Application Note

EndoFree® Plasmid Kit update: more sustainable and quicker DNA preparation for transfection

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Changes to the EndoFree Plasmid Kit make it more eco-friendly, quicker and even better for preparation of plasmid DNA for transfection. Here we show that the updated EndoFree Plasmid Kits deliver pure endotoxin-free DNA (less than 0.04 EU/µg DNA and better than the original kit; EU: endotoxin units), so that not only robust cell lines like HeLa cells but also endotoxin-sensitive cells like CHO cells and even primary blood cells, which are very challenging to transfect, can be transfected even more successfully.

Introduction

Endotoxins negatively influence transfection efficiency in endotoxin-sensitive cell lines like CHO cells or primary cells (1). Increased endotoxin levels can lead to significantly reduced transfection efficiencies of eukaryotic cells.

Endotoxins are lipopolysaccharides (LPS) of the outer cell membrane of gram-negative bacteria such as *E. coli*. These molecules are released during the plasmid DNA purification lysis step from the bacteria's outer membrane into the lysate. A single *E. coli* cell contains about 2 million LPS molecules, which are made of a highly conserved lipid part, a hydrophilic polysaccharide and negatively charged phosphate group. Endotoxins are highly heat stable.

Because of their chemical structure and properties, endotoxin molecules can be co-purified with plasmid DNA. The hydrophobic part of the LPS can bind to the silica matrix competing with the DNA molecules during plasmid isolation using spin columns. During plasmid DNA isolation with gravity flow matrices, the negative phosphate groups of LPS bind to anion-exchange resin. Isolation of low-copy plasmid DNA is even more challenging than the isolation of high-copy plasmid DNA since more bacteria mass is used to obtain the same amount of plasmid DNA. More bacterial mass results in more endotoxins, which can be co-purified with the plasmid.

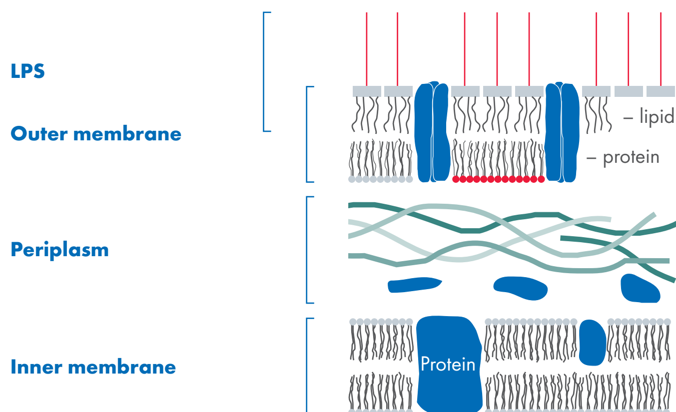

Figure 1. Bacterial cell wall. Structure of the bacterial cell wall. **LPS**: lipopolysaccharides (endotoxins) that can impact transfection especially in endotoxin-sensitive cell lines and primary cells.

Since LPS are membrane components, they can interact with the structure of other membranes and cause membrane damage. This leads to a noncontrollable variable in transfection experiment setup, influencing the outcome and reproducibility of results and making them difficult to compare and interpret.

EndoFree Plasmid Kits provide endotoxin-free plasmid DNA purification. These kits comprise an efficient endotoxin-removal step (using Buffer ER), leading to very low endotoxin levels. Thus, the resulting plasmid DNA is suitable for ▷

transfection of endotoxin-sensitive cells or primary cells. Recently, we updated the composition of Buffer ER. Triton® X-100 detergent was replaced with a more sustainable alternative in line with our commitment to making lab work more sustainable and in compliance with REACH guidelines. During development of the updated kit, we found that we could make the protocol even faster by reducing a 30 minute incubation on ice to a five-minute incubation at room temperature.

