EndotoxDetect™

Colorimetric HEK-Blue™ LPS Detection Kit

Catalog code: rep-lps2

https://www.invivogen.com/endotoxin-lps-detection-assay-kit

A cell-based assay for the detection of biologically active endotoxin

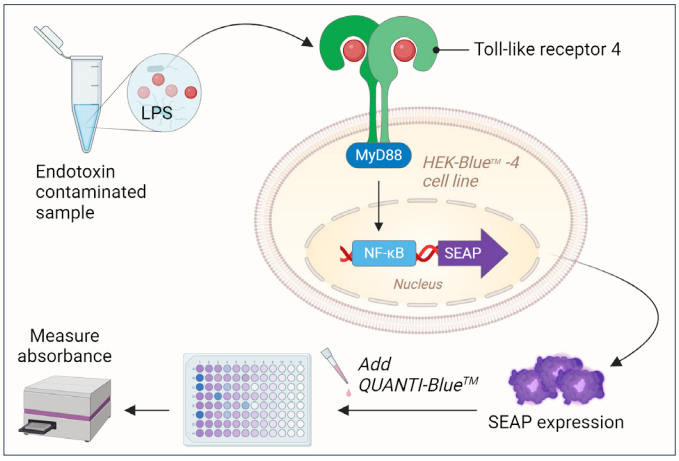
For research use only

Version 24E30-AK

INTRODUCTION

InvivoGen introduces **EndotoxDetect™**, a simple, rapid, and reliable assay to detect the presence of endotoxin (also known as lipopolysaccharide (LPS)) in virtually all biological samples, including particulate compounds, such as vaccine adjuvants and inhibitors of the LAL test.

EndotoxDetect™ is the first cellular assay intended for the detection and quantification of biologically active LPS for research purposes. It is based on the activation of Toll-like receptor 4 (TLR4), the mammalian endotoxin sensor. TLR4 recognizes structurally different LPS from gram-negative bacteria. Proprietary cells engineered to become extremely sensitive to LPS, called HEK-Blue™-4 cells, are the main feature of this endotoxin detection kit. The presence of minimal quantities of LPS, starting as low as 0.01 EU/ml, are detected by the HEK-Blue™-4 cells leading to the activation of NF-κB. Using QUANTI-Blue™, a specific detection medium, NF-κB activation can be observed with the naked eye or measured at 620-655 nm. Since the absorbance is in direct proportion to the amount of endotoxin present, concentrations can be calculated from a standard curve.



This package insert must be read in its entirety before using this product

FOR RESEARCH USE ONLY

InvivoGen
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Diagrams in this protocol are created with BioRender.com.

WHAT'S IN THE BOX

ITEMS	QUANTITY	CONCENTRATION	STORAGE UPON ARRIVAL
HEK-Blue™-4 cells	1 ml	3 - 7 x 10^6 cells/ml	Start cell propagation direct *(see page 5)*
HEK-Blue™ Selection	8 x 1 ml	250X	- 20°C for 12 months
Normocin™	4 x 1 ml	50 mg/ml	- 20°C for 12 months
QUANTI-Blue™ reagent & buffer	2 x 1 ml	100X	- 20°C for 12 months
HEK-Blue™ Endotoxin	lyophilized	Specified on the certificate of analysis	- 20°C for 12 months
HEK-Blue™ water (endotoxin-free)	2 x 50 ml	-	RT for 6 months

HEK-Blue™-4 Cells: Engineered HEK293-derived endotoxin sensor cells. These cells stably express TLR4 and multiple genes from the TLR4 pathway. Additionally, they coexpress an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene.

HEK-Blue™ Selection: a solution containing the required selection antibiotics. These antibiotics guarantee the persistent expression of the various transgenes introduced in HEK-Blue™-4 cells.

Normocin™: an anti-microbial reagent to protect HEK-Blue™-4 cells from any potential contamination, whether caused by mycoplasma, bacteria or fungi.

HEK-Blue™ Endotoxin Standard: a preparation of *E. coli* 055:B5 LPS standardized against FDA approved control standard endotoxin (CSE).

QUANTI-Blue™: a liquid formulation specifically designed for the detection of SEAP. It contains a color substrate that produces a purple/blue color following its hydrolysis by SEAP. QUANTI-Blue™ is provided as 1 ml of QB reagent and 1 ml of QB buffer which is sufficient to prepare 100 ml of QUANTI-Blue™ Solution, a SEAP detection reagent.

Note: Components of the EndotoxDetect™ kit can be purchased separately (see "Related Products" page 10).

ATTENTION!!

HANDLING FROZEN CELLS UPON ARRIVAL

Cells are shipped in dry ice, and upon receipt, should immediately be thawed for culture or stored below -130°C, preferably in liquid nitrogen vapor, for long-term storage.
IMPORTANT: Do not store cell vials at -80°C as this will decrease cell viability and performance. Contact technical support if the cells are not frozen or in dry ice upon arrival. To ensure the highest level of viability and best assay performance, we strongly recommend that you thaw the cells and initiate the culture as soon as possible upon receipt.

