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Quant-iT™ Endotoxin Detection Assay Kit

USER GUIDE

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For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: MAN0029394 A.0 (English)

Revision	Date	Description
A.0	23 May 2023	New document for Quant-iT™ Endotoxin Detection Assay Kit.

The information in this guide is subject to change without notice.

Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Product description

The Quant-iT™ Endotoxin Detection Assay Kit is an efficient, fluorescent endpoint assay that uses amebocyte lysates to quantify endotoxin in various sample types such as nucleic acids, proteins, peptides, antibodies, or water samples (Table 1). Amebocyte lysates are widely used as a sensitive assay for the detection of endotoxin lipopolysaccharide (LPS), the membrane component of gram-negative bacteria. When endotoxin encounters the amebocyte lysate, a series of enzymatic reactions results in the activation of Factor B, Factor C, and pro-clotting enzyme. The activated enzymes catalyze a cleavage event in the substrate to produce a fluorescent signal. After stopping the reaction, the resulting signal can be measured on a fluorescent microplate reader. The fluorescent signal is proportional to the endotoxin concentration in the sample and enables quantification from 0.01–10.0 EU/mL, depending on the sample volume.

Note: This assay is designed to work on fluorescent microplate readers. For lot-specific incubation time, consult the assay kit label or Certificate of Analysis (CofA).

Contents and storage

Table 1 Contents and storage for the Quant-iT™ Endotoxin Detection Assay Kit.

Component	Amount	Storage[1]
Quant-iT™ Endotoxin Reagent (Component A)	2 vials	2–8°C
Quant-iT™ Endotoxin-Free DMSO (Component B)	2 x 100 µL	
Quant-iT™ Lyophilized Endotoxin Standard from *E. coli* (0111:B4) (Component C)	4 vials	
Quant-iT™ Lyophilized Amebocyte Lysate (Component D)	4 vials 40 reactions per vial	
Quant-iT™ Endotoxin-Free Water (Component E)[2]	4 x 50 mL	

[1] Kits are stable for at least 6 months from the date of receipt when stored as directed.

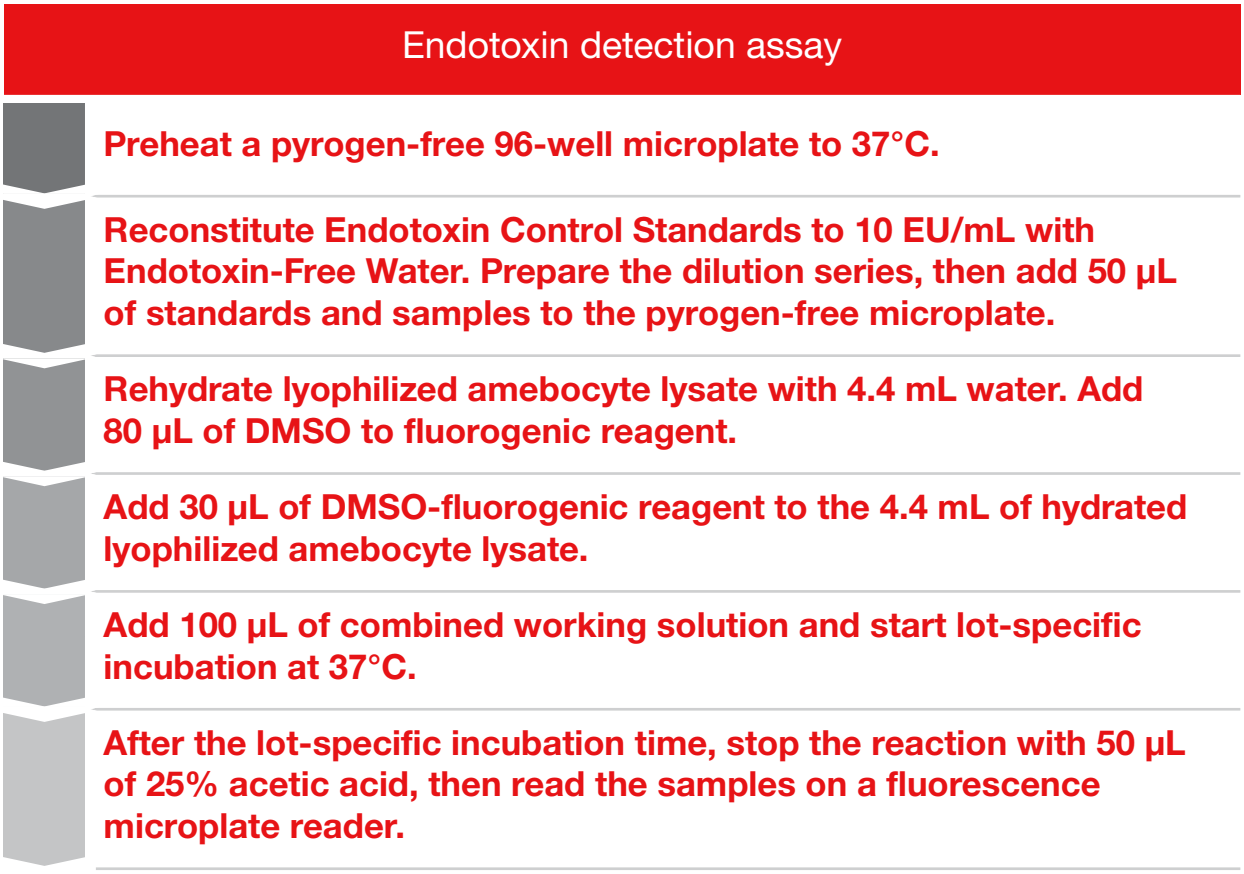
[2] Endotoxin-Free Water can be stored at room temperature for short-term storage.