Material and Methods

Three different transfection experiments were conducted to verify the transfection efficiency of plasmid DNA isolated with the EndoFree Plasmid Kit:

- Transfection of pMax-GFP (coding for green fluorescent protein) and pBRCMVβ (coding for β-galactosidase) with Effectene Transfection Reagent in robust HeLa cells
- Transfection of pMax-GFP and pBRCMVß with Effectene Transfection Reagent in endotoxin-sensitive CHO cells
- Transfection of pMax-GFP with Nucleofection® technology in sensitive Human Peripheral Blood CD3+ Pan T cells (done by a third-party company)

Plasmid DNA isolation

Plasmid DNA was isolated from 7.5 g *E. coli* pellet with pBRCMVβ (low copy) or 4.5 g *E. coli* pellet with pMax-GFP (high copy) using the original or the updated EndoFree Plasmid Purification Kit according to the protocol "Plasmid or Cosmid DNA Purification using EndoFree Plasmid Mega and Giga Kit" in the handbook for the relevant kit. Plasmid DNA was eluted in 100 µL endotoxin-free TE buffer and stored at –20 °C.

Table 1. Comparison of incubation

Kit	Incubation conditions	Incubation time (min)
Original	On ice	30
Updated EndoFree Plasmid Kit	Room temperature	5
Savings	Energy to freeze the ice	25

QC for plasmid DNA

The endotoxin content in the plasmid eluates was determined with the limulus amebocyte lysate (LAL) based Kinetic-QCL® assay (Lonza). The concentration of endotoxin in a sample was calculated with the help of an internal standard curve. A series dilution of 50–0.005 EU/mL endotoxin was used as standard. All samples were diluted 1/100 and analyzed in triplicates on the SpectraMax plus device.

Transfection with Effectene® Transfection Reagent

For transfection of pMax-GFP in permanent cell lines, the QIAGEN supplementary protocols TFP29 and TFP31 "Fast-forward protocol for transient transfection of HeLa or CHO cells in 96-well using Effectene Transfection Reagent" were followed. 2 x 10^5 cells/mL were plated for transfection experiments with plasmid DNA isolated with the EndoFree Plasmid Kit. As a negative control, the transfection protocol was done in parallel without plasmid DNA. After 24 hours, each sample was documented by fluorescence and brightfield images.

For transfection of pBRCMVβ in permanent cell lines, the "Protocol for Transient or Stable Transfection of Adherent Cells" from the *Effectene Transfection Reagent Handbook* was used. It was adapted to 96-well plate format following the recommendations in the handbook. 2 x 10^4 cells per well were seeded on a 96-well plate (Table 1 in the handbook) and incubated for one day at 37°C and 5% CO_2.

On the next day, the transfection was conducted with 4 replicates of 4 different transfection volumes (16 replicates) to optimize transfection according to Table 2 in the handbook. Four different volumes of Effectene (1, 2, 3 or 4 µL as indicated in Figure 1) were premixed with Buffer EC to a total volume of 20 µL. 0.1 µg plasmid DNA was premixed with 0.8 µL Enhancer and Buffer EC to a total volume of 30 µL (Table 3 in the handbook). The DNA mixture was incubated for 5 minutes at room temperature.

After incubation, 20 µL of each Effectene concentration was mixed with 30 µL of each DNA mixture and incubated for 10 minutes at room temperature to allow transfection complex formation. During this incubation the medium from the cells on the 96-well plate was removed and fresh medium was added. The transfection complex was added to the proper wells (except the wells for the negative control (NC) and different β-galactosidase concentrations, see Figure 2). For transfection, the 96-well plate was incubated for 6 hours at 37°C and 5% CO_2. After transfection, the medium was changed and the plate was incubated for 2 days at 37°C and 5% CO_2.

After 2 days, the medium was removed and the cells were washed with PBS. 50 µL of lysis buffer was added to each well and the 96-well plate was incubated for 1 hour at 37°C and 5% CO_2. After incubation, 1 µL of the different β-galactosidase concentration was added to each well (1–2 and 11–12 B–G; see Figure 1). Substrate (50 µL) was added to each well and incubated at room temperature until a color change (yellow) was visible. Stopping buffer (100 µL) was added to each well, and the color change was measured with the SpectraMax Plus device.