Additional materials required:

REAGENTS
Dulbecco's modified Eagle's medium (DMEM), high glucose (4.5 g/L)
Note: If using DMEM without glutamine, add 2 mM glutamine.
Penicillin-Streptomycin solution
Fetal Bovine Serum (FBS) **without endotoxins**
Trypsin-EDTA (0.05% Trypsin, EDTA.4Na)
Phosphate buffered saline (PBS)
Dimethylsulfoxide (DMSO)

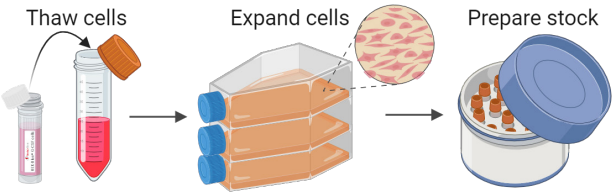
SUPPLIES
Laminar flow hood, CO_2 incubator
Water bath (37°C), centrifuge
Inverted microscope
Microplate reader with 620-655 nm filter
Sterile cell culture plasticware (e.g. 96-well plates, tips, etc.)
Counting cell (e.g. Malassez)
Multichannel pipettes (200/300 µl) and reagent reservoirs

ASSAY OUTLINES

Handling Procedures of Cells *(p.5)*

Cell handling upon arrival

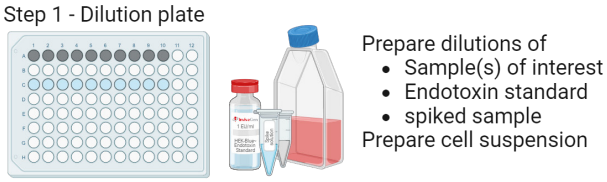
1. Thaw and expand HEK-Blue™-4 cells.
2. Make your frozen stock of HEK-Blue™-4 cells.



Assay Workflow - Endotoxin Detection *(p.7 - 9)*

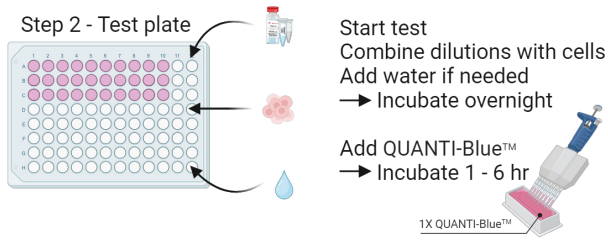
Step 1 - Preparation *(day 1)*

1. Prepare all needed reagents, samples, and cell suspension.
2. In **dilution plate**: Prepare serial dilutions of the samples of interest and the provided (HEK-Blue™) Endotoxin Standard.



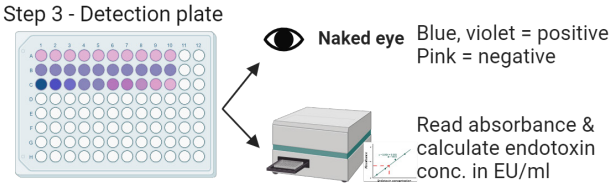
Step 2- Procedure *(day 1 & 2)*

3. In **test plate**: Transfer dilutions of samples of interest and Endotoxin Standard. Add cell suspension → Incubate overnight.
4. In **detection plate**: Transfer HEK-Blue™-4 cell supernatants to 1X QUANTI-Blue™ → Incubate 1-6 hours.



Step 3 - Interpretation & reading *(day 2)*

5. Inspect with the naked eye:
   → purple/blue = positive
   → pink = negative
6. *Optional*: Read the absorbance detection plate at 620-655 nm.
   → Calculate the endotoxin concentration of your sample.



SAFETY CONSIDERATION

The EndotoxDetect™ Kit contains antibiotics and products of biological and bacterial origins that must be handled observing the usual safety precautions (wear appropriate protective equipment, do not ingest, do not inhale). HEK-Blue™-4 cells were derived from HEK293 cells (transformed with adenovirus 5 DNA) that may require Biosafety Level 2 according to CDC guidelines. The biosafety level may vary depending on the country. HEK-Blue™-4 cells are sent with the condition that you are responsible for its safe storage, handling and use. InvivoGen is not liable for damages or injuries resulting from receipt and/or use of an InvivoGen culture. Detailed discussions of laboratory safety procedures are provided in Laboratory Safety: Principles and Practices (Fleming et al., 1995), the ATCC manual on quality control (Hay et al., 1992), the Journal of Tissue Culture Methods (Caputo, 1988), and the U.S. Government Publication, Biosafety in Microbiological and Biomedical Laboratories, 4th ed. HHS publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at: https://www.cdc.gov/safelabs/resources-tools/biosafety-resources-and-tools.html.

Note: InvivoGen recommends for protective gloves and clothing to always be used and a full mask to always be worn when handling frozen vials.

CELL LINE WARRANTIES

- InvivoGen's cells are provided 'AS IS' and their viability is guaranteed upon shipment from our facilities for a period of 30 days, provided that the customer has properly stored and handled the product.
- Our cell lines are guaranteed free of mycoplasma contamination.
- The stability of our cell lines is guaranteed for 20 passages.