Required materials not supplied

- Acetic acid, 25%
- Endotoxin-free 96-well microplates (Cat. No. A43888 or NC9217517)
- Pierce™ Endotoxin-Free Glass Test Tubes (Cat. No. A43889)
- Stable temperature heat block with 96-well plate adaptor
- Plate mixer
- Vortex mixer

Note: Ensure all materials (for example, pipette tips, glass tubes, microcentrifuge tubes, etc.) are endotoxin-free.

Workflow



2 Methods

Procedural guidelines

Assay dynamic range	The assay can be run with variable sample inputs to augment the dynamic range of the assay. • For 50 µL samples, the assay detects endotoxin at 0.01–1.0 EU/mL. • For 25 µL samples, the assay detects 0.02–2.0 EU/mL. • For 5 µL samples, the assay detects 0.10–10.0 EU/mL.
Contamination	To avoid cross-contamination, work from the lowest to highest endotoxin concentration when preparing standards and adding the working solution.
Assay temperature	The Endotoxin Detection Assay requires that the assay be incubated at 37°C throughout the run time. For best results, use a dry-block style heater. Cabinet-style incubators are not recommended for this assay because of insufficient surface area contact to properly heat the 96-well microplate. In addition, never use a water bath as these cannot be maintained as endotoxin-free.
Assay calibration	Assay standards must be run each time the assay is performed. Previous standards and standard curve are not viable because this is a temperature-sensitive enzymatic assay.
More assay considerations	• Accurate pipetting is critical for maintaining consistent results. A repetitive pipettor can help in normalizing volumes between samples. Ensure pipetting order and rate of reagent addition remain consistent from well-to-well. • Endotoxin adheres to glass and plastic surfaces; before pipetting, vortex solutions to ensure the correct endotoxin concentrations are measured. • Glass tubes are recommended for making standard stock solutions. • Each lysate lot is tested for functionality using the United States Reference Standard Endotoxin (EC-6). The assay lot is then matched to a specific lot of Quant-iT™ Lyophilized Endotoxin Standard (Component C) by testing in parallel with the Reference Standard Endotoxin. A Reference Standard Endotoxin to Quant-iT™ Endotoxin Standard correlation assay determines the potency of each new lot when used with each matching lysate lot.

