Technical Report

Cellular Delivery of siRNA & ASO
In vivo Grade Oligo Production, Endotoxin Testing, and Lipid Modification Interference
siRNA, ASO, and Lipid-Conjugated Oligonucleotides
Research to Preclinical Transition
FDA Guidelines — ≤5 EU/mL Endotoxin Threshold
LAL Assay Interference, False-Positive and False-Negative Risks, and Mitigation Strategies

EndoSmart™ *In vivo* Grade Oligos: Endotoxin/LPS Tested

Endotoxin/LPS Tested siRNA and Antisense Oligonucleotides

27-6490-XX [in vivo grade oligos endotoxin LPS tested]

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Endotoxin/LPS Tested siRNA and Antisense Oligonucleotides

for *In vivo* Preclinical Research Applications

Including Lipid-Modified Oligonucleotides: LAL Assay Interference, False-Positive/False-Negative Risks, and Mitigation Strategies

1. Introduction and Background

The use of synthetic oligonucleotides, including small interfering RNA (siRNA) and antisense oligonucleotides (ASO) *in vivo* biological research has expanded substantially over the past two decades. These modalities offer precise gene-silencing and transcript-modulation capabilities, making them valuable tools in both basic research and translational drug discovery. However, successful and reproducible *in vivo* experimentation with synthetic oligonucleotides depends critically on the purity and biological safety profile of the administered materials [1].

A primary and often overlooked concern in the preparation of oligos intended for *in vivo* use is contamination by bacterial endotoxins, specifically lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. Endotoxin contamination at even sub-nanogram concentrations can trigger potent innate immune responses in mammals, confounding experimental results, inducing non-specific inflammatory signaling, and in some cases causing animal morbidity or mortality [2].

The analytical challenge is further compounded when oligonucleotides bear lipid conjugates or are formulated with lipid-based delivery systems. Lipid moieties, including cholesterol, fatty acid chains (C16 palmitoyl, C18 stearoyl, C18:1 oleyl), tocopherol, and components of lipid nanoparticles (LNPs) are inherently amphiphilic and can interact directly with the LAL enzyme cascade, thereby producing both false-positive and false-negative endotoxin test results. This report examines these analytical challenges and outlines practical considerations for valid endotoxin testing of lipid-modified oligonucleotide preparations.

Gene Link's *in vivo* grade oligonucleotide production process is described as a reference model for research-grade materials intended for preclinical study preparation, including considerations specific to lipid-conjugated oligos.

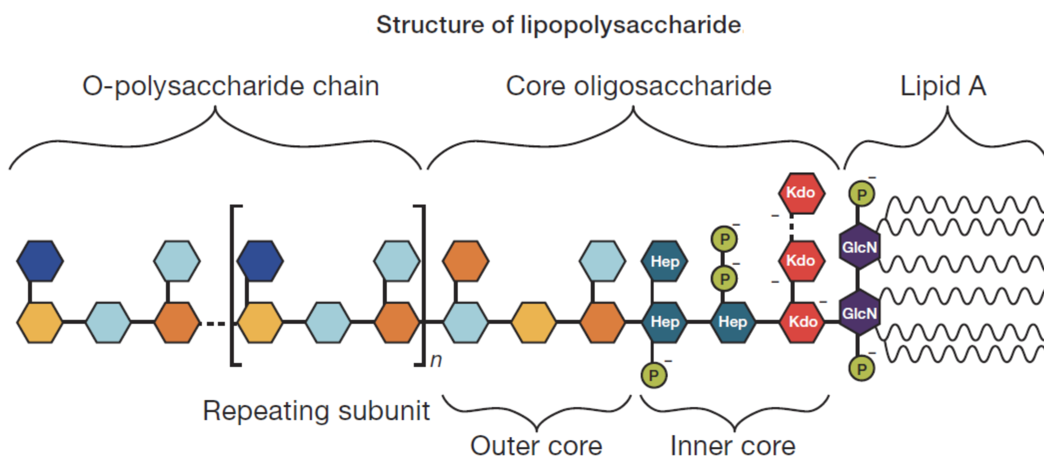
2. Endotoxins and Their Relevance to Oligonucleotide Research

2.1 Biology and Source of Endotoxin

Lipopolysaccharide (LPS), commonly referred to as endotoxin, is a glycolipid present in the outer cell membrane of Gram-negative bacteria such as Escherichia coli, Salmonella, and Pseudomonas species. LPS is composed of three structural regions: lipid A (the bioactive, toxic component), core

oligosaccharide, and O-antigen polysaccharide [5]. The lipid A moiety activates toll-like receptor 4 (TLR4) on innate immune cells, triggering release of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, and interferons [2].

In laboratory settings, endotoxin contamination arises from bacterial presence in water, glassware, reagents, and manufacturing environments. Synthetic oligonucleotide synthesis is conducted in organic solvents and controlled environments, but the final aqueous reconstitution and purification steps introduce risk of endotoxin contamination if endotoxin-free reagents and equipment are not employed throughout.



Endotoxins are complex lipopolysaccharides, which are biologically active structural components of the outer cell membrane of gram-negative bacteria. They consist of a core oligosaccharide chain, O-specific polysaccharide side chain (O-antigen), and a lipid component, lipid A, which is responsible for the toxic effects.