PREPARATION OF REAGENTS

All reagents should be prepared under sterile conditions according to good laboratory practices. All materials coming in contact with the samples or test reagents must be endotoxin-free. These materials should be tested before use. Endotoxins are lurking everywhere. Special caution should be applied to the following potential sources:

WATER
Use high-purity water to prepare media and solutions, and clean glassware.

GLASSWARE
To destroy endotoxins, heat glassware to 250°C for ≥30 min, or 180°C for 3 hrs.

PLASTICWARE
Ask for manufacturer certification of endotoxin levels and absence of pyrogenicity.

MEDIA, SERA & ADDITIVES
Ask for manufacturer certification of endotoxin levels, or test before use.

Cell culture medium for HEK-Blue™-4 Cells

• **Growth Medium** (for thawing and recovery of the frozen cell line)**:**
DMEM, 4.5 g/l glucose, 2 mM L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum (FBS; 30 min at 56 °C), Pen-Strep (100 U/ml-100 µg/ml), 100 µg/ml Normocin™. Do not add selection antibiotics. Warm at 37°C before use and store at 2-8°C.
• **Required Selective Antibiotic(s):** HEK-Blue™ Selection
• **Freezing Medium:** DMEM, 20% (v/v) FBS, 10% (v/v) DMSO. Prepare extemporaneously, no storage. You may also use commercial available serum-free freezing media.
• **Test Medium :** Growth medium without Normocin™.
Note: The use of some FBS might affect the functionality of HEK-Blue™-4 Cells as they may contain endotoxins. Make sure the FBS used is endotoxin-free.

Preparation of diluted Trypsin-EDTA solution

- Mix 10 ml of Trypsin-EDTA (0.05% Trypsin, EDTA.4Na) with 20 ml of PBS.
- Warm at 37°C before use and store at 2-8°C.
The solution is stable for 3 days at 2-8°C and 6 months at -20°C.
Note: HEK-Blue™-4 cells functions are altered by the action of trypsin unless the solution is diluted. We strongly recommend the use of diluted trypsin to pass HEK-Blue™-4 cells. Before the test, use PBS to detach the cells.

QUANTI-Blue™ 1X

- Prepare 100 ml of QUANTI-Blue™ 1X by adding 1 ml of QB reagent (100X) and 1 ml of QB buffer (100X) to 98 ml of sterile water in a sterile glass bottle or flask.
- Mix well by vortexing and incubate at room temperature for 10 min before use.
- Use QUANTI-Blue™ immediately or store at 2-8 °C or -20 °C.
- Resuspended QUANTI-Blue™ is stable for 2 weeks at 2-8°C and for 2 months at -20°C. Avoid repeated freeze-thaw cycles.

HEK-Blue™ Endotoxin Standard

- Each vial contains approximatively 40-100 EU lyophilized endotoxin. The actual value is stated on the Certificate of Analysis.
Example: If the value of the vial is 55 EU, when reconstituted with 1 ml endotoxin-free water it will yield a concentration of 55 EU/ml.
- Reconstitute by adding 1 ml of endotoxin-free HEK-Blue™ water to the content of the tube to obtain an initial stock solution at X EU/ml, where X is the amount of EU per vial.
- Mix vigorously by vortexing as endotoxin tends to attach to glass.
- Prepare a 1.0 EU/ml HEK-Blue™ Endotoxin Standard stock solution by diluting 1/X.
Example: If the initial stock solution is at 55 EU/ml, the dilution is 1/55 or 0.1 ml of initial stock solution into 5.4 ml of endotoxin-free HEK-Blue™ water.
- Store at 2-8°C. Resuspended solutions are stable for 1 week.

Sample preparation

- All powdered samples should be resuspended in endotoxin-free water.
- Samples to be tested should be stored at 2-8°C or frozen to stop all bacteriological activity and activity increase over time.
- Samples containing a phosphatase activity cannot be tested as they can result in false positive results.

HANDLING PROCEDURES OF HEK-BLUE™-4 CELLS

The first propagation of cells should be for generating stocks for future use. This ensures the stability and performance of the cells for subsequent experiments. During the handling process, please note the following points:

CELL RECOVERY
Do not add selective antibiotics until the cells have been passaged twice.

CELL PASSAGE
The cells should be passaged when 60 - 80 % confluency is reached.

CELL PERFORMANCE
Thaw a new vial when cultured cells have reached 20 passages.

CELL STORAGE
Do not store cells at -80°C. Transfer cells in liquid nitrogen for long term storage.

Thawing

1. Thaw the vial in a 37°C water bath. Keep the O-ring and cap out of the water. Thawing should be rapid (~ 2 min).
2. Remove the vial from the water bath as soon as the tube content is thawed, and decontaminate by dipping in or spraying with 70% (v/v) ethanol.
3. Transfer the vial content in a sterile tube containing 20 ml of pre-warmed growth medium and spin at 300 x g (RCF) for 5 min.
4. Remove the supernatant and resuspend the cells with 5 ml of growth medium.
5. Transfer to a 25 cm^2 tissue culture flask or a 75 cm^2 tissue culture flask containing 15 ml of pre-warmed growth medium.
   Do not add selective antibiotics until the cells have been passaged twice.
6. Place the flask at 37°C in a CO_2 incubator overnight.
7. Observe the culture daily with an inverted microscope. When 60 - 80% confluency is reached, continue with cell maintenance.

