Endotoxin & Depyrogenation Tips

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Endotoxin and Depyrogenation Tips

Endotoxin is a common contaminant of many of the formulations submitted to the NCL. In fact, nearly one-third of formulations submitted to the NCL have levels exceeding recommended limits. However, most labs can successfully overcome this hurdle and reduce endotoxin contamination by taking a few precautionary steps during the synthesis and purification procedure.

NCL has collected the attached recommendations and references to aid you in reducing endotoxin contamination. Also, consider detailing your synthetic procedure, noting every step, reagent, reaction vessel and other materials coming into contact with the sample, and handling procedure; this is often helpful to highlight steps where endotoxin can be introduced.

Please note that the methods and kits summarized below are not universal and may not work equally well for all types of nanomaterials. Applying your best knowledge of the nanoparticle formulation, select a product which would be optimal for the given nanoparticle. An educated guess is a good start, but experimentation and empirical confirmation of the optimal performance will be needed. For example, this reference (Nanomedicine (Lond). 2010;5(4):555-62. doi: 10.2217/nnm.10.29) used a Triton X-114 procedure originally described for proteins and found that it works well for endotoxin removal from polymer-based nanoparticles. Please also note, that after purification or depyrogenation, several key physicochemical characterization assays should be conducted to confirm the particle's integrity (e.g., size, zeta potential, PEG density, drug loading) and concentration. (It is expected that some amount of particles will be lost during the purification.)

The NCL does not endorse any of the commercial suppliers mentioned herein. They are provided for informational purposes only. Alternate supplies from other vendors may be substituted.

Recommendations for Minimizing Endotoxin Contamination

1. Wear gloves, spray gloves with Cavicide before touching tubes/reagents etc., and change gloves often during the process.
2. Use pyrogen-free reagents and water (e.g., Lonza LAL grade water, ACC Pyroclear certified LAL reagent water, or other cell culture grade water).
3. When organic solvents are used (e.g. DMSO), it is better to use cell culture grade (e.g. Hybri-Max) as they are endotoxin free.
4. Use sterile, depyrogenated glassware, pipets and other consumables, reagents and equipment. Whenever possible use disposable tools.
5. To depyrogenate glassware and other tools which can tolerate high temperatures, bake them at 230°C for 2 hours or at 200°C overnight. **Note**: Autoclaving *sterilizes* glassware/tools but does *not depyrogenate* it.

6. To depyrogenate plastic tubing, stir bars and other tools which cannot tolerate baking, flush with Cavicide, then thoroughly rinse with sterile, endotoxin-free water. Running acetonitrile through an HPLC system following sterile, depyrogenated water is also helpful to remove any endotoxin stuck to surfaces inside the instrument.
7. Ideally, the entire synthesis should be conducted in a laminar flow hood.
8. Avoid using cellulose-based filters, as these serve as a source of beta-glucans, which can interfere with the LAL assay and generate false-positive results. While the FDA does not regulate beta-glucan content in devices and pharmaceutical products, there is scientific evidence suggesting that beta-glucans can be pro-inflammatory and exaggerate inflammation triggered by other inflammatory substances including endotoxin. Some nanoparticles (especially those containing cationic moieties) can also exaggerate endotoxin-mediated inflammation. This is another reason to avoid beta-glucan contamination in your product.
9. Avoid breathing, coughing and sneezing into the tubes/reaction beakers. Avoid touching your face; wash hands and change gloves immediately if you touch a non-sterile surface. Although these precautions may sound silly, they are a common source of contamination.

Sterility and Endotoxin Test Procedures

The sterility and endotoxin protocols used by the NCL are available on our website: https://ncl.cancer.gov/resources/assay-cascade-protocols

Some of NCL's LAL assays require the Pyros Kinetix or PyrosFlex instruments. If these instruments are unavailable, other reagents and/or kits can be used, e.g. Lonza and ACC endotoxin assay reagents or kits. One of the most easily adapted protocols is the end-point chromogenic LAL protocol, found here: https://ncl.cancer.gov/sites/default/files/protocols/NCL_Method_STE-1.1.pdf. This protocol is based on the QCL-1000 kit (Lonza Corp) and USP standard 85 (found here).