2.2 Biological Consequences of Endotoxin Contamination in Animal Studies

Endotoxin contamination in oligonucleotide preparations administered to animal models can have far-reaching biological consequences that obscure experimental interpretation:

- Activation of the innate immune system via TLR4 signaling, resulting in systemic inflammation independent of the oligo's intended mechanism of action [2].
- Induction of interferon-stimulated genes (ISGs), potentially confounding gene expression studies or RNAi knockdown efficiency assessments [2].
- Hepatic and renal toxicity at higher endotoxin doses, complicating safety profiling of therapeutic candidates [6].
- Altered pharmacokinetics due to cytokine-mediated changes in liver enzyme activity and organ perfusion.
- Septic shock in sensitive animal strains or immunocompromised models at endotoxin concentrations as low as 1 EU/kg body weight [6].

2.3 FDA Endotoxin Limits and Regulatory Framework

The U.S. Food and Drug Administration (FDA) has established endotoxin limits for injectable products under 21 CFR and associated guidance documents [6]. For non-pyrogenic injectable formulations, the general limit is 5 EU/mL. For research-grade materials used in preclinical *in vivo* animal studies, the same threshold of ≤5 EU/mL is widely adopted. Gene Link's *in vivo* grade oligonucleotide production process is calibrated to this FDA-referenced standard, with a certification criterion of <5 EU/mL as measured by chromogenic LAL endotoxin assay at the final product stage [7].

3. Limulus Amebocyte Lysate (LAL) Testing: Principles and Methods

3.1 History and Mechanism of LAL Testing

The Limulus Amebocyte Lysate (LAL) assay is the gold standard method for endotoxin detection, derived from the blood cells (amebocytes) of the horseshoe crab (Limulus polyphemus) [5,8]. Upon contact with endotoxin, a serine protease cascade is activated within the LAL reagent, culminating in the cleavage of a chromogenic or turbidimetric substrate. This biological cascade provides exquisite sensitivity to LPS, with detection limits as low as 0.001 EU/mL in optimized kinetic formats [5].

3.2 Charles River Endosafe nexgen-PTS Platform

The Endosafe nexgen-PTS (Portable Test System) is an FDA-licensed, kinetic chromogenic LAL-based instrument developed by Charles River Laboratories for rapid, point-of-use endotoxin testing [9]. It integrates sample analysis, reagent dispensing, and result quantification into a single cartridge-based format with minimal hands-on time.

3.2.1 Instrument Design and Cartridge Technology

The nexgen-PTS utilizes single-use, pre-loaded test cartridges containing lyophilized LAL reagent and chromogenic substrate [9]. Each cartridge contains four channels: two for sample analysis and two for built-in spike controls, enabling concurrent sample quantification and spike recovery validation in a single test run. This design eliminates the need for separate standard curve preparation and reduces technician variability.

3.2.2 Kinetic Chromogenic Detection Principle

In the kinetic chromogenic format, endotoxin activates the LAL clotting cascade, which cleaves the synthetic chromogenic substrate pNA (para-nitroaniline) from Boc-Leu-Gly-Arg-pNA [9,5]. The release of yellow pNA is measured photometrically at 405 nm. The reaction is monitored kinetically, with the onset time of color development inversely correlated with endotoxin concentration.

3.2.3 Performance Characteristics

Parameter	Performance Specification
Detection Range	0.001 – 100 EU/mL (cartridge-dependent)
Test Time	~15–20 minutes per cartridge
Sample Volume	25 µL per channel
Spike Recovery Criteria	50–200% (standard acceptance)
Sensitivity (PTS20 cartridge)	0.005 EU/mL
Regulatory Status	FDA-licensed for lot release testing
Interference Check	Built-in spike control per cartridge
Data Output	EU/mL with pass/fail interpretation

3.2.4 Advantages Over Conventional LAL Methods

- Rapid turnaround: results within 15–20 minutes versus 60+ minutes for tube-based methods.
- Integrated spike controls: simultaneous endotoxin recovery validation.
- Small sample volume: 25 µL per channel, critical for low-yield oligonucleotide samples.
- Audit-ready data logging with time-stamped, operator-linked records.
- Minimal training requirement and reduced contamination risk.

4. Gene Link *In vivo* Grade Oligonucleotide Production Process

Gene Link, Inc. provides research-grade oligonucleotides specifically manufactured and quality-controlled for *in vivo* biological applications. Gene Link recommends *in vivo* grade oligos for antisense oligonucleotides (ASO) or siRNA testing at a research level before entering preclinical studies. The production pipeline integrates endotoxin control at every stage of synthesis, processing, purification, QC analysis, normalization to final shipping.

4.1 Endotoxin Testing and Certification

All stages of the *in vivo* oligo production process are subjected to endotoxin testing and certified either to be below the limit of detection or to fall within FDA guideline thresholds, with a final concentration specification of <5 EU/mL as determined by a chromogenic LAL assay. All *in vivo* oligos are shipped dried (lyophilized) and, upon request, may be supplied at a normalized reconstituted concentration in endotoxin-free water or TE buffer. *In vivo* oligonucleotides are not certified as sterile.

4.2 Quality Grade Levels and Purification Methods

Quality Grade	Purification Method	Final Filtration	Recommended Use
Desalted	Gel filtration / size-exclusion chromatography	0.22 µm prior to lyophilization	Short oligos, basic *in vivo* knockdown studies
Reverse Phase Purified (RPC)	Reverse phase chromatography (C18)	0.22 µm prior to lyophilization	Higher purity siRNA duplexes
PAGE Purified	Polyacrylamide gel electrophoresis & desalting	0.22 µm prior to lyophilization	Full-length enriched; critical preclinical work

4.2.1 Desalted Grade

All desalted grade *in vivo* oligos are desalted to remove residual salts by gel filtration/size-exclusion chromatography. The final step is filtration using a 0.22 µm filter prior to lyophilization.

