Endotoxins and their influence on transfection efficiency during CRISPR workflows

Introduction

Gene editing experiments using CRISPR/Cas9 are complex, multistep procedures. Gene editing efficiency and accuracy is not 100% and depends on various factors, e.g. experimental reliability.

Transfection of eukaryotic cells is a very important step in many CRISPR workflows, as the CRISPR components required to induce the desired genetic manipulation need to be present in the target cells/organism. Mammalian expression vectors are frequently used to clone gRNA and Cas9 into a plasmid, which is introduced into cells for transient or stable expression. Reliable and efficient transfection is critical for the success of the overall gene editing experiment.

Isolated plasmid DNA can contain endotoxins from the bacterial cell, which are co-purified. These endotoxins can negatively influence the transfection of eukaryotic cells, decreasing the transfection efficiency.

What are endotoxins?

Endotoxins are lipopolysaccharides, also known as LPS, and are located in the outer membrane of gram-negative bacteria. In humans they stimulate an immunogenic response to a bacterial infection.

Endotoxins are made of a highly conserved lipid part and a polysaccharide (Figure 2). The phospholipid on the right side of the figure is the part responsible for the toxic effect. The polysaccharide on the left side has structural functions. ▷

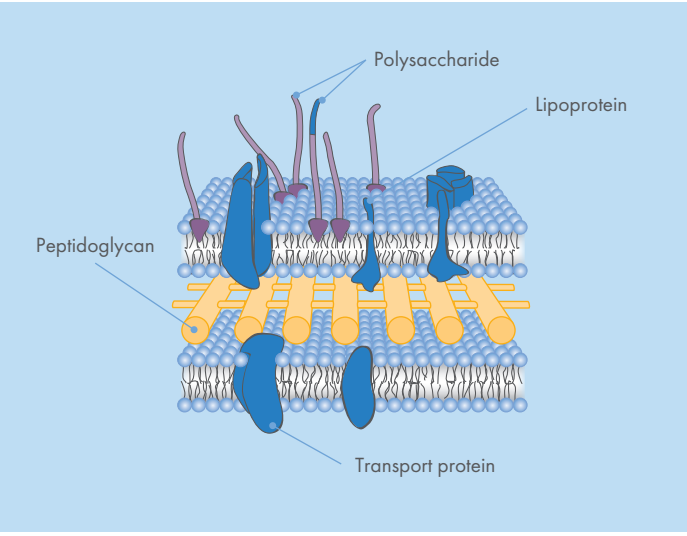


Figure 1. Lipopolysaccharides/endotoxins are located in the outer membrane of Gram-negative bacteria.

