Evaluation of a novel fluorescence-based approach to sensitive bacterial endotoxin testing

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Key takeaways

- **Endotoxin can decrease transfection efficiency; as little as 0.5 EU/µg can reduce transfection efficiency by 50% for sensitive cell lines. Therefore, it is recommended that users routinely test plasmid preparations for endotoxin contamination.**
- **The Invitrogen™ Qubit™ Endotoxin Detection Assay offers a novel, fluorescence-based approach to determine endotoxin levels and ensure verification of safe sample use in downstream applications such as cell culture transfection studies.**

Invitrogen™ Qubit™ Flex Fluorometer

Introduction

Endotoxin contamination is a common problem with recombinant proteins and nucleic acids used in cell and gene therapy workflows. These heat-stable lipopolysaccharides (LPS) are associated with the outer membranes of certain gram-negative bacteria and often introduce themselves into the experimental workflow during the lysis of plasmid DNA preparations. In cell-based experiments, contamination with endotoxin can trigger toxic shock, activation of nonspecific immune responses, or premature cell death. These effects can significantly compromise cell-based studies.

Current approaches to endotoxin testing primarily utilize amebocyte lysate in which an enzymatic cascade is activated in the presence of endotoxin to produce a colorimetric or turbidimetric readout. These assays are a mainstay of endotoxin detection, but often require cumbersome workflows, cost-prohibitive instrumentation, and lengthy manual data analysis. Here we highlight a new endotoxin detection system, the Qubit Flex Fluorometer and Qubit Endotoxin Detection assay which utilizes a fluorogenic reporter to improve sensitivity and dynamic range with a workflow that is more streamlined than existing approaches. Furthermore, we demonstrate the impact of endotoxin contamination in a model cell study where known amounts of endotoxin with a GFP encoding plasmid are titrated into primary cells.

Materials and methods

HUH-7 cells were grown in Gibco™ DMEM media + 10% FBS and HEK293 cells were grown in MEM + 10% FBS. In preparation for transfection, cells were plated overnight in a 96-well plate (Greiner Bio-one, 07000166) at 12,000 cells/well and 5,000 cells/well, respectively. Cells were transfected with control plasmid DNA (pDNA) (Invitrogen™ pJTI™ R4 Exp CMV EmGFP pA Vector) and pDNA spiked with variable levels of endotoxin (eBioscience™ Lipopolysaccharide (LPS) Solution (500X)) (0.1-10 EU/µg DNA) using Invitrogen™ Lipofecatmine™ 3000 (LF3K) transfection reagent following provider protocol. To assess transfection efficiency, cells were stained with Invitrogen™ Hoechst 33342 and imaged after 24 hrs. post-transfection using an Invitrogen™ EVOS™ M7000 microscope. All reagents were supplied by Thermo Fisher Scientific unless otherwise specified.

Cell transfection results

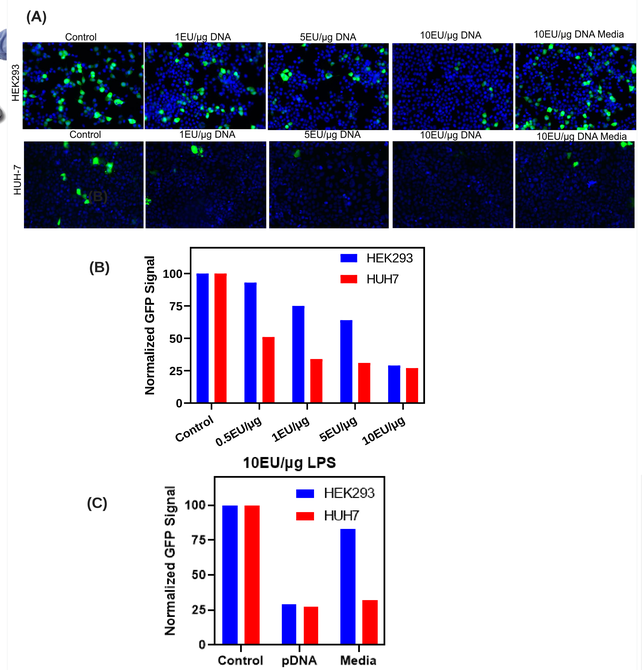

Figure 1. Visualization of endotoxin impact on transfection efficiencies. **(A)** Fluorescence images of HEK293 and HUH-7 cells transfected with mGFP pDNA spiked with variable amounts of endotoxin. Cells were labeled with Hoechst 33342 and imaged on EVOS M7000 system using a 20X objective. **(B)** HUH-7 and HEK293 cells both showed ~80% loss of transfection, as measured by GFP expression, when the pDNA was treated directly with endotoxin at levels of 10 EU/µg DNA. Signal was compromised at lower levels, showing 70% and 30% expression at 1 EU/µg DNA, respectively. **(C)** Spiking 10 EU/µg DNA directly into media post LF3K + pDNA complexing resulted in 20% decreased transfection efficiency in HEK293 and 65% decreased efficiency in HUH-7 cells. Data reveals that even low levels of endotoxin (1 EU/µg DNA) can significantly affect transfection efficiency in some cell lines.