4.2.2 Reverse Phase Purified (RPC) Grade

All RPC grade *in vivo* oligos, after RPC purification, are filtered using a 0.22 µm filter prior to lyophilization. RPC effectively removes truncated failure sequences, free nucleotides, and hydrophilic synthesis byproducts.

4.2.3 PAGE Purified Grade

All PAGE purified grade *in vivo* oligos, after gel purification and desalting, are filtered using a 0.22 µm filter prior to lyophilization. PAGE purification provides the highest resolution for full-length sequence enrichment and is recommended for IND-enabling preclinical stages.

5. Lipid-Modified Oligonucleotides: Delivery Chemistry and LAL Assay Interference

The conjugation of lipid moieties to oligonucleotides is a widely used strategy to enhance cellular delivery, pharmacokinetic stability, tissue targeting, and endosomal escape. However, the amphiphilic character of lipid-conjugated oligos introduces a significant analytical complication: many lipid modifications can directly interfere with the LAL enzyme cascade, producing either false-positive (elevated apparent endotoxin) or false-negative (suppressed or undetected endotoxin) readings. Understanding the mechanism and magnitude of this interference is critical for designing valid endotoxin testing protocols for lipid-modified *in vivo* grade oligonucleotide preparations.

Click to view list of Gene Link Cellular Delivery Modifications

Click to view list of Gene Link ASO & siRNA Modifications

5.1 Overview of Common Lipid Modifications Used for siRNA/ASO Delivery

Lipid conjugation strategies for oligonucleotides span a range of chemical classes, each with distinct physicochemical properties relevant to LAL assay behavior:

5.1.1 Cholesterol Conjugates

Cholesterol is one of the most extensively studied and clinically validated lipid modifications for siRNA delivery. Attachment via a C3-OH linker to the 3′ end of the sense (passenger) strand greatly improves cellular uptake through interaction with lipoprotein particles (LDL, HDL) and membrane cholesterol microdomains (lipid rafts) [10]. Cholesterol-siRNA conjugates (e.g., siRNA-Chol) have been used in numerous *in vivo* studies targeting the liver, CNS, and muscle [10]. However, cholesterol is a sterol with well-characterized surfactant-like behavior at lipid-water interfaces and presents a significant risk of LAL cascade disruption (see Section 5.3).

5.1.2 Saturated Fatty Acid Conjugates: C16 (Palmitoyl) and C18 (Stearoyl)

C16 palmitoyl and C18 stearoyl conjugates are attached to the 5′ end of the sense strand or to the 2′ position of internal residues via phosphodiester-compatible linker chemistries. These saturated long-chain fatty acids promote membrane anchoring, improve albumin-mediated circulation time, and facilitate liver-targeted delivery via scavenger receptor pathways. C16, C18 and C22 modifications create a strongly hydrophobic tail that can cause micelle or aggregate formation in aqueous reconstitution buffers, directly impacting sample homogeneity and LAL reagent access.

5.1.3 Unsaturated Fatty Acid Conjugates: C18:1 (Oleyl/Oleoyl)

The monounsaturated C18:1 oleyl chain (cis-9-octadecenoic acid) is commonly used in place of saturated C18 because the double bond reduces tight lipid packing, enhancing membrane fluidity and endosomal membrane disruption. Oleyl conjugates are a component in several LNP and lipoplex formulations. The C18:1 kink introduced by the cis double bond still confers strong hydrophobicity but modifies the aggregate morphology compared to saturated C18, with implications for LAL interference (see Section 5.3.3).

5.1.4 Tocopherol (Vitamin E) Conjugates

Alpha-tocopherol conjugated siRNA was pioneered for hepatocyte-targeted delivery via the apolipoprotein E (ApoE)/LDL receptor pathway [11]. Tocopherol has an isoprenoid lipid tail and a chroman ring head group and is highly lipophilic (log P ~10). Tocopherol-siRNA conjugates can form compact nanostructures with surfactant-like behavior and are a documented source of LAL interference [3]. Like cholesterol, the chroman ring system and polyisoprene tail interact with hydrophobic enzyme binding pockets.

5.1.5 Docosanoic Acid (C22, Docosyl/Behenyl) and Other Long-Chain Conjugates

Longer saturated chains such as C20 (arachidic), C22 (behenic/docosanoic), and C24 (tetracosanoic/lignoceric) have been explored for enhanced receptor-mediated uptake and lymph node targeting following subcutaneous administration. These very long chain fatty acids are poorly soluble in aqueous buffers and may precipitate or partition into micelles at concentrations relevant to LAL testing, causing sample matrix heterogeneity.

5.1.6 Lipid Nanoparticle (LNP) and Lipoplex Formulations

Lipid nanoparticles are the dominant clinical delivery vehicle for therapeutic siRNA (e.g., patisiran/ONPATTRO) [12,13]. LNPs contain four primary lipid components: an ionizable or cationic lipid (e.g., DLin-MC3-DMA, DLin-KC2-DMA, C12-200), a helper lipid (DOPE or DSPC), cholesterol, and PEG-lipid (e.g., PEG2000-C-DMG or PEG-DSPE) [12]. This complex lipid mixture creates a nanoparticulate matrix that severely complicates LAL testing: the LNP surface sequesters both endotoxin and LAL enzymes, particle surfaces scatter 405 nm light (interfering with chromogenic readout), and individual LNP components (particularly ionizable lipids and cholesterol) each independently interact with the LAL cascade [4].