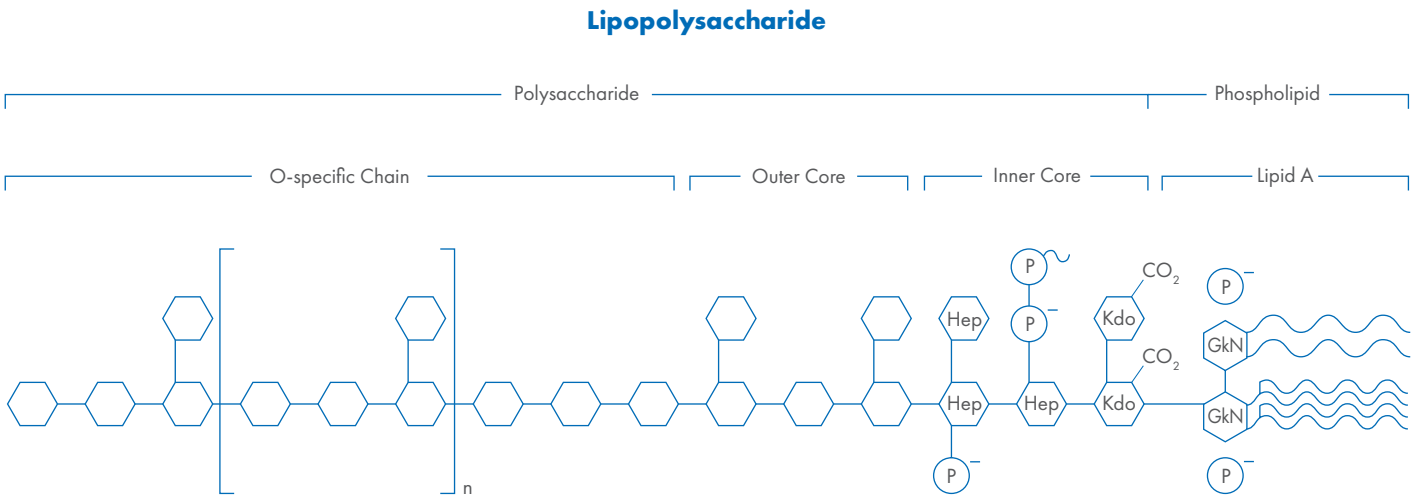


Figure 2. Lipopolysaccharide diagram.

Why are endotoxins so important and how do they influence eukaryotic cells?

The effects are very complex and can vary significantly between different cells. One very obvious effect is membrane damage. As LPS is a membrane component, it can also interact with the structure of other membranes. This can influence the function of the membrane and can lead to morphological changes.

Further interactions are due to the structural nature of the molecule. The phosphate groups of the inner core can interact with cationic proteins of the transfected cell. And the fatty acid chain can interact with lipid membranes and hydrophilic regions of proteins.

In addition, endotoxins can cause mitogenic effects, by activating cell division of B-cells for example, as well as inhibitory effects on cell division depending on the origin of the endotoxin and on the concentration.

How sensitive are different cell lines to LPS and how do they react to endotoxin exposure?

Permanent cell lines derived from carcinogenic sources are less sensitive than primary cells or cells in suspension. The difference between these two types is, that permanent cells have undergone through a high number of passages and are theoretically immortal.

Table 1. Cell line features, characteristics and origin

	Features	Origin	Abbreviated name	Complete name	Endotoxin sensitivity
Primary Cells	Viable for a few cell passages	Directly isolated from tissue	HUVEC	Human umbilical vein endothelial cells	Very sensitive
Permanent Cells	Adherent cells, growing in monolayers; can be passaged multiple times	Theoretically immortal, either by nature or by design; some are of carcinogenic origin	Hela S3	Human cervix carcinoma pithelial cells	Moderate
			CHO-K1	Chinese hamster ovary fibroblasts	Sensitive
			Huh7	Human hepatoma cells	Very sensitive

Primary cells are derived from fresh tissue and have gone only a few passages. They are more differentiated than permanent cell lines and not as well characterized. Primary cells are very sensitive to endotoxins. The sensitivity of permanent cells can vary and depends on the cell type. For example, the human cervix carcinoma epithelial cell line Hela S3 is a more robust cell line, whereas the Chinese hamster ovary fibroblasts ChO-K1 is sensitive and the human hepatoma cell line Huh7 is very sensitive.

How can plasmid DNA be contaminated with LPS?

The LPS is released when bacteria die or are destroyed. This is the case during plasmid purification and during this process, endotoxins can be co-purified. During plasmid purification using a silica matrix, endotoxins can be co-purified, because the fatty acyl chains of the LPS are hydrophobic and are able to bind to the silica membrane competing with the DNA molecules.

Just like DNA, LPS have negative phosphate groups that bind anion-exchange matrices during plasmid purification using this technology.

How can LPS be removed from purified DNA?

Thus, endotoxins must be removed from the plasmid DNA to guarantee optimal transfection results to enable expression of the gene editing machinery in the transfected cells. There are two ways to do this. One method involves removal of endotoxins during purification and the other involves removal after purification.