Before you begin

- Obtain the lot-specific incubation time for the assay from the Certificate of Analysis or the outside kit label.
- Ensure that the endotoxin-free water, DMSO, and endotoxin control are equilibrated to room temperature. The remaining materials can stay refrigerated until needed or stored at room temperature for up to 30 minutes before use.
- Before starting, warm a thermal block to 37 ± 1°C to ensure the heat is stable and consistent. *(Optional)*: Start preheating pyrogen-free 96-well microplate.

Prepare endotoxin control stock solutions

Each vial of Quant-iT™ Lyophilized Endotoxin Standard contains 10–50 EU of lyophilized endotoxin from *E. coli* (0111:B4) (Component C). Lot-specific potency is printed on the label and also available on the CofA. Reconstitute the material with room temperature Quant-iT™ Endotoxin-Free Water (Component E) by adding the amount of endotoxin-free water indicated on the vial to make a 10 EU/mL solution. For example, reconstituting a vial with a potency of 15 EU with 1.5 mL of endotoxin-free water yields a concentration of 10 EU/mL.

1. Mix thoroughly by vortexing at 1,500 rpm or greater for at least 10 minutes.

   Note: Reconstituted stock solution is stable for 4 weeks at 2–8°C. Before subsequent use, warm the solution to room temperature and mix thoroughly by vortexing for 15 minutes to ensure that no endotoxin adheres to the sides of the glass vial.

2. Prepare Endotoxin Standards using pyrogen-free materials using the serial dilution described in Table 2.

   Note: For optimal results, mix thoroughly by vortexing each solution for at least 30 seconds before use in preparing the subsequent dilution.

Table 2 Serial dilutions for endotoxin standard stock solutions.

Standard	Endotoxin concentration	Previous dilution volume	Endotoxin-free water volume (Component E)
Standard 4	1.0 EU/mL	200 µL of reconstituted 10 EU/mL Endotoxin Standard (Component C) prepared in step 1	1,800 µL
Standard 3	0.1 EU/mL	200 µL of 1.0 EU/mL standard	1,800 µL
Standard 2	0.01 EU/mL	200 µL of 0.1 EU/mL standard	1,800 µL
Standard 1	0.00 EU/mL	0 µL	2,000 µL

Plate standards and samples

1. Add 50 µL of each endotoxin standard solution to a pyrogen-free 96-well microplate using the desired number of replicates (n ≥ 3 recommended). For optimal results, mix the samples vigorously before pipetting.

2. Add 5, 25, or 50 µL of each sample into the appropriate well. For best results, vigorously vortex each solution for 10–30 seconds before transfer. If using less than 50 µL of sample, first add endotoxin-free water, then the sample to the appropriate well to ensure that the final volume is 50 µL. See the following table for the effect of sample input on assay detection range.

Table 3 Sample volume input and the corresponding assay detection range.

Sample volume	Volume of endotoxin-free water to add	Assay range
50 µL	0 µL	0.01–1.0 EU/mL
25 µL	25 µL	0.02–2.0 EU/mL
5 µL	45 µL	0.1–10.0 EU/mL

- If needed, adjust the sample pH to 6–8 using endotoxin-free 0.1 M NaOH or 0.1 M HCl. Avoid pH-electrode contamination of the sample by testing the pH of a small sample taken from the bulk sample.
- Components of undiluted serum interfere in the assay. For example, serum must be completely free of red blood cells, and the diluted sample may need to be heat-shocked (70°C for 15 minutes). For more information about the assay tolerance for contaminants see “Contaminants and interfering factors” on page 13.
- To stop all bacteriological activity in test samples, store samples to be tested at 2–8°C for <24 hours or –20°C for >24 hours.

Note: Run each sample in replicate with dilution controls.

3. Preheat the 96-well plate containing the standards and samples at 37 ± 1°C for 10 minutes using a heat block.

Note: Use of cabinet-style incubators and water baths is not recommended because of possible improper heating or contamination.

4. Continue heating the 96-well microplate containing the standards and unknowns at 37 ± 1°C for 10 minutes using a heat block.