5.1.7 GalNAc Conjugates with Lipid Linker Regions

N-Acetylgalactosamine (GalNAc) clusters are the leading liver-targeting motif for clinical ASO and siRNA development, exploiting the asialoglycoprotein receptor (ASGPR). While GalNAc itself is a carbohydrate and does not directly interfere with LAL, several commercial GalNAc-siRNA platforms incorporate lipid spacer or linker domains (e.g., C6 or C12 aliphatic linkers, or phospholipid-mimetic spacers) that can contribute to micellar behavior at high concentrations. The endotoxin testing of GalNAc-conjugated oligos should be evaluated for matrix effects at each concentration used.

5.1.8 Peptide-Lipid and Polymer-Lipid Hybrid Modifications

Cell-penetrating peptide (CPP) conjugates that include lipidated peptides (e.g., stearylated penetratin, myristoylated Tat peptide) are used for CNS and muscle delivery. Similarly, polyethylene glycol (PEG)-lipid conjugates improve circulation time and reduce non-specific uptake. Both CPP-lipid and PEG-lipid modifications can introduce amphiphilic behavior that inhibits or enhances the LAL clotting cascade in a concentration-dependent manner.

5.2 Structural Basis of Lipid Interference with the LAL Cascade

To understand why lipid modifications, interfere with LAL-based endotoxin testing, it is necessary to consider the structural mechanism of LPS detection and the sites at which lipophilic compounds can disrupt it.

The LAL cascade is initiated when LPS, specifically the lipid A domain, binds to Factor C, a serine protease zymogen in the horseshoe crab hemocyte lysate [5,3]. Lipid A binds to Factor C via hydrophobic and electrostatic interactions with the lipid A's fatty acid chains and negatively charged phosphate groups. This cascade proceeds: Factor C → activated Factor C → Factor B activation → pro-clotting enzyme activation → coagulin gel (gel-clot) or chromogenic substrate cleavage (chromogenic formats) [5,8].

Lipid modifications on oligonucleotides can interfere at multiple points in this cascade through several distinct mechanisms:

5.2.1 Competitive Binding to Factor C (False Negatives — Inhibition)

Structurally, the lipid A moiety of LPS presents two phosphorylated glucosamine residues bearing four to six fatty acyl chains. The Factor C binding site accommodates this specific lipid topology [5,3]. Exogenous lipid tails conjugated to oligonucleotides, particularly saturated or monounsaturated long-chain fatty acids (C16, C18, C18:1) can competitively occupy the Factor C hydrophobic binding groove, preventing LPS from initiating the cascade. This produces a false-negative result: the sample contains endotoxin, but the LAL assay fails to detect it because the lipid-conjugated oligo has occupied the receptor site [3]. The spike recovery control will show suppressed recovery (<50%), flagging the inhibition if the testing protocol is correctly executed [5].

5.2.2 Detergent-Like Disruption of the Cascade Enzymes (Biphasic Effects)

Cholesterol, tocopherol, and C16/C18 fatty acid conjugates all exhibit surfactant properties above their critical micelle concentration (CMC) [3,4]. At concentrations above CMC, these lipid structures form micelles or mixed micelles that can solubilize and partially denature the serine proteases of the LAL cascade (Factor C, Factor B, pro-clotting enzyme). This denaturation-mediated enzyme inactivation produces inhibition of the chromogenic signal, i.e., false negatives. Below CMC, the monomeric lipid may activate Factor C weakly via structural mimicry of lipid A, potentially contributing to elevated baseline signal (false positives) [3].

5.2.3 Optical Interference at 405 nm (False Positives in Chromogenic Assays)

Lipid nanoparticles, lipoplexes, and highly aggregated lipid-oligo conjugates scatter visible light across a broad spectrum that includes 405 nm — the detection wavelength of the kinetic chromogenic LAL assay [14,4]. This Mie scattering contribution increases the measured absorbance independently of pNA release, generating an apparent positive signal that is not due to endotoxin. This is a particularly insidious false-positive mechanism because it may not be corrected by sample dilution in a simple manner and may not be fully mitigated by standard spike recovery testing [4]. Turbid LNP formulations require specialized approaches (see Section 5.4).

5.2.4 Endotoxin Sequestration into Lipid Aggregates (False Negatives)

Cholesterol-bearing, C16-, C18-, and LNP-formulated oligonucleotides can sequester LPS molecules into hydrophobic lipid aggregates or within lipid nanoparticle cores [3,4]. Sequestered LPS is physically inaccessible to Factor C in the aqueous phase, producing a false-negative result even when endotoxin is present. This mechanism is especially relevant for LNP-encapsulated siRNA preparations, where the multi-layered lipid shell provides substantial sequestration capacity [4]. Pre-treatment strategies to disrupt lipid aggregation are required before LAL testing of these samples.

5.2.5 pH and Ionic Strength Perturbation

Some lipid-conjugated oligo formulations contain ionizable lipid components (e.g., DLin-MC3-DMA, pKa ~6.4) that require low-pH buffers for encapsulation and high-pH buffers for endosomal escape [12]. Residual acidic or basic buffer components can shift the assay pH outside the LAL-optimal range (6.0–8.0), perturbing enzyme kinetics and producing both inhibitory and enhancing effects on the chromogenic signal [5].

