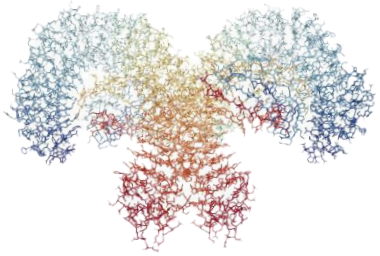
Caithness BIOTECHNOLOGIES

High quality reagents for innate immunity research, vaccine development and drug discovery

Recombinant proteins with a focus on low PAMP content

Background



Recombinant proteins are essential tools for the investigation of many research questions in the life sciences. However, a recurring problem with their use, particularly in the fields of immunity and inflammation, is the contamination of reagents by bacterial pathogen-associated molecular patterns (PAMPs), such as bacterial lipopeptide (BLP) and lipopolysaccharide (LPS), which are stimulants of Toll-like receptor (TLR) 2 and TLR4, respectively. The presence of such contaminants is a frequent contributor to inappropriate cellular activation and false positive signals, which can result in the misinterpration of results [1]. These problems are particularly common with recombinant proteins that are produced in the Gram-negative bacterium *Escherichia coli*.

Our primary focus is to address these limitations by producing recombinant proteins with the lowest possible levels of PAMP contamination. This is achieved through expression in mammalian cells, rigorous testing of reagents for PAMP content using the most appropriate methods and stringent attention to protocols that minimise the potential for re-entry of PAMPs into the product during purification and packaging.

Low PAMP content for reliable investigation

Many reports have highlighted the benefit of utilising proteins with low levels of PAMP contamination when inflammatory signalling may confound the results. For example, numerous mammalian proteins, including heat-shock proteins (HSP) 60 and HSP-70, high mobility group box-1 (HMGB1) and beta-amyloid peptide, were reported to promote inflammation by directly stimulating TLR2 and/or TLR4 [1]. However, when low PAMP expression systems were used for their production, subsequent studies found that these proteins had no capacity to stimulate TLR-signalling [2-5].

We take three main approaches to minimise the PAMP content of our recombinant proteins: (i) expression in mammalian cells, (ii) stringent use of methods that prevent the re-introduction of PAMPs to the product during purification or packaging and (iii) continuous testing of reagents and products for PAMP content using the most sensitive and appropriate assays. This last point is achieved using the HEK-293 cell TLR transfection system to detect and quantify TLR2 and TLR4 stimulating contaminants. As shown in Figure 1, these efforts can result in proteins with much lower levels of TLR2 or TLR4 stimulants than some equivalent proteins from competitors.

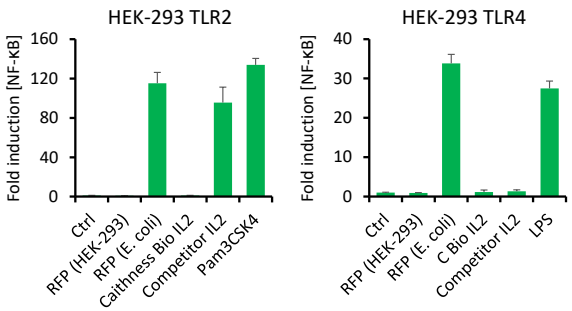


Figure 1: TLR2- and/or TLR4-stimulants can be abundant in recombinant proteins expressed in E. coli. Example proteins made in E. coli or HEK-293 cells were measured at 1 µg/ml using HEK-293-TLR2 or TLR4 bioassays (RFP - red fluorescent protein, IL2 - interleukin-2)

Key advantages of our recombinant proteins

All of our proteins are expressed in mammalian cells for:

- ✓ Extremely low levels of non-specific PAMP contaminants, validated by HEK-293 TLR2 / TLR4 bioassays
- ✓ Human glycosylation patterns for authentic structure and antigenicity
- ✓ Superior folding and disulphide bond formation in comparison to proteins expressed in *E. coli*

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Recombinant proteins with a focus on low PAMP content

Advantages of the TLR testing method

The most common method for testing whether reagents may be contaminated with bacterial products is the limulus amoebocyte lysate (LAL) assay. However, although the LAL assay is easy to use and the method most commonly chosen for detecting LPS contamination, it has certain limitations. First, it is confounded by the presence of beta glucans, a fungal product that is often present in some samples (such as serum). Second, the limulus assay utilises PRRs from the horseshoe crab, which renders it incapable if distinguishing whether PAMPs may have agonist activity in the context of human PRRs. For example, some molecules which are antagonist of human TLR4 are agonist of the limulus enzyme system. Finally, the limulus assay cannot detect BLP, which is also a near ubiquitous contaminant of proteins expressed in *E. coli*, and which can also trigger inappropriate activation of the many types of immune cell that express TLR2.