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Figure 2. Pipetting scheme. Transfection of pBRCMVβ in HeLa and CHO cells in a 96-well plate. **Columns 1, 2 11 and 12:** β-galactosidase standards. Volumes of Effectene reagent used for transfection are indicated; amounts of DNA and Enhancer were kept constant; see materials and methods.

Transfection with Nucleofection

Primary blood cells were transfected and analyzed by a third-party company. Cells were transfected in replicates of 6 with plasmid DNA isolated using the EndoFree Plasmid Kit. Transfection was conducted with voltage for half of the replicates and without voltage for the other half. As a negative control, 3 replicates without pDNA (without voltage) were done in parallel and analysis was by flow cytometry and microscopy.

Analysis of transfection efficiency

Transfected cells produce the enzyme β-galactosidase, which cleaves the added substrate into galactose and a yellow dye. The higher the transfection efficiency, the more β-galactosidase is released during lysis and the stronger the yellow coloring.

Results

The results of the Kinetic-QCL® assay (Table 2) indicate that plasmid DNA isolated with the updated EndoFree Plasmid Kit yields endotoxin levels below 0.001 EU/µg, which is 2–40x below the limit of 0.04 EU/µg DNA that is recommended in the area of vaccinations (1). Even for challenging low-copy plasmids (pBRCMVβ), the endotoxin content is clearly below the concentration necessary for effective transfection.

Table 2. Endotoxin content in plasmid DNA isolated using the EndoFree Plasmid Kit

	Endotoxin (EU/µg)	
Kit	**pMax-GFP**	**pBRCMVβ**
Original kit	0.001	0.019
Updated EndoFree Plasmid Kit	0.001	0.001

HeLa cells

Transfection efficiency with pBRCMVβ isolated using the EndoFree Plasmid Kit in HeLa cells was measured with the β-Gal assay. Figure 3 shows that the transfection efficiency with the different plasmid lots/purification rounds was comparable in robust HeLa cells.

Transfection of pMax-GFP in HeLa cells shows that transfection of cell lines works well with plasmid DNA isolated with the EndoFree Plasmid Kit (Figure 4). There were no morphological changes observed in transfected cells.

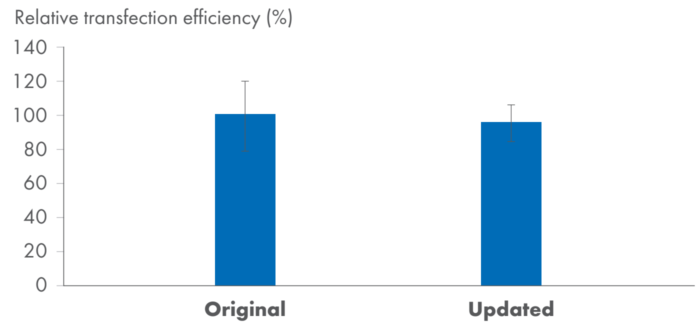

Figure 3. Comparable transfection efficiencies of pBRCMVβ in HeLa cells. For relative transfection efficiency, the mean value of the absorption of the color change for each type of kit (as indicated) was calculated and the original was used as reference (100%).