Importantly, the maximum valid dilution (MVD) for commercial kits or reagents may be different from the MVD calculated for NCL assays, because the sensitivity (lambda) of the assays may be different from our protocols. Please refer to our protocols or USP BET 85 for directions on calculating the MVD.

Common Depyrogenation Methods

1. Baking: 230 °C for at least 30 min or overnight at 200 °C; works great for all glassware, metallic supplies and any component which can tolerate high temperature.
2. Ethylene oxide sterilization: 12% ethylene oxide, 88% Freon, 50% humidity and 3.5 psig for 6.5 hr
3. Acid hydrolysis: 0.05N HCl for 30 min at 100°C
4. Alkylation: 0.25N NaOH at 56 °C for 1 hr
5. Alkylation: 0.1N NaOH in either 95% ethanol or in 80% DMSO for at least 1 hr
6. Acid hydrolysis: 1% glacial acetic acid for 2-3 hr at 100°C
7. Gamma irradiation (from 60 Co source)
8. Hydrogen peroxide gas-plasma sterilization (Sterrad 100 S sterilizer)
9. Soft hydrothermal process (requires special equipment): http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2725499/pdf/0122-09.pdf

Commercial Reagents, Kits and Services for Endotoxin Removal

1. Pall's Mustang E filters: https://shop.pall.com/us/en/laboratory/sterile-filtration-and-clarification/endotoxin-removal/acrodisc-units-with-mustang-e-membrane-zidgri78lpj
2. ThermoFisher's spin columns: https://www.thermofisher.com/order/catalog/product/88274

   and resins https://www.fishersci.com/shop/products/thermo-scientific-pierce-high-capacity-endotoxin-removal-resin-columns-resin-slurry-10ml/pi88270
3. Sigma's endotoxin removal solution: https://www.sigmaaldrich.com/catalog/product/sigma/e4274?lang=en&region=US&gclid=EAIaIQobChMIxfr9xfOH3AIVDrnACh21BgYTEAAYAyAAEgLQ_fD_BwE
4. GenScript's endotoxin removal resin: https://www.genscript.com/kit/L00402-ToxinEraser_sup_TM_sup_Endotoxin_Removal_Resin.html
5. BioRad's Proteus endotoxin removal kits: https://www.bio-rad-antibodies.com/proteus-endotoxin-removal-kits.html
6. CRO specializing in endotoxin removal and testing: https://www.creative-biogene.com/Services/Endotoxin-Detection-Removal.html?gclid=EAIaIQobChMImpjJjtyX3AIVUTuBCh1BrwRLEAMYASAAEgKBSPD_BwE
7. CRO specializing in endotoxin removal and testing: http://www.arvysproteins.com/EndotoxinRemoval.html?gclid=EAIaIQobChMIwImOnd2X3AIV2BKBCh221QJ8EAAYAiAAEgLzv_D_BwE

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Please also see references authored by NCL, listed on our website: https://ncl.cancer.gov/resources/ncl-scientific-bibliography#block-views-be9a2b20b4b702cba70125f859fb92c3

Additionally, NCL has published a video protocol on "Detection of Endotoxin in Nano-formulations Using Limulus Amoebocyte Lysate (LAL) Assays" in the Journal of Visualized Experiments (JoVE, **2019**, 143, e58830, https://www.jove.com/t/58830/detection-endotoxin-nano-formulations-using-limulus-amoebocyte-lysate)

Contact

For more information or to discuss questions pertaining to your specific nanoformulation, please reach out to NCL's Head of Immunology, Dr. Marina Dobrovolskaia:

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