QIAGEN offers plasmid kits that enable effective and convenient endotoxin reduction/removal directly during the plasmid DNA purification procedure. Anion-exchange purification combined with an endotoxin removal buffer wash directly after cell lysis, as provided with QIAGEN's EndoFree® Plasmid Kits, lead to endotoxin levels of less than 0.04 EU/µg DNA, which is suitable for very sensitive cells, such as HUVEC cells, and complying with FDA specifications for endotoxins in vaccines (<0.04 EU/µL). Endotoxin levels between 1–3 EU/µg DNA, sufficient for transfection of sensitive cells such as Huh7 cells, can be achieved using the QIAGEN Plasmid *Plus* Kits, that include an endotoxin wash step after the plasmid DNA was bound to the column.

Other commercial suppliers offer kits with an additional endotoxin removal step after the actual plasmid purification process. The ZymoPURE™ II Plasmid Maxiprep Kit procedure claims endotoxin reduction after plasmid purification, by adding the lysate to a dedicated spin column, the EndoZero™ Spin column and centrifugation. Our data show that this procedure does not result in an efficient endotoxin removal. ▷

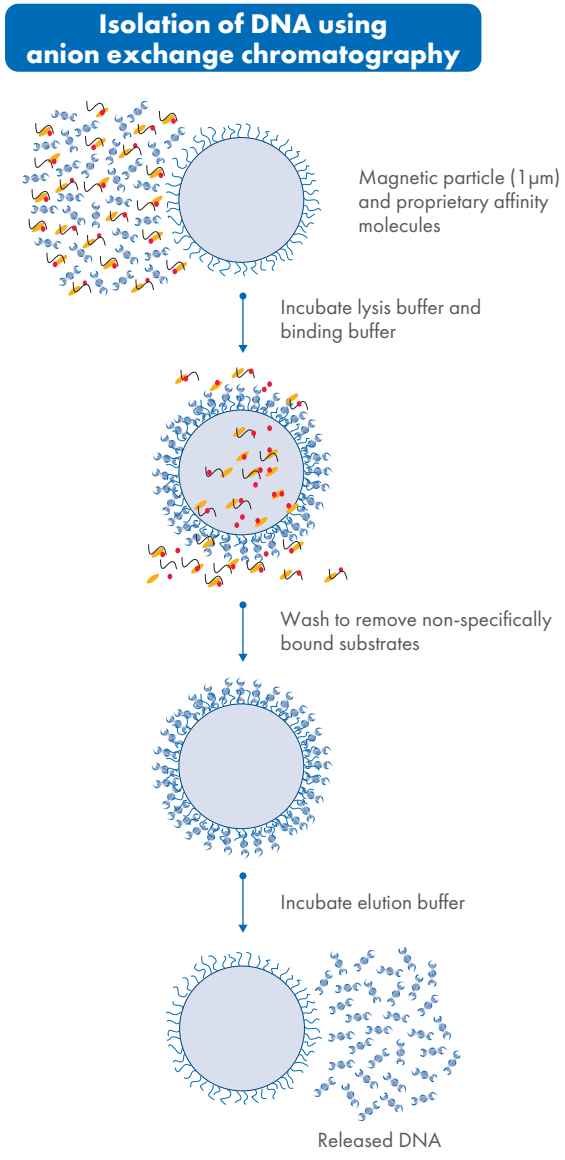
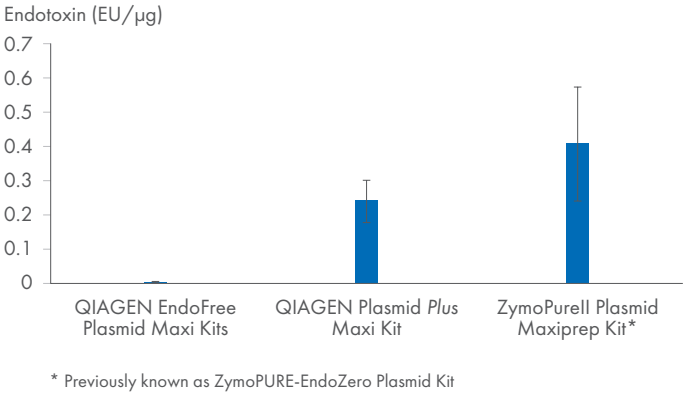


Figure 3. Isolation of DNA using onion exchange chromatography.