Prepare Assay Working Solution

1. Dissolve the Quant-iT™ Endotoxin Reagent (Component A) by adding 80 µL of the Quant-iT™ Endotoxin-Free DMSO (Component B). Mix thoroughly to fully dissolve the material.

   Note: Remaining reconstituted Quant-iT™ Endotoxin Reagent can be stored for 4 weeks at 2–8°C. Before subsequent use, warm the solution to room temperature and mix thoroughly to ensure homogeneity.

2. Reconstitute Quant-iT™ Lyophilized Amebocyte Lysate (Component D) using 4.4 mL of Quant-iT™ Endotoxin-Free Water (Component E). To mix, swirl gently to dissolve the powder. **Avoid foaming and do not vortex the solution.** If more than 1 vial is needed, pool 2 or more vials before use. For best results, let stand for 1–2 minutes after the first mixing to enable the solution to settle. Use within 10 minutes of preparation.

   Note: Ensure that you recover all powder from the sides and the cap of the vial by gently inverting end-over-end. Extreme care must be taken not to touch the inside part of the cap to avoid contamination.

   Note: Reconstituted Quant-iT™ Lyophilized Amebocyte Lysate (Component D) solution is stable for 1 week at –20°C or colder if frozen immediately after reconstitution. On thawing, the reconstituted lysate solution can be used only one time. Once thawed, gently swirl the reagent to mix before use.

3. Prepare the Assay Working Solution by adding 30 µL of the DMSO-Endotoxin Reagent Solution (from step 1) to the hydrated amebocyte lysate from step 2 to create the Assay Working Solution. Mix by inversion and do not shake because this can create unwanted bubbles.

   Note: If using multiple vials of the Quant-iT™ Lyophilized Amebocyte Lysate (Component D), add 30 µL of the DMSO-Endotoxin Reagent solution to each vial and pool the Assay Working Solutions before use to ensure a homogeneous solution. For optimal results, use the Assay Working Solution within 10 minutes of preparation and do not store for future use.

Start assay

1. Keeping all samples and standards at 37 ± 1°C, add 100 µL of the Assay Working Solution to each well. Start timing as the Assay Working Solution is added to the first sample. The lot-specific incubation time (T) is printed on the kit label and CofA (typically 20 ± 5 minutes).

   Note: Ensure pipetting order and rate of reagent addition remain consistent from well-to-well and row-to-row throughout the procedure. A repeater or multichannel pipette is recommended.

2. After the Assay Working Solution has been added to all standards and samples, thoroughly mix all samples and standards by tapping the plate several times or using a plate mixer.

3. After mixing, continue incubating the well plate at 37 ± 1°C for the lot-specific time (T), as indicated on the kit label and CofA.

   Note: For optimal results, do not immediately transfer into a plate reader because direct contact heating is essential for proper assay performance. If desired, transfer into a microplate reader preheated to 37 ± 1°C after 10 minutes of incubation on the direct contact heating block. Ensure the overall incubation time is closely monitored because this is critical to assay performance.

4. After the incubation time has elapsed, immediately add 50 µL of the Stop Solution (25% acetic acid in water) into each standard and sample well. For optimal results, add the Stop Solution to the standards and samples in the same order and at the same speed as the lysate reagent was added. Failure to stop the reaction can compromise the results.

5. Briefly mix the samples by tapping the plate several times or using a plate mixer.

Acquire and analyze data

1. Using a fluorescent plate reader to read the sample plate. Recommended settings include:
   - Ex/Em = 490 nm/525 nm
   - Bandwidth ±10 nm
   - Optimal gain

2. Use a software analysis program to obtain a regression for the standards. Various Pharmacopeia associations suggest performing this analysis using log-transformed concentration and RFU data, then performing a linear regression fit to ensure that the correlation coefficient (*r*) is ≥0.980. After this is verified, the best results for calculating the sample concentrations can be obtained using a log-transformation on the concentration and background-corrected RFUs using a quadratic curve fitting.