Cell maintenance

HEK-Blue™-4 cells grow as adherent cells in a monolayer.

1. Detach the cells in the presence of pre-warmed 1X PBS at 37°C or by using diluted trypsin for 2-3 min at room temperature (RT).
   Note: The response of HEK-Blue™-4 cells can be altered by the prolonged action of trypsin. Do not incubate with trypsin at 37 °C and no longer than 3 min.
2. Maintain and subculture the cells in growth medium supplemented with 1X HEK-Blue™ Selection.
3. Renew selection medium 2 to 3 times a week.
4. **Cells should be passaged when a 60 - 80% confluency is reached.** Do not let them grow to 100% confluency as it might affect cell performance.

Frozen stock preparation

Use cells at 80% confluency to prepare frozen stocks.

1. Detach the cells in the presence of pre-warmed 1X PBS at 37°C or by using diluted trypsin for 2-3 min at room temperature (RT).
2. Wash cells in growth medium by centrifuging at 300 x g (RCF) for 5 min.
3. Resuspend cells at 5 - 8 x 10^6 cells/ml in freezing medium.
   Note: A T-75 culture flask typically yields for 3 - 4 frozen vials.
4. Dispense 1 ml of cell suspension per cryotube.
5. Place vials in a freezing container and store at -80 °C overnight.
6. **Transfer vials to liquid nitrogen for long term storage.**
   *Note: If properly stored, cells should remain stable for years. To ensure a maximal efficiency of the HEK-Blue™-4 cell line, thaw a new tube when the cultured cell line has reached **20 passages.***

NOTES BEFORE STARTING THE ASSAY

Cytotoxicity

1. We recommend to test the absence of toxicity of the test sample on HEK-Blue™-4 cells before running the endotoxin detection test. If a cytotoxic effect is observed, the sample may require further dilution until the cytotoxicity is overcome.
2. Avoid testing of pure samples soluble only in ethanol or DMSO. These solutions are toxic to the cell line and can result in false negative results.

Test interferences

- Inhibition / enhacement

  Sample inhibition or enhancement occurs when substances in the test sample interfere with the activation of the HEK-Blue™-4 cells. This interference affects the final absorbance, leading to false-negative or false-positive results. The lack of sample interference should be determined for each specific sample. To verify the lack of interference, a second serial dilution of the test sample is spiked with a known amount of endotoxin (e.g. 0.1 EU/ml). The dilutions spiked sample are assayed along the unspiked sample dilutions. The absorbances of the spiked sample dilutions should equal to 50-200% of the absorbance of the spike *(see page 11 for detailed protocol)*.

Limitations

- Toxic products

Toxic compounds may interfere with the HEK-Blue™-4 cells at high concentrations. The lack of product cytotoxicity should be determined for each specific sample, either undiluted or at different dilutions.

- Particulate samples

Particulate samples, such as vaccine adjuvants, should not affect the HEK-Blue™-4 endotoxin assay. Particulate samples are recognized by the HEK-Blue™-4 cells equally well as soluble samples.

- Colored samples

Samples which possess significant color may require special attention. A quick visual inspection of the detection plate prior to incubation may be sufficient to determine if a product's intrinsic color is of concern.

The endotoxin concentration of colored samples should be determined using the absorbance of the sample dilutions that have a color similar to the blank prior to the incubation of the detection plate.

- NF-κB inducers

Compounds known to activate the NF-κB pathway may interfere with the HEK-Blue™-4 endotoxin assay by activating the HEK-Blue™-4 cells in a TLR4-independent manner. Such compounds include ionomycin, phorbol 12-myristate 13-acetate (PMA), cytokines (e.g. TNF-α, IL-1β), and ligands of TLR3, TLR5 or NOD1.

FREQUENTLY ASKED QUESTIONS

Q: What are endotoxins?

A: Endotoxins are small bacterially-derived hydrophobic lipopolysaccharide (LPS) molecules that can easily contaminate labware and whose presence can significantly impact both in vitro and in vivo experiments.

Q: What are the effects of endotoxin contamination?

A: Endotoxins can alter cellular morphology, proliferation, and cytokine production as well as induce fever, multiple organ failure, and fatal septic shock.

Q: What are the acceptable limits of endotoxins in cell culture?

A: Current FDA limits require eluates from medical devices to be less than 0.5 EU/ml (0.06 EU/ml for cerebrospinal fluid devices).

Q: How to prevent endotoxin contamination?

A: It is essential to use endotoxin tested reagents, as well as proper aseptic techniques. Thoroughly rinse and sterilize all cell culture plasticware and consumables like pipettes and conical tubes before culturing cells.

Q: How to remove endotoxins if cultures become contaminated?

