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# Evaluation of endotoxin contamination testing for LNP and AAV gene delivery systems

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## Key challenges and takeaways

- Evaluation of potential endotoxin contamination is often a required step prior to using LNP or AAV treatments in living models.
- Particle interference is a common concern for LNP and AAV sample types. The cause of which is likely electrostatic interactions with LPS (endotoxins) rather than the often-cited light scattering.
- Invitrogen™ Qubit™ and Quant-iT™ Endotoxin Detection Assays offer a user-centric approach with results that are consistent with other assays.
- Data indicates that *highly concentrated* LNPs and AAVs can interfere with endotoxin evaluation using the fluorescent Qubit and Quant-iT assays, but dilutions can effectively alleviate the issue.

Invitrogen™ Qubit™ Flex Fluorometer

## Methods and materials

AAV samples were produced using Gibco™ AAV-MAX Helper Free AAV Production System. Post-transfection 72 hours, cells were lysed to extract AAV particles and followed by purification using POROS™ CaptureSelect AAVX resin. AAV titer was measured using TaqMan™ qPCR assay to approximately $1 \times 10^{12}$ (vg/mL).

LNP samples were prepared using SM-102 core lipid and helper lipid mix in a molar ratio of 50:10:38.5:1.5 (core lipid: DSPC: Cholesterol: DMG-PEG2000). The lipid mix and fLuc mRNA were mixed at a 1:3 volume ratio to form LNPs on the Nanoassemblr® Ignite™ into either 1X PBS and stored at 4C or PBS (+) 10% sucrose at -80C until use. Additional details are in **Figure 1** below.

| Sample | Lipid content (mg/mL) | PDI | Size (nm) | Particles/mL | RNA (ng/mL) | Buffer Storage |
|---|---|---|---|---|---|---|
| LNP A | 1.55 (0.17%) | 0.041 | 97 | $3.6 \times 10^{12}$ | 155 | PBS 4C |
| LNP B | 0.78 (0.09%) | 0.069 | 132 | $7.7 \times 10^{11}$ | 155 | |
| LNP A+ | 1.55 (0.17%) | 0.041 | 97 | $3.5 \times 10^{12}$ | 116 | PBS + 10% sucrose -80C |
| LNP B+ | 0.78 (0.09%) | 0.069 | 132 | $7.4 \times 10^{11}$ | 116 | |

Endotoxin testing was performed with Qubit and Quant-iT endotoxin detection assays using a spike control test methodology in accordance with Pharmacopeia guidelines (USP 85/1085). Values were confirmed using a compendial assay test that uses LAL and a chromogenic response. Unless otherwise noted, results were consistent between the two test methods.

## Dilutions and release criteria

Pharmacopeia guidelines suggest ensuring that your dilutions are "valid" by confirming that the endotoxin levels in the sample are not being diluted past the assay detection limit. This can be determined through either 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.

Example. A 50 ng/uL (or 50,000 ng/mL) LNP sample needs to be diluted to avoid interference. Only 5 µL of sample can be used per test. The assay limit for this input is 0.1 EU/mL and an example release criteria is 5 EU/mL.

- MVD = (Endotoxin limit × Potency) / (Test sensitivity) → $0.1\frac{\text{EU}}{\text{mL}} \times 50{,}000\frac{\text{ng}}{\text{mL}} \div 5\frac{\text{EU}}{\text{mL}} = 1{,}000$
- MVC = (Test sensitivity) / (Endotoxin limit) → $5\frac{EM}{\text{mL}} \div 0.1\frac{\text{EU}}{\text{mL}}$ = 50 ng/mL or 0.5 ng/µL

## Qubit Flex Endotoxin Detection Assay

**Figure 2. Streamlined endotoxin assay workflow.** 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 µL.

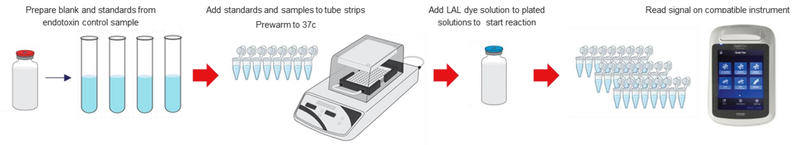
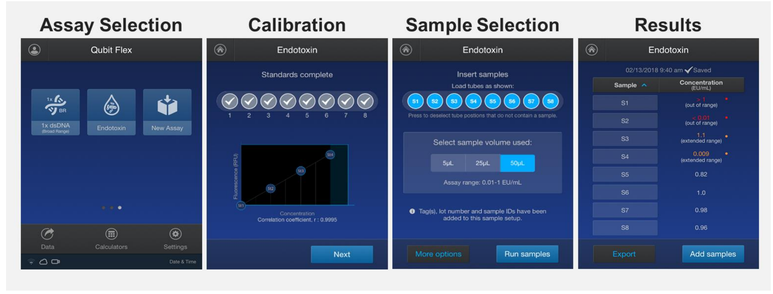
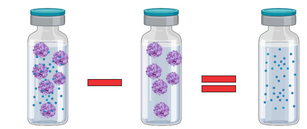