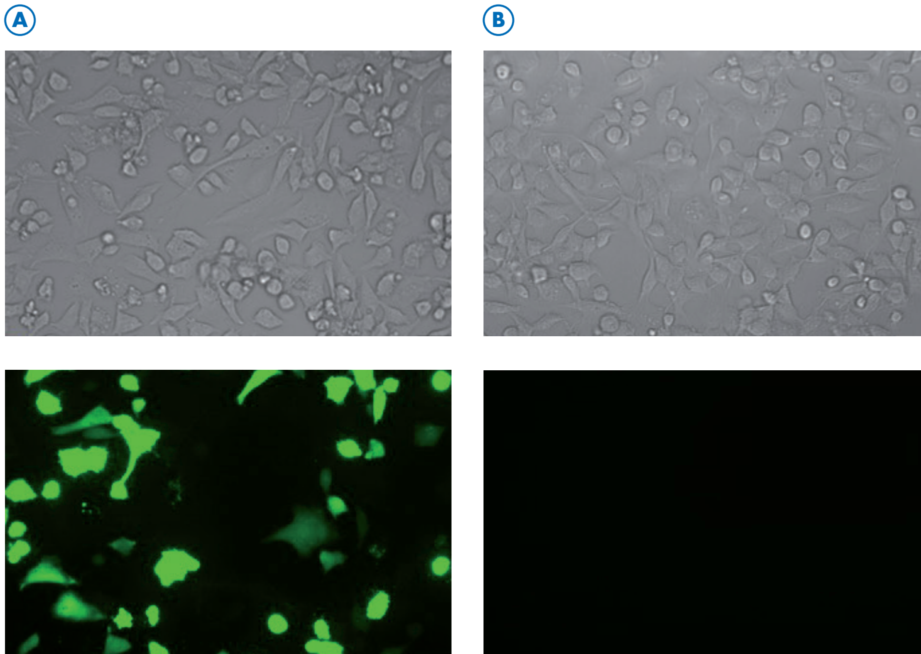

Figure 4. No morphological changes. Transfection of pMax-GFP with the Effectene Transfection Reagent in HeLa cells (20x magnification). Micrographs under bright-field (upper row) and UV light to show GFP (lower row). **A** Transfection of pMax-GFP isolated with the EndoFree Plasmid Kit. **B** Control/transfection without pMax-GFP.

CHO cells

Transfection efficiency with pBRCMVβ isolated using the original and the updated EndoFree Plasmid Kit in CHO cells was measured with the β-Gal assay. Figure 5 shows that the transfection efficiency with the different lots was comparable even for the endotoxin-sensitive cell line CHO. The transfection of pMax-GFP in CHO cells shows that transfection of endotoxin-sensitive cell lines works well with plasmid DNA isolated using the EndoFree Plasmid Kit (Figure 6). There were no morphological changes observed in transfected cells.

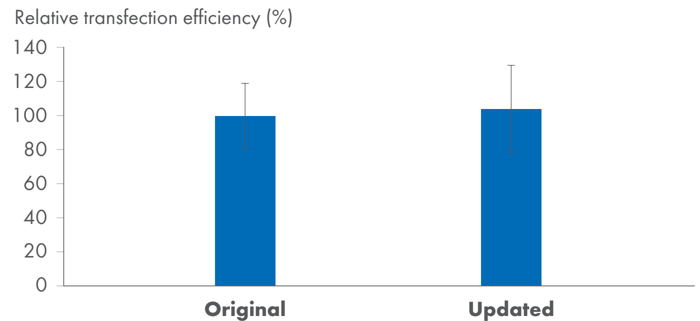

Figure 5. Relative transfection efficiency of pBRCMVβ in CHO cells. For the relative transfection efficiency, the mean value of the absorption of the color change for each type of kit (as indicated) was calculated and the original EndoFree Kit was used as reference and set at 100%.