5.3 Lipid Modification-Specific Interference Profiles

Lipid Modification	Primary Interference Mechanism	Effect on LAL Signal	Risk Level
Cholesterol (Chol)	Factor C competitive binding + detergent disruption + LPS sequestration	Inhibition (false negative) predominant; false positive at low conc.	HIGH
C16 Palmitoyl	Factor C competitive binding; detergent disruption above CMC	Inhibition (false negative); CMC-dependent	HIGH
C18 Stearoyl	Factor C competitive binding; poor aqueous solubility causes aggregation	Inhibition (false negative); aggregation may also sequester LPS	HIGH
C18:1 Oleyl/Oleoyl	Micellar disruption of serine proteases; less rigid than C18	Inhibition (false negative); less severe than C18 at equivalent conc.	MODERATE–HIGH
C22 Docosanoyl	Very low CMC; near-complete aggregation at testing concentrations	Severe inhibition (false negative)	HIGH

Lipid Modification	Primary Interference Mechanism	Effect on LAL Signal	Risk Level
Tocopherol (α-Toc)	Chroman ring + isoprenoid tail: Factor C binding; enzyme denaturation	Inhibition (false negative); may cause false positive at sub-inhibitory conc.	HIGH
LNP (ionizable lipid + Chol + DOPE/DSPC + PEG-lipid)	Multi-mechanism: optical scattering + LPS sequestration + Factor C disruption	False positive (optical) + false negative (sequestration): unpredictable	VERY HIGH
GalNAc + C6/C12 linker	C6/C12 aliphatic linker: weak hydrophobic effect; minimal at low conc.	Low-level inhibition at high oligo concentration	LOW–MODERATE
PEG-lipid (e.g., PEG-DSPE)	PEG shell reduces aggregation; DSPE lipid tail causes mild inhibition	Mild inhibition; PEG corona may reduce Factor C access to LPS	MODERATE
Stearylated CPP (e.g., stearyl-Arg8)	C18 stearyl tail: detergent disruption + cationic headgroup electrostatic effects	Inhibition + potential enhancement; highly variable	HIGH
Myristoyl (C14)	Shorter chain; moderate CMC; milder than C16/C18	Mild-to-moderate inhibition	MODERATE
Docosahexaenoic acid (DHA, C22:6)	Highly unsaturated; oxidation-prone; reactive species may activate cascade non-specifically	Enhancement (false positive) risk from oxidative products	MODERATE

⚠ **ASSAY INTERFERENCE WARNING:** Cholesterol-, C16-, C18-, tocopherol-, and LNP-conjugated oligonucleotides carry a HIGH to VERY HIGH risk of producing invalid LAL endotoxin results if standard testing procedures are applied without interference mitigation. Standard spike recovery testing alone is insufficient for turbid LNP preparations — optical interference must be separately assessed.

5.4 Interference Mitigation Strategies for Lipid-Modified Oligonucleotides

A systematic approach to interference mitigation is required for all lipid-conjugated oligonucleotide preparations. The strategy depends on the specific lipid modification, its concentration in the test sample, and whether the primary concern is inhibition, enhancement, or optical interference. The following hierarchy of mitigation approaches is recommended:

5.4.1 Minimum Valid Dilution (MVD) Testing — First-Line Approach

The most straightforward mitigation for inhibitory lipid interference is sample dilution. Diluting the sample reduces both the lipid concentration and the endotoxin concentration proportionally. The MVD is calculated as:

MVD = (Endotoxin Limit × Sample Concentration) / λLOD

For lipid-conjugated oligos, the MVD should be determined empirically by testing a minimum of three dilutions (1:10, 1:100, 1:1000) and selecting the lowest dilution at which spike recovery falls within 50–200%. Note that for highly lipophilic conjugates (C18, cholesterol, tocopherol), even 1:100 or 1:1000 dilutions may not achieve acceptable spike recovery, indicating that additional interference removal steps are required.

5.4.2 Detergent Addition for Lipid Disaggregation

Non-ionic detergents at sub-CMC concentrations can be used to solubilize and disaggregate lipid-modified oligonucleotide samples prior to LAL testing, without themselves significantly activating or inhibiting the LAL cascade. The most validated approaches include:

- Triton X-100 at 0.1–0.25% (v/v): effective for cholesterol and fatty acid conjugate disaggregation; however, Triton X-100 itself must be validated for LAL compatibility at each concentration used, as it can inhibit the cascade above 1% [3].
- Tween-80 (Polysorbate 80) at 0.1%: used in LNP disruption; validated in FDA-approved endotoxin testing protocols for lipid emulsions and liposomal products [4]. Each lot of Tween-80 should be verified to be endotoxin-free (<0.05 EU/mL) prior to use.
- CHAPS at 0.1%: a zwitterionic detergent effective for tocopherol and C16/C18 solubilization with lower LAL interference than anionic surfactants [3].

The detergent concentration, pre-treatment time, and temperature should be optimized and validated for each lipid-modified oligonucleotide product. Detergent-treated samples must be retested for spike recovery to confirm interference removal.

5.4.3 Recombinant Factor C (rFC) Assay as an Orthogonal Method

Recombinant Factor C (rFC)-based fluorescence assays (e.g., PyroGene, Lonza [15]; EndoZyme II, bioMérieux [16]) use a single-component recombinant enzyme rather than the full LAL cascade, reducing susceptibility to interference from lipid modifiers that disrupt downstream cascade components (Factor B, pro-clotting enzyme). The rFC assay also uses a fluorogenic rather than chromogenic substrate, eliminating 405 nm optical interference from LNP light scattering.

The rFC assay is recommended as an orthogonal or confirmatory method for lipid-conjugated oligonucleotide samples where LAL inhibition is suspected but cannot be fully mitigated by dilution [15,16]. Note that rFC assays are not yet recognized in the USP <85> monograph as equivalent to LAL methods for batch release, but they are acceptable as supplementary methods in a research QC context and are under consideration for regulatory acceptance in multiple jurisdictions [5].