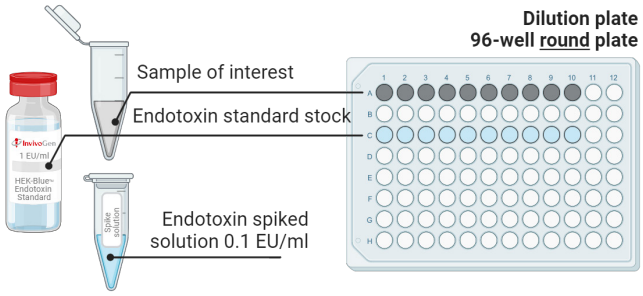
A: Start with new reagents and cells and discard all contaminated reagents and cells. Endotoxin removal solutions are also an option. One can use Triton X-114 to remove LPS endotoxins from samples.

ASSAY WORKFLOW - ENDOTOXIN DETECTION PROCEDURE

This protocol details the procedure to be performed in 96-well plates. Warm the samples and all the test reagents at 37°C before use. We recommend to run the standard and samples in duplicate on separate plates.

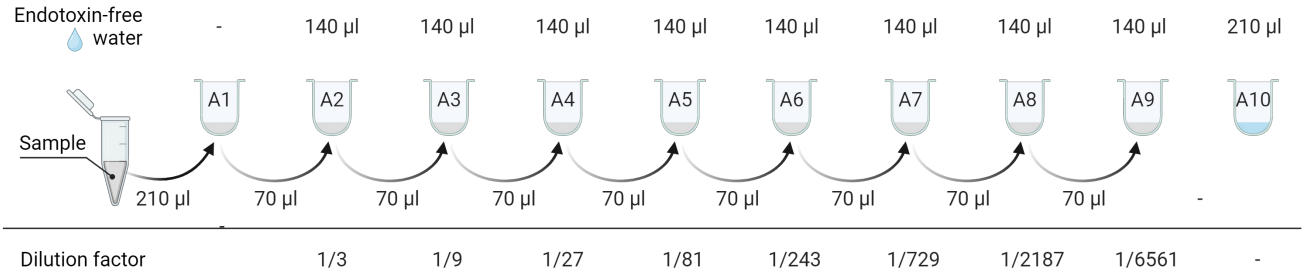
Step 1: Preparation of samples

REAGENTS AND MATERIAL REQUIRED
HEK-Blue™-4 cells (50 - 80% confluent; passage < 20)
Cell culture maintenance (PBS, test medium, etc.)
Sample(s) of interest (210 µl per plate)
HEK-Blue™ Endotoxin Standard stock solution (1.0 EU/ml)
HEK-Blue™ water (endotoxin-free)
96-well round and flat bottom plates (1 x round, 2 x flat)
Cell culture plasticware (pipette tips, 1.5 ml tubes, etc.)
Lab equipment (vortex machine, pipettes, cell counting device)



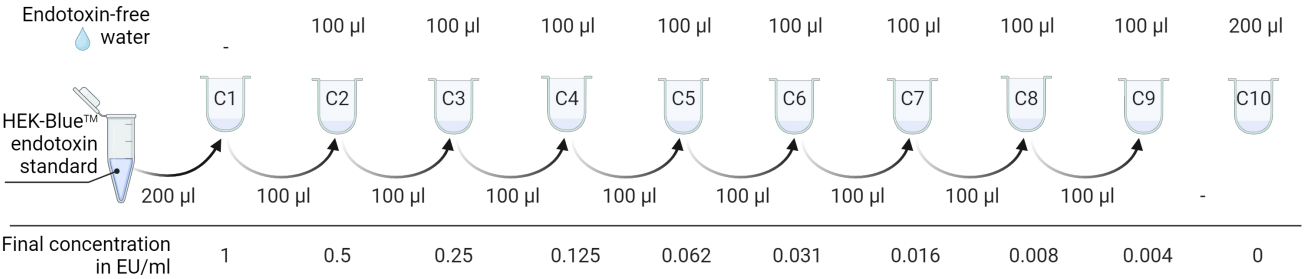
Preparation of sample dilution

1. Use a 96-well round bottom plate as dilution plate.
2. Mix 200 µl of your sample of interest (by vortex or pipette).
3. In dilution plate in row **A2** - A10: Add endotoxin-free water.
4. In dilution plate in row A1- A9: Prepare a **three-fold serial dilution** of the test sample:



Preparation of HEK-Blue™ Endotoxin Standard dilution

1. Mix vigorously by vortexing the HEK-Blue™ Endotoxin Standard stock solution (1 EU/ml).
2. In dilution plate I in row **C2** - C10: Add endotoxin-free water.
3. In dilution plate I in row C1 - C9: Prepare a **two-fold serial dilution** of the HEK-Blue™ Endotoxin Standard stock solution:



Preparation of the spike solution (0.1 EU/ml of endotoxin standard)