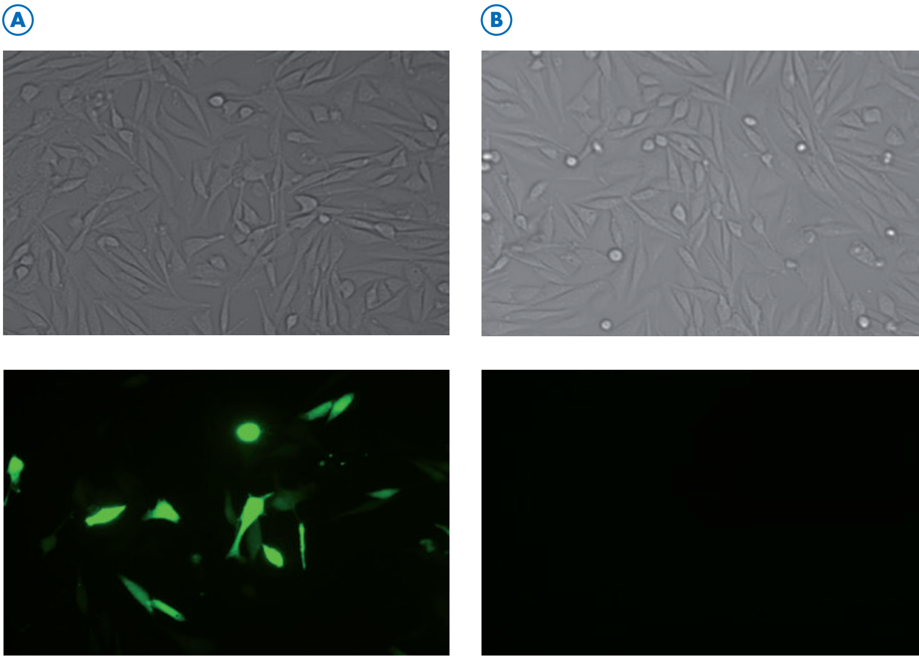

Figure 6. No morphological changes. Transfection of pMax-GFP with the Effectene Transfection Reagent in CHO cells (20x magnification). Micrographs under bright-field (upper row) and UV light to show GFP (lower row). **A** transfection of pMax-GFP isolated with the updated EndoFree Plasmid Kit. **B** Control/transfection without pMax-GFP.

Human Peripheral Blood CD3+ Pan T-cells

- Transfection efficiency with pMax-GFP in endotoxin-sensitive primary cells was measured with flow cytometry. Data were sorted for relevant parameters in the following order according to Figure 7. Lymphocytes are determined by Forward Scatter (FSC)/Sideward Scatter (SSC)
- Lymphocytes were further gated on single cells (FSC height and area)
- Single cells are selected for CD3 via antibody screening
- Viable CD3+ cells are determined by DAPI staining
- Transfected CD3+ cells were detected by GFP fluorescence

By separating CD3 cells and DAPI staining, the total number of viable CD3 cells with and without transfection program could be analyzed. As expected, non-transfected samples have a higher viability (~50%) than transfected cells (Figure 8A) because of the voltage applied for transfection. But the transfection efficiency of 42% (data not shown) measured with GFP demonstrates that the transfection of endotoxin-sensitive primary cells was very high.

Transfection of pMax-GFP in Human Peripheral Blood CD3+ Pan T cells shows that transfection of endotoxin-sensitive primary cells works well with plasmid DNA isolated using the EndoFree Plasmid Kit (Figure 9). There were no morphological changes observed in transfected cells.

Lymphocytes

SSC-A (10^3)

1000
500
0.01

0.01 500 1000

FSC-A (10^3)

Singlets

FSC-H (10^3)

1000
500
0.01

0.01 500 1000

FSC-A (10^3)

CD3+

FSC-A (10^3)

1000
500
0.01

10^0 10^1 10^2 10^3 10^4 10^5 10^6

CD3 - APC-A

viable CD3+

FSC-H (10^3)

1000
500
0.01

$-5{,}00 \times 10^3$ -10^3 0 10^3 10^4 10^5 10^6

DAPI-A

viable CD3+ GFP+

CD3 - APC-A

10^6
10^5
10^4
10^3

-10^2 10^2 10^3 10^4 10^5 10^6

GFP-A

Figure 7. Gating strategy. Flow cytometry with transfected Human Peripheral Blood CD3+ Pan T cells.