**Figure 3. Qubit Flex instrument workflow.** The endotoxin assay is selected from the home page of the Qubit Flex Fluorometer. Standards are used to generate a 4-point standard curve that is used to measure endotoxin concentration. Up to 8 samples can be measured at a given time using Qubit Flex Pyrogen Free Assay Tube Strips. Endotoxin levels are reported on the instrument with alerts if values fall out of the assay detection range. The assay is also compatible with fluorescence microplate readers using the Invitrogen™ Quant-iT™ Endotoxin Detection Assay.




## Interference testing validation with spike tests

**Figure 4. An overview of sample-spike testing.** Outlined by various Pharmacopeia organizations, sample-spike testing is the preferred method to evaluate sample interference. In brief, this test evaluates for interfering substances potentially present in the sample matrix by comparing controls to samples 'spiked' with a known amount of endotoxin.



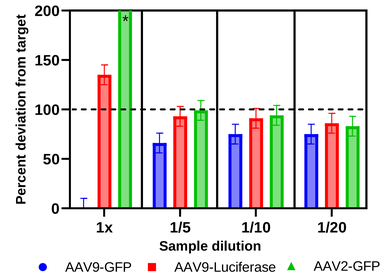
A. Sample + known endotoxin | B. Sample only (unknown endotoxin levels) | C. Endotoxin only (reference)

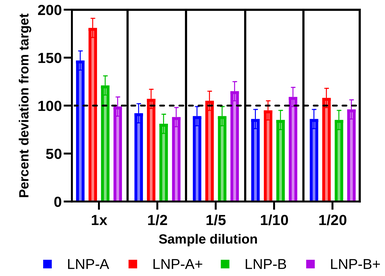
$$\frac{(Sample\ spiked\ with\ endotoxin\ reference) - (Sample\ only)}{Endotoxin\ reference} = 50 - 200\%$$

The spiked amount of endotoxin is dependent on the testing method specified at half of the observable test range to obtain maximum sensitivity. For this assay, that value is 0.1 EU/mL. Any deviation outside of 50-200% from expected is considered to interfere, however this is more ideally suited to 75-125%. Testing of LNP and AAV samples was performed at various dilutions using the Qubit and Quant-iT endotoxin detection assay.

## Interference testing

**Figure 5.** Interference testing for AAV and LNP samples. Spike recovery tests were performed in replicates using 5 µL of sample and 50 µL of 0.1 EU/mL endotoxin reference controls. Sample dilutions were prepared in endotoxin free water using pyrogen-free materials.




**AAV samples (left)** suggest divergent interference patters for undiluted samples. This was observed with AAV9 and AAV2 samples, suggesting that the interference is not specified to this designation. Once diluted, sample interference is then successfully and consistently mitigated. The AAV buffer was tested by itself (data not shown) at 1X and determined to not interfere with results (90-110%).

**LNP samples (right)** suggest inconsistent interference for undiluted samples. Once diluted, sample interference is then successfully and consistently mitigated. Interference was not seemingly affected by the contents of the buffer, PBS vs PBS with 10% sucrose (+ vs otherwise), or the increased lipid content (A vs B sample types).

**Overall**, this testing suggests that most AAV or LNP samples should be diluted $^1/_2$ - $^1/_5$ to mitigate potential interference unless otherwise established. For cell and gene therapy applications, this is well within the valid dilution range and the endotoxin testing results are well below common endotoxin release thresholds.

## Conclusion

Here, 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 while showing the validation process for endotoxin testing of LNP and AAV sample types. Results of the subset of samples tested suggest that high concentrations of particles can interfere with the endotoxin determination. To negate potential interference, utilization of valid dilutions is demonstrated to be effective and consistent. Other efforts, such as use of Triton X100, BSA, and buffer were inconclusive and inconsistent.

## Additional resources

For additional information on Qubit and Quant-it Endotoxin Detection Assay Kits, please visit thermofisher.com/qubit or thermofisher.com/endotoxin. Purified AAV particles were prepared by Cell Biology Custom Services, Thermo Fisher Scientific in Carlsbad, CA. For additional information on services contact GEMservices@lifetech.com. For more information about other transfection services visit thermofisher.com/Vivofectamine.



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