   Nonlinear curve fitting of the standards, such as 4PL or quadratic models, will provide the best results. However, performing a linear curve fitting can be done first to ensure that the correlation coefficient (*r*) is satisfactory before continuing to the quadratic curve fit.

   Table 4 Example of data processing using a nonlinear quadratic model.

Standard	Concentration	Average RFU	Corrected average RFU	log concentration	log RFU
STD1	0.00 EU/mL	8.4	0.0	—	—
STD2	0.01 EU/mL	15.1	6.7	–2	0.827
STD3	0.10 EU/mL	96.5	88.1	–1	1.945
STD4	1.00 EU/mL	614.5	606.1	0	2.783
Correlation coefficient (*r*) = 0.997					

3. Use the curve-fitting model to determine the unknown sample concentrations.

Example of data processing using log-transformed data from Table 4 and quadratic curve fitting.

Sample EU/mL =

$$\frac{10^{(-b+\sqrt{(b^2-4a(c-\log(sample-STD1)}}}{2a}$$

with a = –0.140, b = 0.697, and c = 2.783 being determined by the curve fitting of the standards.

Table 5 Example data results.

Standard	Concentration	Calculated concentration
STD1	0.00 EU/mL	—
STD2	0.01 EU/mL	0.010 EU/mL
STD3	0.10 EU/mL	0.100 EU/mL
STD4	1.00 EU/mL	1.000 EU/mL

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Troubleshooting

Observation	Possible cause	Recommended action
Non-linear standard curve with correlation coefficient below desired 0.980	Endotoxin Standard solution and dilutions were not mixed well.	Vortex the Endotoxin Standard solution for 15 minutes before each use.
		Vortex all Endotoxin Standard dilutions for 1–2 minutes before each use.
		Before adding into sample wells, vortex the Endotoxin Standard dilutions for 2 minutes if they were sitting for >10 minutes after preparation.
	Pipetting order and rate of reagent addition were not correct.	Ensure pipetting order and rate of reagent addition remain consistent from well-to-well.
		Use a repetitive or multichannel pipettor.
	Incubation times were not correct.	Ensure correct incubation times are used and start the timer when adding reagent into the first well.
Greater signal in blank than standard dilutions	Materials (for example, tips, vials, or microplate) were contaminated.	Use endotoxin-free materials.
Higher signal in sample than in standards	Test sample concentration was >1.0 EU/mL.	If the sample is too high, dilute the sample 5-fold in endotoxin-free water or use a lower sample volume. Repeat the test.

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Supplemental information

Contaminants and interfering factors

The presence of interfering substances in test samples can cause inhibition leading to false negatives. Common interfering substances include detergents, buffers, serum, or media (Table 6). If unknown whether the sample contains interfering substances, or how their presence affects the assay results, determine the potential product inhibition for each sample type undiluted or at an appropriate dilution. This type of test is often referred to as a Positive Product Control (PPC) or spike recovery test.

To confirm potential inhibition, add a known amount of endotoxin to an aliquot or test sample dilution (for example, 5 µL of the 1.0 EU/mL prepared standard). Assay the spiked sample and an unspiked sample to determine the respective endotoxin concentrations. Interference is said to be negligible if the recovery value is between 50–200%. If the recovery value is outside of that range, then use a sample dilution to reduce potential interference.

Table 6 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.

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%
Sucrose	1%	2%	10%
EDTA (pH 8.0)	1 mM	2 mM	10 mM

[1] Supplemented with Penicillin-Streptomycin-Glutamine (100x) diluted to 1X and containing phenol red.

[2] Dilutions in this row are expressed in the form of a ratio where 1:100 means a 100-fold dilution of contaminant to the standard samples.

[3] Sheared salmon sperm DNA (15632011)

[4] pBR322 (SD0041)

Sample dilutions

If samples require dilutions to be in the detection range of the assay, or to reduce interference factors identified by a PCC or spike recovery test, confirm the validity of that dilution by calculating the maximum valid dilution (MVD) or minimum valid concentration (MVC) of the sample. In either case, the endotoxin limit of the target (EU/unit) as well as the potency of sample or its concentration (units/mL) must be determined by the user or their application. For the Quant-iT™ Endotoxin Detection Assay Kit, the endotoxin limit is 0.01 EU/mL when using 50 µL or 0.1 EU/mL when using 5 µL samples.