* Previously known as ZymoPURE-EndoZero Plasmid Kit

Figure 4. Comparison of Endotoxin levels. Endotoxin levels in plasmid DNA samples prepared with the EndoFree Plasmid Kit and QIAGEN Plasmid *Plus* Kit from QIAGEN and the ZymoPureII Endo-Zero Plasmid Kit from Zymo was measured using the Limulus Amebocyte Lysate (LAL) test according to the manufactures Instruction (Lonza). For each kit a 1:100 dilution of two samples were tested in triplicate. Standard curve of the test ranged between 0.005 EU/mL and 50 EU/mL. Plasmid DNA recovered with the Plasmid *Plus* Kit showed a significant reduction in endotoxins, while endotoxins were nearly undetectable in the resulting plasmid DNA using the EndoFree Plasmid Kit. In contrast, the ZymoPureII resulted in plasmid DNA with higher amounts of endotoxins. Yield and quality of plasmid DNA as assessed by agarose gel electrophoresis were comparable for both kits (data not shown).

What quality of plasmid DNA is required for the different CRISPR workflow steps?

Molecular biology-grade DNA can be obtained by purification using selective absorption to silica-gel membranes under controlled ionic conditions, such as the QIAprep® Spin Miniprep Kit. The purified plasmid DNA is sufficient for cloning and transformation of bacterial cells, but not for optimal for transfection. The QIAprep Spin Miniprep Kit can therefore be used for cloning of the CRISPR components, during validation of gene editing and analysis of the edited cells.

QIAGEN Plasmid Plus chemistry provides plasmid DNA suitable for the transfection of robust and sensitive cell lines and is therefore the right choice for transfection of the CRISPR components into cell lines, such as HeLa S3 or CHO-K1.

QIAGEN's EndoFree Plasmid Kits using anion-exchange chromatography in combination with an endotoxin removal buffer result in DNA of the highest quality with lowest endotoxin levels, suitable for transfection into very sensitive cell lines. They enable transfection of CRISPR components into such as HUVEC cells, as well as primary and suspension cells.

Table 2. A comparison of products for use in removing endotoxins from cell lines being transfected

	Endotoxin free	Transfection grade	Molecular biology grade
Method	Anion exchange plus endotoxin removal buffer	Plasmid *Plus* Technology	Silica
Endotoxin levels	<0.04 EU/µg DNA	<1 EU/µg DNA	<100 EU/µg DNA
Products	EndoFree Plasmid Kits	QIAGEN Plasmid Plus Kits	QIAprep Spin MiniPrep Kit
CRISPR workflow step(s) to be used for	• Transfection of very sensitive cell lines	• Transfection of robust and sensitive cell lines	• Cloning of CRISPR components

Influence of endotoxins on transfection efficiency

To measure the efficiency of transfection, pCMVβ including the β-galactosidase gene was transfected into highly passaged Huh7 cells. β-galactosidase activity was measured using the β-gal assay.

As shown in the figure, a correlation between different parameters can be observed, quality of DNA, amount of DNA, and amount of transfection reagent. The QIAGEN EndoFree Plasmid Kits give the best result in all experiments.