5.4.4 Endotoxin Removal from Lipid-Modified Oligo Preparations

For cholesterol-, tocopherol-, and C16/C18-conjugated oligonucleotides, endotoxin removal can be achieved using polymyxin B-conjugated agarose beads or EndoTrap HD columns (Lionex GmbH/Hyglos), which bind lipid A via polymyxin B's cyclic peptide structure [17]. This approach is effective for removing LPS from aqueous phases but has limitations for amphiphilic preparations where LPS may be sequestered in lipid aggregates [17]. Endotoxin removal efficiency must be independently validated using spiked recovery studies at each product lot [3].

Alternatively, for LNP-formulated siRNA, a two-step approach is recommended: (1) disrupt LNP structure with organic solvent (e.g., isopropanol or methanol extraction of lipid components), recovering the oligonucleotide in the aqueous phase, and (2) test the aqueous fraction by standard nexgen-PTS LAL testing.

5.4.5 Gel-Permeation or Ultrafiltration Separation

Size-exclusion chromatography (SEC) or ultrafiltration (molecular weight cut-off membranes, 10–30 kDa) can separate the high-molecular-weight lipid-oligonucleotide conjugate from lower-molecular-weight free lipid impurities, reducing the lipid concentration in the final test sample while concentrating the oligonucleotide. However, for covalently conjugated lipid-oligos (e.g., siRNA-Chol, siRNA-C16), the lipid remains attached through the separation and continues to present interference risk.

5.4.6 Validation of the Testing Method for Each Unique Lipid Modification

Critically, the interference profile of a lipid-modified oligonucleotide is not predictable a priori from the lipid modification type alone, it depends on the oligo sequence, the nature of the linker chemistry, the modification position (5′ vs. 3′, sense vs. antisense strand), the oligo concentration tested, and the formulation matrix. Each unique lipid-oligo combination should be independently validated for LAL assay compatibility using the following protocol:

- Test at three concentrations spanning the expected QC testing range.
- Run spike recovery in the nexgen-PTS cartridge spike channels at each concentration.
- If spike recovery is outside 50–200% at any concentration, implement a mitigation strategy and revalidate.
- Run parallel rFC assay as an orthogonal confirmation.
- Document the validated testing concentration, dilution factor, and any pre-treatment used in the method SOP.

5.5 Special Considerations: LNP-Formulated siRNA

Lipid nanoparticle-encapsulated siRNA represents the most analytically challenging category of lipid-modified oligonucleotides for endotoxin testing. The following special considerations apply:

- LNPs typically contain 40–50 mol% ionizable lipid, 10–20 mol% helper lipid (DSPC or DOPE), 30–40 mol% cholesterol, and 1–2.5 mol% PEG-lipid [12]. Each component can independently interfere with LAL [4].
- Particle sizes typically range from 60–120 nm, within the Mie scattering regime for 405 nm light, making direct chromogenic LAL testing unreliable without optical correction or turbidity controls [14,4].
- Endotoxin removal from LNP preparations must occur before the lipid assembly step (i.e., at the siRNA drug substance level) to avoid endotoxin encapsulation within the LNP core, which makes post-formulation removal virtually impossible.
- Gene Link's endotoxin testing and certification of siRNA at the drug substance level (before LNP formulation) is therefore the appropriate stage for QC release testing of LNP-encapsulated products. Post-formulation endotoxin testing requires specialized LNP disruption methods.
- For pre-formulation siRNA from Gene Link, the <5 EU/mL specification applies to the unformulated siRNA duplex tested by chromogenic LAL. Researchers are responsible for endotoxin monitoring of excipients (ionizable lipid, cholesterol, PEG-lipid, helper lipid) and the LNP formulation process itself.

> ℹ **NOTE:** When Gene Link *in vivo* grade siRNA is formulated into LNPs by the researcher, the certified <5 EU/mL endotoxin status applies to the siRNA drug substance only. All LNP excipients must be independently verified as endotoxin-free, and the LNP formulation process must be conducted in an endotoxin-controlled environment to maintain the overall endotoxin specification of the final drug product.

5.6 Recommended Testing Decision Tree for Lipid-Modified Oligonucleotides

The following decision framework is recommended for endotoxin testing method selection based on the lipid modification type:

Sample Type	Initial Test Approach	If Spike Recovery Fails	Confirmatory Method
Unmodified siRNA or ASO	Direct nexgen-PTS at MVD dilution	Dilute further; check pH/ionic strength	Repeat LAL
GalNAc-siRNA (carbohydrate linker only)	Direct nexgen-PTS at 1:10–1:100 dilution	Dilute to 1:500; retest	rFC assay
Cholesterol-siRNA	nexgen-PTS + spike recovery at 1:100–1:1000	0.1% Tween-80 pre-treatment; retest	rFC assay (recommended)
C16-siRNA or C18-siRNA	nexgen-PTS + spike recovery at 1:100–1:1000	CHAPS 0.1% pre-treatment or SEC separation	rFC assay
C18:1 (Oleyl)-siRNA	nexgen-PTS at 1:100; spike recovery check	0.1% Triton X-100 pre-treatment	rFC assay
Tocopherol-siRNA	nexgen-PTS at 1:100–1:1000; spike recovery	Triton X-100 0.1% or IPA extraction	rFC assay (required)
LNP-formulated siRNA	Test siRNA before formulation; for post-LNP: IPA disruption of LNP then test aqueous phase	rFC assay on disrupted LNP aqueous phase	Both LAL + rFC required
Stearyl-CPP conjugate	nexgen-PTS at 1:100 with CHAPS 0.1%	Increase dilution; polymyxin B endotoxin removal step	rFC assay