To counter these issues, all of our proteins are tested using a HEK-293 cell transfection system combining human TLR2 or TLR4/MD2 receptors with a sensitive NF-κB reporter. By combining this approach with reference curves using defined BLP and LPS standards, we are able to quantify the two molecules most commonly problematic in recombinant protein production - namely BLP and LPS (Figure 2). For our flagellin and flagellin fusion products, we also test their capacity to stimulate TLR5 in similar systems.

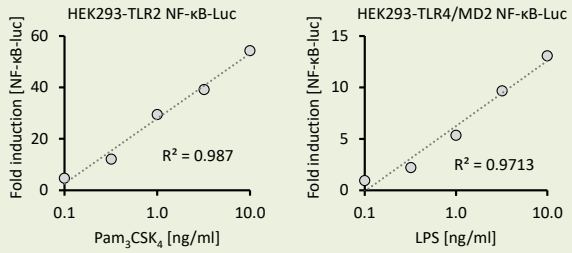
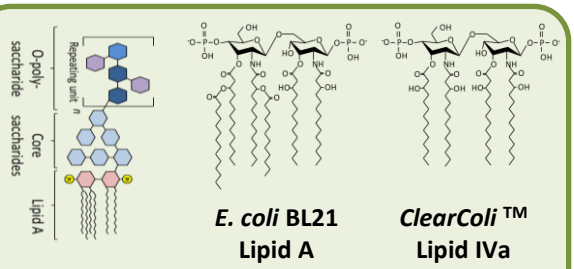


Figure 2: Typical HEK-293-TLR assay calibration curves

HEK-293 cells are transiently transfected with plasmids coding for NF-κB reporter, human CD14 and human TLR2 or human TLR4/MD2. Test proteins are then added and the induction of NF-κB signalling compared to a respective calibration curve using defined ligands of TLR2 (Pam_3CSK_4) or TLR4 (*E. coli* LPS).

Other advantages of mammalian expression

Many companies now offer recombinant proteins expressed in an *E. coli* strain (*ClearColi*™) which has been genetically modified to alter the lipid A domain of its LPS from the wild-type hexa-acyl form to a tetra-acyl form, referred to as Lipid IVa. As Lipid IVa is not an agonist of human TLR4/MD2, it is thought that proteins made using this system have effectively eliminated concerns over LPS contamination. However, although lipid IVa is not an activator of human TLR4/MD2, it is an agonist of mouse TLR4/MD2 [6], and also of human caspase-4 - an alternative inflammatory sensor of LPS [7] (Fig 3). Moreover, *ClearColi*™ cells do not lack lipopeptide, so retain the possibility of TLR2 stimulants co-purifying with expressed proteins.



	E. coli BL21 Lipid A	*ClearColi* ™ Lipid IVa
Acyl chains	6	4
Human TLR4/MD2	Agonist	Antagonist
Mouse TLR4/MD2	Agonist	Agonist
Human Caspase-4	Agonist	Agonist

Figure 3: Inflammatory properties of lipid IVa

Finally, many mammalian proteins require appropriate disulphide bond formation and glycosylation to achieve optimal structure, stability and biological activity. As *E. coli* is typically unable to perform these necessary post-translational modifications, and yeast or insect cell glycosylation often differs greatly, mammalian cell expression may be preferable when such properties are critical. For example, authentic glycosylation may be necessary for appropriate antigenicity when using proteins in immunoassay or vaccine development.

References: [1] Erridge C. J Leukoc Biol 87:989-99 (2010) [2] Gao B, Tsan MF. J Biol Chem 278:22523-22529 (2003) [3] Bausinger H, *et al*. Eur J Immunol 32:3708-3713 (2002) [4] Hreggvidsdottir HS, *et al*. J Leukoc Biol 86:655-662 (2009) [5] Youn JH, *et al*. J Immunol 180:5067-5074 (2008) [6] Saitoh S, *et al*. Int Immunol 16:961-9 (2004) [7] Lagrange B, *et al*. Nat Commun 9:242 (2018)

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