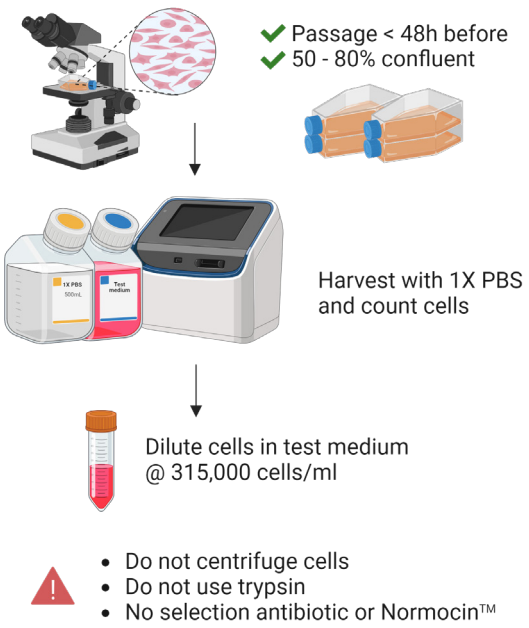
1. For the enhancement/inhibition test: Prepare a 1.5 ml tube with spike solution (0.1 EU/ml final concentration): Mix 50 µl of HEK-Blue™ Endotoxin Standard stock solution (1 EU/ml) + 450 µl of endotoxin-free water.

HEK-Blue™-4 cell suspension preparation

To ensure the best results of the test:

- Preparation of the cells should be as short as possible to prevent any damage resulting from the prolonged stay at RT without 5% CO_2
- Prepare test plate from STEP 2: TEST PROCEDURE - Day 1 *(see below)* before preparing cell suspension.
- Use HEK-Blue™ -4 cells that have been **passaged less than 20 times.**
- Use a culture showing 50-80% confluency and that has been passaged at least 48 h before the test.

1. Prepare a suspension of HEK-Blue™-4 cells by gently rinsing the cells once with pre-warmed 1X PBS. Detach the cells from the flask in the presence of 1X PBS. *Note: Do not use trypsin to detach the cells. Do not centrifuge cells.*
2. Estimate the cell concentration. Avoid air bubbles formation.
3. Dilute the cells with pre-warmed test medium at 315,000 cells/ml.
4. Transfer cell suspension into a sterile reagent reservoir if using a multichannel pipette and continue as soon as possible with TEST PROCEDUCE.



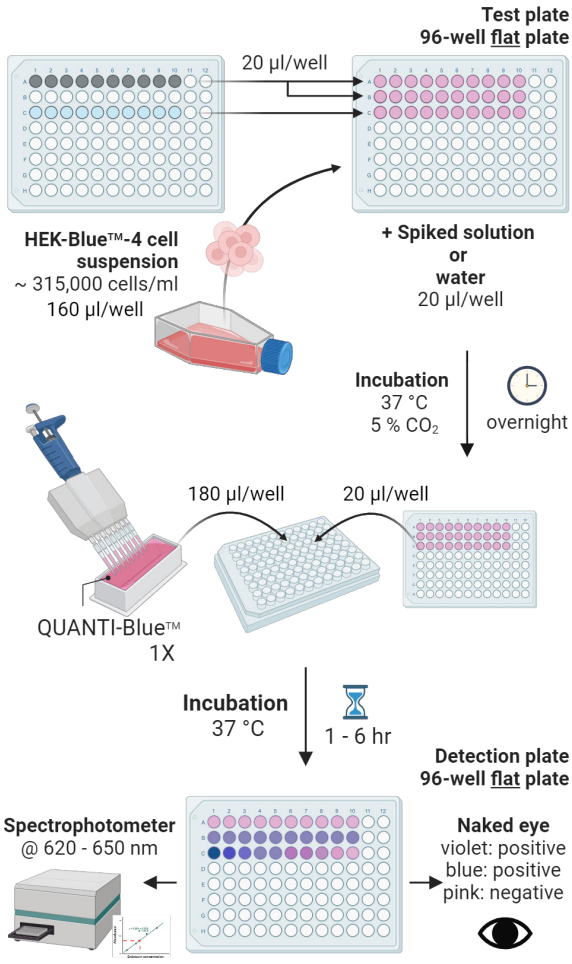
Step 2: Test procedure

Day 1

1. Use a new 96-well flat bottom plate as **test plate.**
2. In **test plate** in row **A1 - A10 and B1 - B10**: Transfer 20 µl of the sample serial dilution from the **dilution plate**:
   Example: 20 µl from well A1 of dilution plate into wells A1 and B1 of test plate, 20 µl from well A2 of dilution microplate into wells A2 and B2 of test plate, and until wells A10 and B10.
3. In **test plate** in row **C1 - C10**: Transfer 20 µl of the HEK-Blue™ Endotoxin Standard serial dilution:
   Example: 20 µl from well C1 of dilution plate into well C1 of test plate, 20 µl from well C2 of dilution plate into well C2 of test plate until C10.
4. Add 20 µl of endotoxin-free water into wells A1 to A10 of test plate. **Row A** corresponds to the unspiked sample dilutions.
5. Add 20 µl of 0.1 EU/ml HEK-Blue™ Endotoxin Standard spike solution into wells B1 to B10 of test plate.
   Row B corresponds to the spiked sample dilutions.
6. Add 20 µl of endotoxin-free water into wells C1 to C10 of test plate. **Row C** corresponds to the HEK-Blue™ Endotoxin Standard dilutions.
7. Prepare the HEK-Blue™ -4 cell suspension as described above.
8. Add 160 µl/well (max. 50,000 cells/well) of the HEK-Blue™-4 cell suspension to the **test plate** wells. Use new tips for each well to avoid cross-contamination.
9. Incubate the test plate at 37°C in a CO_2 incubator for 18-24 hours.