6. Testing Protocol: Endosafe nexgen-PTS for Oligonucleotide Samples

6.1 Sample Preparation Considerations

Oligonucleotide samples present specific analytical challenges for LAL-based endotoxin testing that must be addressed to ensure valid results. High-concentration nucleic acid solutions can exhibit inhibitory or enhancing effects on the LAL cascade, and the pH and ionic strength of the sample must be compatible with the assay reagents. The following preparative steps are recommended:

- Reconstitute lyophilized samples in LAL Reagent Water (LRW, ≤0.005 EU/mL) at a defined concentration.
- Adjust sample pH to 6.0–8.0 if outside this range.
- For lipid-modified samples: apply relevant pre-treatment (see Section 5.4) prior to dilution.
- Prepare dilution series (minimum 1:10 and 1:100) to identify the MVD yielding spike recoveries within 50–200% [5,3].
- Gene Link tests *in vivo* grade oligo samples in duplicate with the integrated spike controls on the nexgen-PTS cartridge.

6.2 Acceptance Criteria and Result Interpretation

Parameter	Acceptance Criterion	Action If Failed
Endotoxin Concentration	<5 EU/mL (final product)	Reject lot; investigate source
Spike Recovery	50–200%	Repeat at higher dilution or investigate matrix interference
Duplicate CV%	≤25%	Repeat test; review cartridge handling
Negative Control (LRW)	<0.05 EU/mL	Investigate LRW and environmental contamination
Lipid-modified samples: rFC orthogonal	Consistent with LAL result (±2-fold)	Investigate discordance; report both values; apply conservative result
Cartridge Lot Validity	Within expiry; stored 2–8°C	Replace cartridge lot

7. *In vivo* Study Design Considerations

7.1 Route of Administration and Endotoxin Sensitivity

The route of administration of oligonucleotides in animal models significantly influences the tolerable endotoxin threshold. Intravenous (IV) and intrathecal (IT) routes carry the greatest endotoxin sensitivity [6]. Intraperitoneal (IP) and subcutaneous (SC) routes are generally more tolerant, though endotoxin-mediated inflammation can still confound results. The <5 EU/mL standard is a conservative threshold appropriate for the most sensitive routes encountered in preclinical research [7]. For cholesterol- or lipid-conjugated siRNA administered IV, the practical endotoxin sensitivity may be further elevated due to the lipid's own inflammatory potential via TLR4 (cholesterol oxidation products are known TLR4 ligands) [2], reinforcing the importance of rigorous endotoxin control.

7.2 Selection of Oligonucleotide Grade for Study Type

Study Type	Recommended Grade	Purification	Rationale
Target validation (in vitro to *in vivo*)	Desalted *in vivo* grade	Size-exclusion + 0.22 µm	Cost-effective; adequate for initial feasibility
Dose-response and efficacy studies	RPC *in vivo* grade	RPC + 0.22 µm	Higher consistency; reduced truncation products
Toxicology and IND-enabling preclinical	PAGE *in vivo* grade	PAGE + desalt + 0.22 µm	Highest purity; full-length enriched
Cholesterol- or lipid-conjugated siRNA *in vivo*	RPC or PAGE *in vivo* grade	RPC or PAGE + 0.22 µm	Lipid conjugate QC requires validated interference-mitigation testing protocol
LNP-formulated siRNA	PAGE *in vivo* grade (pre-formulation)	PAGE + desalt + 0.22 µm	Test at siRNA drug substance level; separate excipient QC required
ASO CNS (IT/ICV)	PAGE *in vivo* grade	PAGE + desalt + 0.22 µm	CNS routes: highest endotoxin sensitivity

7.3 Handling and Reconstitution of *In vivo* Grade Oligos

- Reconstitute only in certified endotoxin-free water (LRW, ≤0.005 EU/mL) or endotoxin-free TE buffer.
- For lipid-conjugated oligos: use endotoxin-free PBS or other physiological buffer appropriate for the lipid modification; avoid aqueous solutions with organic co-solvents that may not have been endotoxin-tested.
- Use sterile, single-use, endotoxin-free plasticware for all reconstitution, dilution, and dosing steps.
- For cholesterol-siRNA or C18-siRNA, gentle sonication or heating (37°C, 5–10 min) may be required to achieve homogeneous reconstitution; perform in endotoxin-free tubes.
- Re-test reconstituted lipid-modified samples by nexgen-PTS (with validated interference-mitigation protocol) if there is any doubt about endotoxin status.

8. Quality Documentation and Regulatory Compliance

8.1 Certificate of Analysis (CoA) Requirements

In vivo grade oligonucleotides from Gene Link are accompanied by a Certificate of Analysis documenting the endotoxin test result (EU/mL), the method (chromogenic LAL), the acceptance threshold (<5 EU/mL), the purification grade, and mass/OD yield data. For lipid-modified oligos, the CoA may additionally specify the validated testing dilution, any pre-treatment applied (e.g., Tween-80 disaggregation).

8.2 Alignment with GLP Principles

Although Gene Link's *in vivo* grade oligos are manufactured for research use and are not GMP certified, the production process incorporates key GLP-aligned elements, including instrument calibration and qualification records for the chromogenic LAL testing system, use of certified reference standard endotoxin (RSE or CSE) for standard-curve generation, traceability of test records to specific production lots, and defined acceptance criteria with explicit pass/fail thresholds. For lipid-modified oligos, method-validation records, including spike recovery across dilution series and documentation of interference-mitigation strategies should also be included in the batch record.