Qubit Flex Endotoxin Detection Assay

The Qubit Endotoxin Detection Assay utilizes a streamlined single-incubation step workflow with a detection range of 0.01 - 10 EU/mL based on variable input volume ranging between 5 - 50 uL. The working solution is prepared from rehydrated amebocyte lysate in water (4.4 mL) and dye substrate in DMSO (30 uL).

Assay kit components:

- Amebocyte lysate reagent
- Endotoxin standards
- Endotoxin free water
- Fluorescence substrate
- DMSO

Pre-warm Invitrogen™ Qubit™ Flex Pyrogen- Free Assay Tube Strips

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Add samples & standards to the tube strips

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Prepare & add working solution and incubate at 37ºC for lot-specific time

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Stop reaction with 25% acetic acid then read samples on the Qubit Flex

Figure 2: Qubit Endotoxin Detection Assay workflow.

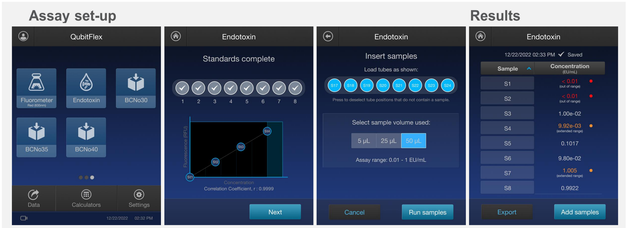

Figure 3: Qubit Flex workflow for detection of endotoxin. The endotoxin assay is selected from the home page of the Qubit Flex Fluorometer. Standards are used to generate a 4-point standard calibration curve that is used to measure endotoxin concentration. Up to 8 samples can be measured at a given time using the Qubit Flex Pyrogen Free Assay Tube Strips. Endotoxin levels are reported on the instrument with alerts if values fall out of the assay detection range. The Invitrogen™ Qubit™ Flex SAE Software for 21 CFR Part 11 Support is also available to support compliance with 21 CFR Part 11 FDA guidelines for security, auditing, and electronic (SAE) signatures.

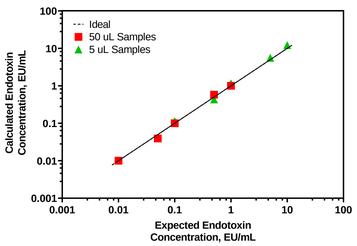

Figure 4: Endotoxin assay dynamic range. Representative fluorescence intensity readout from Qubit Endotoxin Detection Assay across 0.01 - 10 EU/mL detection range (left).

Contaminant	Concentration in 50-µL sample	Concentration in 25-µL sample	Concentration in 5-µL sample
Complete Ham's F-12K Medium supplemented with 1% fetal bovine serum[1]	1:10[2]	1:5	1:1
Complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% fetal bovine serum[1]	1:10[2]	1:5	1:1
DNA[3]	0.1 µg/mL	0.2 µg/mL	1.0 µg/mL
Plasmid DNA[4]	1.0 µg/mL	2.0 µg/mL	10.0 µg/mL
Glycerol	0.1%	0.2%	1.0%
Triton™ X-100 Detergent	0.01%	0.02%	0.1%
Triton™ X-100 Detergent reduced	0.01%	0.02%	0.1%
SDS	0.001%	0.002%	0.010%
Tween™-20 Detergent	0.005%	0.010%	0.050%

Figure 5: Endotoxin assay tolerance. The assay quantifies endotoxin in common sample types such as cell culture media and plasmid DNA. Listed are the known tolerance of potential interfering factors to achieve a valid endotoxin spike recovery of 50–200%. Concentrations listed refer to the actual concentration in the sample that produced no decrease in quantification values when spiked with 0.5 EU/mL endotoxin.

Conclusions

Here we demonstrate the impact of endotoxin contamination in lipid-based cell transfection and highlight the importance of testing reagents to ensure minimal contamination. We introduce the Qubit Flex Fluorometer and Qubit Endotoxin Detection Assay Kit as a platform for streamlined and robust endotoxin testing for medium throughput needs. The assay is also compatible with fluorescence microplate readers using the Invitrogen™ Quant-iT™ Endotoxin Detection Assay.

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