Day 2

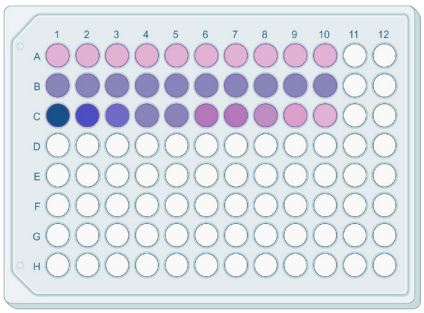
1. Prepare QUANTI-Blue™ *(see page 5)* and warm at 37°C for 30 min.
2. Use a new 96-well flat bottom plate as **detection plate.**
3. Transfer 20 µl of supernatant from each well of the **test plate** into the corresponding well of a new plate **(detection plate)**.
4. Add 180 µl of QUANTI-Blue™ and incubate 1 - 6 hours at 37°C.
5. Read the absorbance of the plate at 620-655 nm.



Step 3: Interpretation and reading

Presence of endotoxin in a sample can be observed with the naked eye. If needed, the concentration can be calculated by measuring the absorbance using a spectrophotometer.

Detection plate



Naked eye interpretation

The test was successful if after 1 - 6 hours incubation:

- the HEK-Blue™ Endotoxin Standard (row C1-C10) displays a blue/purple/pink gradient.
- the blank (wells A10 and C10) remains pink or light purple. The blank might appear as a light purple color without altering the interpretation of the test. However, if the blank results in a deep purple color, the test cannot be validated and should be repeated.

All samples resulting in a purple or blue color should be considered as positive and containing ≥ 0.01 EU/ml endotoxin.

Endotoxin standard curve calculation

1. If samples were run in duplicates, calculate the average optical density (OD) values to get the mean absorbance for the standard (table 1) and test sample. (table 2).
2. Subtract the averaged blank OD values from the averaged OD values to calculate mean Δ absorbance.
3. **For the standard curve:** Plot the mean Δ absorbance OD values of the standard against the known concentration of endotoxin (EU/ml). Fit a linear trendline to these points and display the equation and the coefficient of correlation (R-squared value) on chart. *Note: HEK-Blue™ Endotoxin Standard curve is reliable when the coefficient of correlation (R^2 value) is ≥ 0.98. No less than 4 standard concentrations spanning the desired concentration range should be assayed.*

Table 1. Example results of a standard curve:

Endotoxin conc. in EU/ml	Absorbance OD at 650 nm (duplicate 1)	Absorbance OD at 650 nm (duplicate 2)	Mean Absorbance	Mean Δ Absorbance (Absorbance - Blank)
1	1.217	1.305	1.261	1.093
0.5	0.914	1.001	0.958	0.790
0.25	0.676	0.730	0.703	0.535
0.125	0.483	0.533	0.508	0.341
0.062	0.352	0.369	0.361	0.193
0.031	0.254	0.307	0.281	0.113
0.016	0.207	0.231	0.219	0.052
0.008	0.181	0.192	0.187	0.019
0.004	0.180	0.192	0.186	0.019
0 (Blank)	0.165	0.170	0.168	-

Regression analysis of the HEK-Blue™ endotoxin standard curve, calculated using the example values from table 1 in bold:

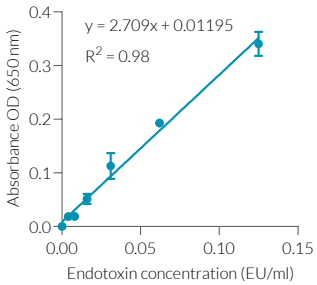


Figure 1. Representative standard curve, calculated using the example values from table 1 in bold (see above).

y = 2.709x + 0.01195

y : Mean Δ Absorbance value

x : Endotoxin concentration of dilution

X : Endotoxin concentration of sample in EU/ml

→ X = x / dilution factor

Calculating endotoxin concentration in test sample

1. Choose y values of the sample dilutions that are in the linear range of the standard curve.
2. Calculate the corresponding x value(s) using the equation of the standard curve.
3. Example calculation using regression analysis:

   y = 2.709x + 0.01195 or x = (y - 0.01195)/2.709

   • 1/2187 sample dilution:

   x = (0.295 - 0.01195)/2.709 = 0.104

   → X = 0.104 × 2187 = 228 EU/ml

   • 1/6561 sample dilution:

   x = (0.121 - 0.01195)/2.709 = 0.0403

   → X = 0.0403 × 6561 = 264 EU/ml

   → Result: Sample contains an average of **246 EU/ml.**

Table 2. Example results of a test sample run:

Dilution factor	Absorbance (duplicate 1)	Absorbance (duplicate 2)	Mean Absorbance	Mean Δ Absorbance
1	2.584	2.508	2.546	2.378
1/3	2.520	2.461	2.491	2.323
1/9	2.411	2.329	2.370	2.202
1/27	2.088	2.056	2.072	1.904
1/81	1.516	1.462	1.489	1.321
1/243	1.104	1.036	1.070	0.902
1/729	0.613	0.577	0.595	0.427
1/2187	0.485	0.441	0.463	**0.295**
1/6561	0.302	0.276	0.289	**0.121**
0 (Blank)	0.171	0.165	0.168	-

Determination of non-inhibitory sample dilution

To determine if a sample interferes with the performance of the EndotoxDetect™ assay, we recommend to perform a inhibitory/enhancement assay using spiked samples *(see page 8)*.