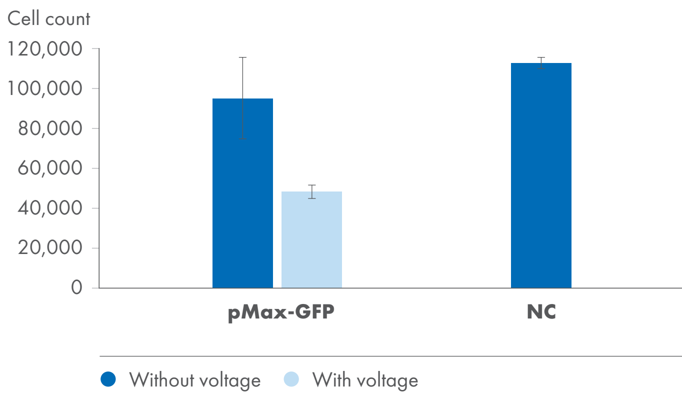

Figure 8. Transfection with EndoFree plasmid DNA. Effective transfection of endotoxin-sensitive primary cells. Total number of viable Human Peripheral Blood CD3+ Pan T cells after transfection with pMax-GFP with and without voltage and negative control without pMax-GFP and without voltage.

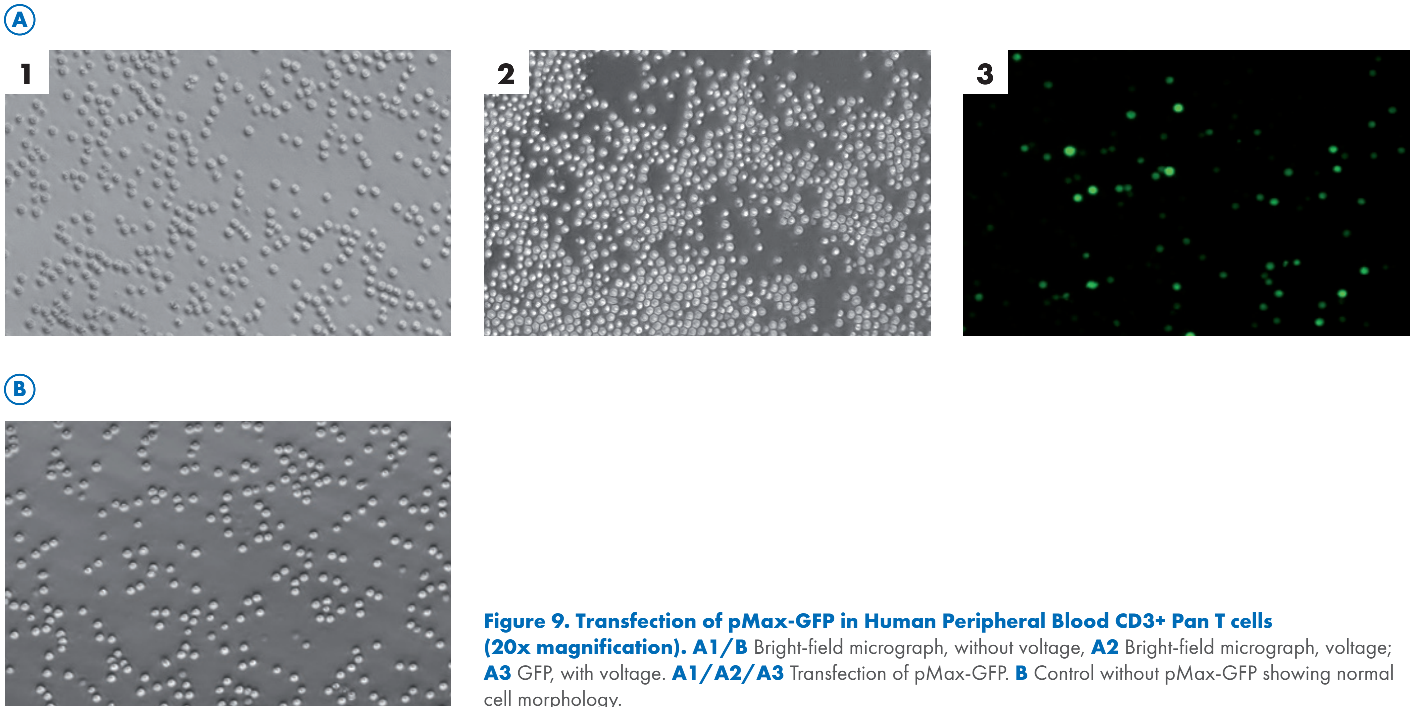

Figure 9. Transfection of pMax-GFP in Human Peripheral Blood CD3+ Pan T cells (20x magnification). A1/B Bright-field micrograph, without voltage, **A2** Bright-field micrograph, voltage; **A3** GFP, with voltage. **A1/A2/A3** Transfection of pMax-GFP. **B** Control without pMax-GFP showing normal cell morphology.

