding the appropriate filter-sterilized antibiotic (e.g. ≤ 30 mg/L kanamycin; ≤ 100 mg/L ampicillin or carbenicillin; ≤ 30 mg/L chloramphenicol).

Pour approximately 25 mL per Petri plate.

Related Lucigen Products

- OverExpress® Competent Cells
- *E. cloni*® EXPRESS BL21(DE3) Competent Cells
- *E. cloni*® 10G Competent Cells

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ClearColi Competent cells are subject to US Patent 8,303,964 and other US and foreign pending patents.

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