1. The spiked samples are assayed along with the unspiked samples.
2. Subtract the OD values of the spiked dilution from the OD values of the unspiked dilution to calculate Δ difference in absorbance.
3. Fix the percentage of the Δ OD value of the blank *(here 0.40)* as 100 % of absorbance value and determine the remaining values using the rule of three.
4. Spiked samples with absorbance values equal to **50-200%** of the absorbance of the spike are considered non-interfering and can be used for the determination of the endotoxin concentration.

Table 3. Example results of a spiked test sample run:

Dilution factor	Absorbance of unspiked samples	Absorbance of spiked samples	Δ Difference (spiked - unspiked)	Δ absorbance values in percentage
1	2.546	2.606	0.06	15 %
1/3	2.491	2.571	0.08	20 %
1/9	2.370	2.58	0.21	53 %
1/27	2.072	2.322	0.25	63 %
1/81	1.489	1.769	0.28	70 %
1/243	1.070	1.36	0.29	73 %
1/729	0.595	0.905	0.31	68 %
1/2187	0.463	0.823	0.36	90 %
1/6561	0.289	0.669	0.38	95 %
0 (Blank)	0.168	0.568	0.40	100 %

→ Results *(see table above last column):* Undiluted sample and first sample dilution (in red) are inhibitory.
The other values (in green) are non-inhibitory. Thus, they can be used to determine the endotoxin concentration in the sample of interest.

RELATED PRODUCTS

Product	Description	Quantity	Cat. Code
HEK-Blue™ Selection	Selective antibiotic	10 x 1 ml	hb-sel
QUANTI-Blue™	SEAP detection reagent	5 ml	rep-qbs
HEK-Blue™ Endotoxin Standard	Standardized *E. coli* O55:B5 LPS	10 x 50 EU	rep-hbes-10
LPS-RS Ultrapure *(R. sphaeroides)*	TLR4 antagonist	1 mg	tlrl-prslps

USE RESTRICTIONS

HEK-Blue™ -4 cells are distributed for research purposes only.
Third party distribution of this cell line is discouraged, since this practice has resulted in the unintentional spreading of cell lines contaminated with inappropriate animal cells or microorganisms.
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Tel: 858-457-5873. Fax: 858-457-5843.

TROUBLESHOOTING GUIDE

PROBLEM	REASON	SOLUTION
All wells of the detection plate are blue	• Cell culture was over confluent • Cells have been subcultured more than 30 times • Cells used for the test have been under stress before the test • Cells have been under stress during the test (e.g. long time at 20-25°C without 5% of CO_2, cold PBS, centrifugation) • Excessive number of cells per well in the test plate • Cell culture medium contains endotoxins (typically in the FBS or trypsin)	• Use cells at 60-80% confluence • Start a new culture from the frozen stock • Use healthy cells that have been passaged at least 48 h before the test • Prepare the cell suspension as fast as possible using warm reagents and avoid excessive pipetting and centrifugation • Do not use more than 50,000 cells per well of a 96-well plate • Use FBS and trypsin that is certified endotoxin-free
Blank gives a blue color	• See above	• See above
HEK-Blue™ Endotoxin Standard dilutions stay pink	• Endotoxins adhere to the inner surface of the tube • QUANTI-Blue™ was stored for more than 2 weeks at 2-8°C • HEK-Blue™-4 cells are not in a healthy state • Cells are not HEK-Blue™-4 cells	• Warm and vortex extensively the endotoxin standards before use • Use a new vial to prepare QUANTI-Blue™ • Start a new culture from the frozen stock • Start a new culture from the frozen stock
False negatives	• Samples are toxic to the HEK-Blue™-4 cells • Endotoxins in the samples adhere to the inner surface of the tube	• Dilute samples in endotoxin-free water and test their cytotoxicity on HEK-Blue™-4 cells before running the EndotoxDetect™ assay • Vortex the sample extensively before use
False positives	• Presence of a phosphatase activity in the sample • Sample contains an NF-κB inducer	• Test the presence of a phosphatase activity in your sample by adding 20 µl of your sample to 180 µl of QUANTI-Blue™. If a purple/blue color appears after 1-3h at 37°C your sample contains a phosphatase activity and cannot be tested using the EndotoxDetect™ kit • This sample cannot be tested with EndotoxDetect™
Negative using EndotoxDetect™ endotoxin assay but positive using a LAL assay	• Sample contains a LPS that does not activate TLR4 (e.g. LPS from *Rhodobacter sphaeroides*)	• Such LPS may act as an endotoxin inhibitor

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