8.3 Endotoxin Testing Within a Broader QC Framework

Endotoxin testing is a critical but not exclusive quality parameter. A complete QC framework for *in vivo* grade oligonucleotide release should additionally include mass spectrometry or HPLC-based purity and identity confirmation, OD_{260} quantification, analytical HPLC or PAGE for truncation product assessment, and stability assessment. For lipid-modified oligos, lipid conjugation efficiency should be confirmed by analytical methods (e.g., HPLC, mass spectrometry) to ensure the lipid modification is present and uniform before commencing *in vivo* studies.

9. Summary and Conclusions

The use of endotoxin-controlled, *in vivo* grade siRNA and ASO oligonucleotides is an essential prerequisite for reliable and interpretable *in vivo* preclinical research. Bacterial endotoxin contamination in oligonucleotide preparations can trigger potent non-specific immune responses, introduce confounding variables, and cause direct animal toxicity. The Charles River Endosafe nexgen-PTS provides a rapid, sensitive, FDA-licensed platform for endotoxin quantification using the kinetic chromogenic LAL principle.

The incorporation of lipid modifications — including cholesterol, C16 palmitoyl, C18 stearoyl, C18:1 oleyl, tocopherol, and multi-component LNP systems — significantly complicates endotoxin testing. These modifications can cause false-negative results through competitive binding to Factor C, micelle-mediated enzyme denaturation, and LPS sequestration, or false-positive results through optical interference (LNPs) or sub-CMC cascade activation. Standard LAL testing without interference validation is insufficient for lipid-modified oligonucleotide preparations.

A validated, modification-specific testing strategy is required for each lipid-conjugated oligo, including empirical determination of the minimum valid dilution, pre-treatment with appropriate non-ionic detergents where needed, and orthogonal confirmation by recombinant Factor C (rFC) assay for high-risk modifications such as cholesterol, tocopherol, and LNP-formulated siRNA.

Gene Link's in vivo grade production process certifying <5 EU/mL at the drug substance level by chromogenic LAL, with three purification grades (Desalted, RPC, and PAGE) and universal 0.22 µm final filtration—provides a well-documented and technically appropriate starting point for both unmodified and lipid-conjugated siRNA and ASO oligonucleotides intended for preclinical in vivo research.

References and Resources

Primary References and Technical Resources

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Regulatory Guidance Documents

- ICH Q6A: Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products (Chemical Substances).
- FDA 21 CFR Part 610.13: Sterility and Pyrogenicity Standards for Biological Products.
- USP <85>: Bacterial Endotoxins Test — including Inhibition/Enhancement Testing requirements.
- FDA Guidance: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials (endotoxin dose calculations).
- EMA Reflection Paper on the Use of Recombinant Factor C in Place of LAL Assay for Batch Release Testing of Medicinal Products (EMA/CHMP/BWP/245500/2020).

This technical report is prepared for informational and research planning purposes. Gene Link in vivo grade oligonucleotides are intended for research use only and are not sterile certified. Researchers should consult their institutional IACUC, regulatory affairs, and quality assurance teams when designing formal preclinical studies. Endotoxin testing method validation for lipid-modified oligonucleotides is the responsibility of the end user and must be performed independently for each unique lipid conjugate and formulation.

Non-Silencing/NTC Control SmartBase™ siRNA Duplexes			
	Catalog #	Qty	Product
NTC Control SmartBase™ siRNA for human, mouse and rat No cellular delivery ligand			
❑	27-6740-05	5 nmols	SmartBase™ siRNA NTC PAGE Purified 5 nmols
❑	27-6740-10	10 nmols	SmartBase™ siRNA NTC PAGE Purified 10 nmols
❑	27-6740-20	20 nmols	SmartBase™ siRNA NTC PAGE Purified 20 nmols
NTC Control SmartBase™ siRNA for human, mouse and rat Trivalent GalNAc delivery ligand			
❑	27-6741-05	5 nmols	SmartBase™ siRNA NTC with Trivalent GalNAc PAGE Purified 5 nmols
❑	27-6741-10	10 nmols	SmartBase™ siRNA NTC with Trivalent GalNAc PAGE Purified 10 nmols
❑	27-6741-20	20 nmols	SmartBase™ siRNA NTC with Trivalent GalNAc PAGE Purified 20 nmols
NTC Control SmartBase™ siRNA for human, mouse and rat Cholesterol-TEG delivery ligand			
❑	27-6742-05	5 nmols	SmartBase™ siRNA NTC with Cholesterol-TEG PAGE Purified 5 nmols
❑	27-6742-10	10 nmols	SmartBase™ siRNA NTC with Cholesterol-TEG PAGE Purified 10 nmols
❑	27-6742-20	20 nmols	SmartBase™ siRNA NTC with Cholesterol-TEG PAGE Purified 20 nmols
NTC Control SmartBase™ siRNA for human, mouse and rat α-tocopherol delivery ligand			
❑	27-6743-05	5 nmols	SmartBase™ siRNA NTC with α-tocopherol PAGE Purified 5 nmols
❑	27-6743-10	10 nmols	SmartBase™ siRNA NTC with α-tocopherol PAGE Purified 10 nmols
❑	27-6743-20	20 nmols	SmartBase™ siRNA NTC with α-tocopherol PAGE Purified 20 nmols

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