For example, a plasmid preparation yields 1,000 µg/mL of DNA. The endotoxin limit of this assay is 0.010 EU/mL when a 50 µL sample is being tested. The target for the endotoxin limit in the sample is 0.1 EU/µg in their sample. The following equations are used to find the MVD and MVC values:

- MVD = (Endotoxin limit x Potency) / (Test sensitivity)
- MVC = (Test sensitivity) / (Endotoxin limit)

Entering the example values:

- MVD = (1,000 µg/mL) x (0.1 EU/µg) / (0.01 EU/mL) = 10,000
- MVC = (0.01 EU/mL) / (0.1 EU/µg) = 0.1 µg/mL

Quant-iT™ assay kits and accessories

Table 7 Bulk reagents and kits.

Product	Quantity	Cat. No.
Quant-iT™ PicoGreen™ dsDNA Assay Kit	1 mL assay kit 10 x 100 µL	P7589 P11496
Quant-iT™ PicoGreen™ dsDNA Reagent	1 mL reagent 10 x 100 µL	P7581 P11495
TE Buffer (20X), RNase-free	100 mL	T11493
Quant-iT™ RiboGreen™ RNA Assay Kit	1 mL assay kit	R11490
Quant-iT™ RiboGreen™ RNA Reagent	1 mL reagent	R11491
Quant-iT™ RediPlate™ 96 RiboGreen™ RNA Quantitation Kit	1 plate	R32700
Quant-iT™ OliGreen™ ssDNA Assay Kit	1 mL assay kit	O11492
Quant-iT™ OliGreen™ ssDNA Assay Reagent	1 mL reagent	O7582

Table 8 Microplate reader assays.

Product	Dynamic Range	Quantity	Cat. No.
Quant-iT™ 1X dsDNA Assay Kit, High Sensitivity	200 pg–100 ng	1,000 reactions	Q33232
Quant-iT™ 1X dsDNA Assay Kit, Broad-Range	4 ng–2 µg	1,000 reactions	Q33267
Quant-iT™ DNA Assay Kit, High Sensitivity	200 pg–100 ng	1,000 reactions	Q33120
Quant-iT™ DNA Assay Kit, Broad-Range	4 ng–1 µg	1,000 reactions	Q33130
Quant-iT™ RNA Assay Kit	5–100 ng	1,000 reactions	Q33140
Quant-iT™ RNA Reagent	5–100 ng	1,000 reactions	Q32884
Quant-iT™ RNA Assay Kit, Broad Range	20 ng–1 µg	1,000 reactions	Q10213
Quant-iT™ RNA XR Assay Kit	200 ng–10 µg	1,000 reactions	Q33225
Quant-iT™ microRNA Assay Kit	1–100 ng	1,000 reactions	Q32882
Quant-iT™ Protein Assay Kit	250 ng–5 µg	1,000 reactions	Q33210
Microplates for Fluorescence-based Assays, 96-well	—	10 plates	M33089

Table 9 Additional Qubit™ and Quant-iT™ products.

Product	Dynamic range	Quantity	Cat. No.
Qubit™ Flex Fluorometer	—	1 each	Q33327
Qubit™ Flex Endotoxin Starter Kit	—	1 each	Q32894
Qubit™ Flex SAE Software for 21 CFR Part 11 Support	—	1 each	Q31994
Qubit™ Endotoxin Detection Assay Kit	0.01–10 EU/mL	80 reactions	Q32891
Quant-iT™ Endotoxin Detection Assay Kit	0.01–10 EU/mL	160 reactions	Q32892
Qubit™ Flex Pyrogen-Free Assay Tube Strips	—	120 tube strips	Q32893

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Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

Documentation and support

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

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- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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