Conclusion

- Replacement of the detergent used in Buffer ER of the updated EndoFree Plasmid Kit has made the kit more eco-friendly than the original kit while removing the need for ice and making the protocol 25 minutes faster.
- Three transfection experiments with different cell lines were conducted to verify the transfection efficiency with different plasmid DNA isolated using the EndoFree Plasmid Kit.
- Transfection was successful in robust HeLa cells with pMax-GFP (high copy) as well as with pBRCMVβ (low copy) and also for endotoxin-sensitive CHO cells with both plasmids. Even for highly sensitive primary cells (Human Peripheral Blood CD3+ Pan T cells), good transfection efficiency (almost 50%) could be measured for pMax-GFP. There were no differences observed in morphology of transfected and non-transfected cells.
- Endotoxins can negatively influence the transfection efficiency in endotoxin-sensitive cell lines (like CHO) and primary cells. Increased endotoxin levels can lead to significantly reduced transfection efficiencies of eukaryotic cells. Buffer ER in EndoFree Plasmid Kits is especially designed to remove endotoxin (less than 0.04 EU/µg DNA) during plasmid isolation, which can be crucial for transfection of endotoxin-sensitive cells or primary cells. The results of these experiments indicate that plasmid DNA isolated using the EndoFree Plasmid Kit contains endotoxin levels below the threshold that inhibits transfection. The endotoxin level was far below the value recommended (2) for vaccines of 0.04 EU/µL (2–40x less) even for low-copy plasmids. There is so little endotoxin in the plasmid DNA isolated using the QIAGEN EndoFree Plasmid Kit that even highly sensitive primary cells can be successfully transfected with a very good transfection efficiency and a healthy morphology.
- The EndoFree Plasmid Kit is the product of choice for plasmid DNA isolation for any kind of transfection experiment. For further information, see also reference 3.

Ordering Information

Product	Contents	Cat. no.
EndoFree Plasmid Maxi Kit (10)	10 QIAGEN-tip 500, Reagents, 10 QIAfilter Maxi Cartridges, Endotoxin-free Buffer	12362
EndoFree Plasmid Mega Kit (5)	5 QIAGEN-tip 2500, Reagents, 5 QIAfilter Mega-Giga Cartridges, Endotoxin-free Buffers	12381
EndoFree Plasmid Giga Kit (5)	5 QIAGEN-tip 10000, Reagents, 5 QIAfilter Mega-Giga Cartridges, Endotoxin-free Buffers	12391
Effectene Transfection Reagent (1 mL)	For 40 transfections in 60 mm dishes or 160 transfections in 12-well plates	301425
Effectene Transfection Reagent (4 x 1 mL)	For 160 transfections in 60 mm dishes or 640 transfections in 12-well plates	301427

References

1. Zang-Gandor MO (1997) Improved transfection of CHO cells using endotoxin-free plasmid DNA. QIAGEN News (4)1997, 1,16–18.
2. Guidance for Industry: Considerations for Plasmid DNA Vaccines for Infectious Disease Indications (fda.gov) https://www.fda.gov/files/vaccines,%20blood%20%26%20biologics/published/Guidance-for-Industry--Considerations-for-Plasmid-DNA-Vaccines-for-Infectious-Disease-Indications.pdf Accessed June 25 (2024)
3. QIAGEN. Endotoxins and their influence on transfection efficiency during CRISPR workflows. November 2019. www.qiagen.com/PROM-15302)

For more details and to order EndoFree Plasmid Kits, visit **www.qiagen.com/endofree-kits**

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