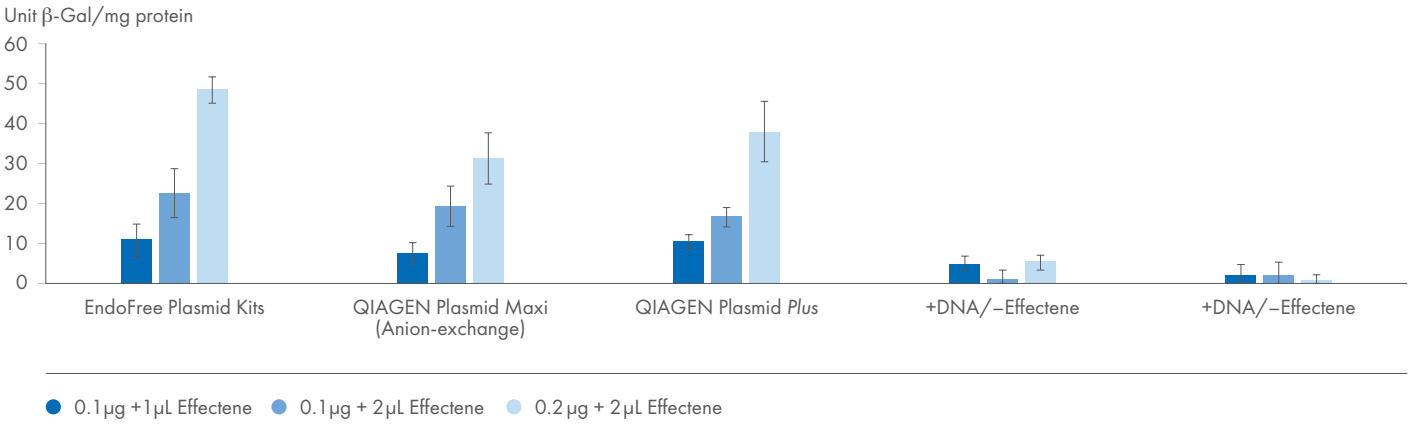


Figure 5. Transfection of Huh7 cells with plasmid DNA with different endotoxin levels. Influence of plasmid DNA quality on transfection efficiency using Effectene® Transfection Reagent. One day before transfection, 2 x 10^4 Huh7 cells were seeded per well in 96-well plates. Cells were transfected using 0.1 and 0.2 µg of a β-galactosidase-reporter plasmid and enhancer (DNA enhancer ratio of 1:8) and 1 µL and 2 µL Effectene Transfection Reagent in the presence or absence of serum. Each bar represents the average efficiency from four replicates 48 hours post-transfection.

Conclusion

Transfection of eukaryotic cells is a very important step in many CRISPR workflows and reliable and efficient transfection is critical for the success of the overall gene editing experiment. Endotoxins deriving from the bacterial cell are co-purified during plasmid preparation and can negatively influence the transfection of eukaryotic cells, decreasing the transfection efficiency and therewith, gene editing efficiency.

QIAGEN offers dedicated kits for sample purification that comply with the different needs along the CRISPR workflow steps. The QIAprep Spin Miniprep Kit is ideal for cloning of the CRISPR components, validation of gene editing and analysis of the edited cells.

QIAGEN Plasmid Plus Kits provide plasmid DNA suitable for the transfection of robust and sensitive cell lines with reduced endotoxins levels.

QIAGEN's EndoFree Plasmid kits result in DNA of the highest quality with lowest endotoxin levels, suitable for transfection of very sensitive cell lines and enable transfection of CRISPR components into such as HUVEC cells, as well as primary and suspension cells.

CRISPR ethical statement

QIAGEN solutions are used in almost every laboratory researching CRISPR-Cas9 and other gene modification technologies, primarily aiming to prevent and treat many diseases. Tight regulations and ethical rules about the use of genome editing are necessary to prevent misconduct and avoid harm to people and the ecosystem in which we live. QIAGEN endorses the principles and proposals of scientific organizations and advisory groups – such as the American Society of Human Genetics (ASHG) and the European Society of Human Genetics (ESHG) – that have issued cautionary guidelines. We also support the careful development of guidelines by scientific and societal leaders, with involvement and transparency for diverse elements of society with a stake in the issue. Any confirmation of QIAGEN products being used in a non-compliant manner will be passed to the U.S. Food and Drug Administration (FDA), or relevant regional regulatory authority.

For further information on gene editing ethics see QIAGEN's statement at **https://corporate.qiagen.com/about-us/sustainability/gene-editing-ethics/**.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

For more information, please look at the full statement on our website.