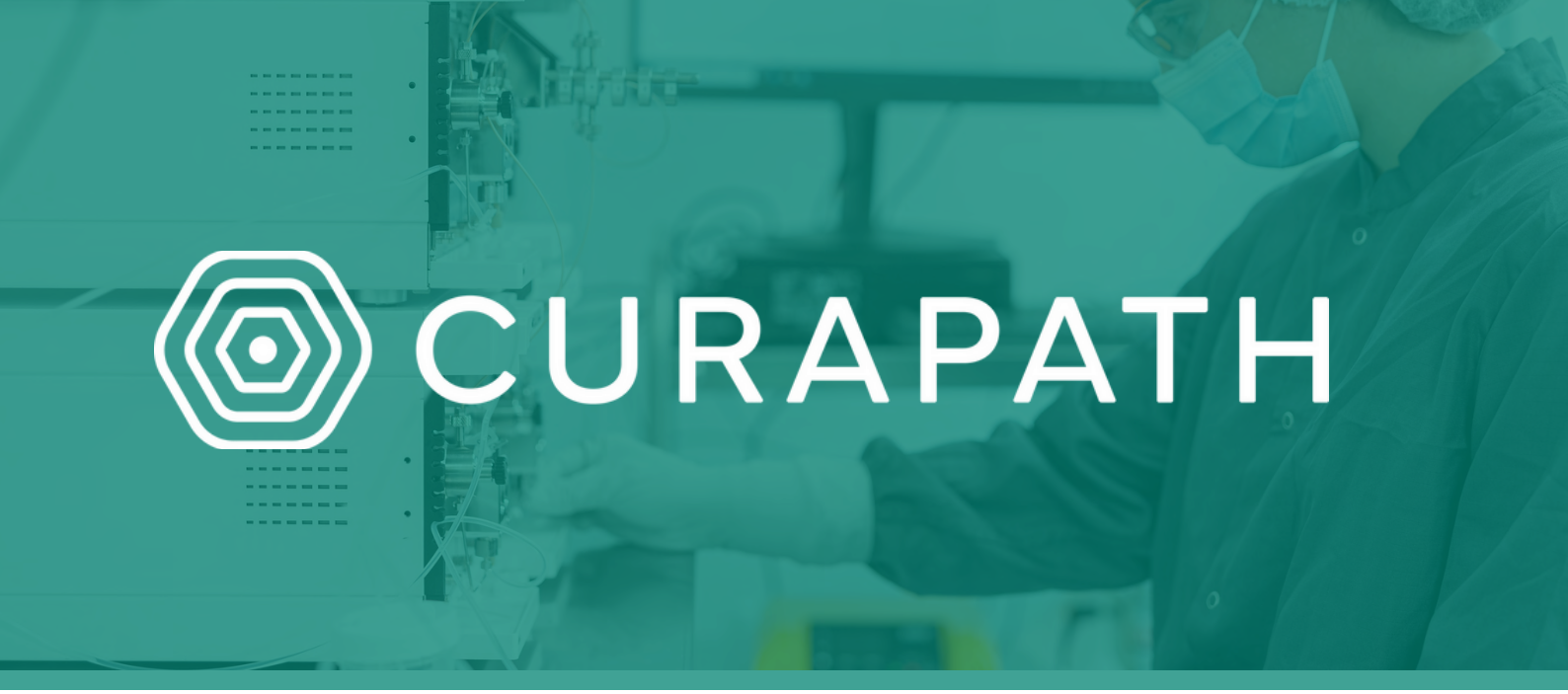

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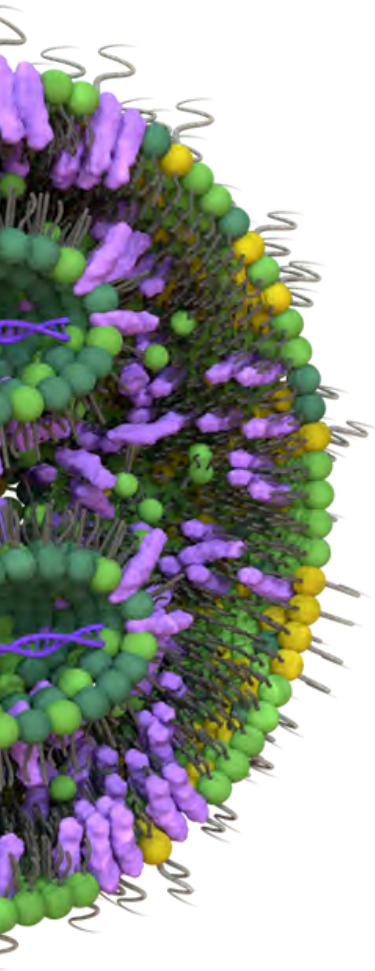

LNP Analytical Methods

Application Note

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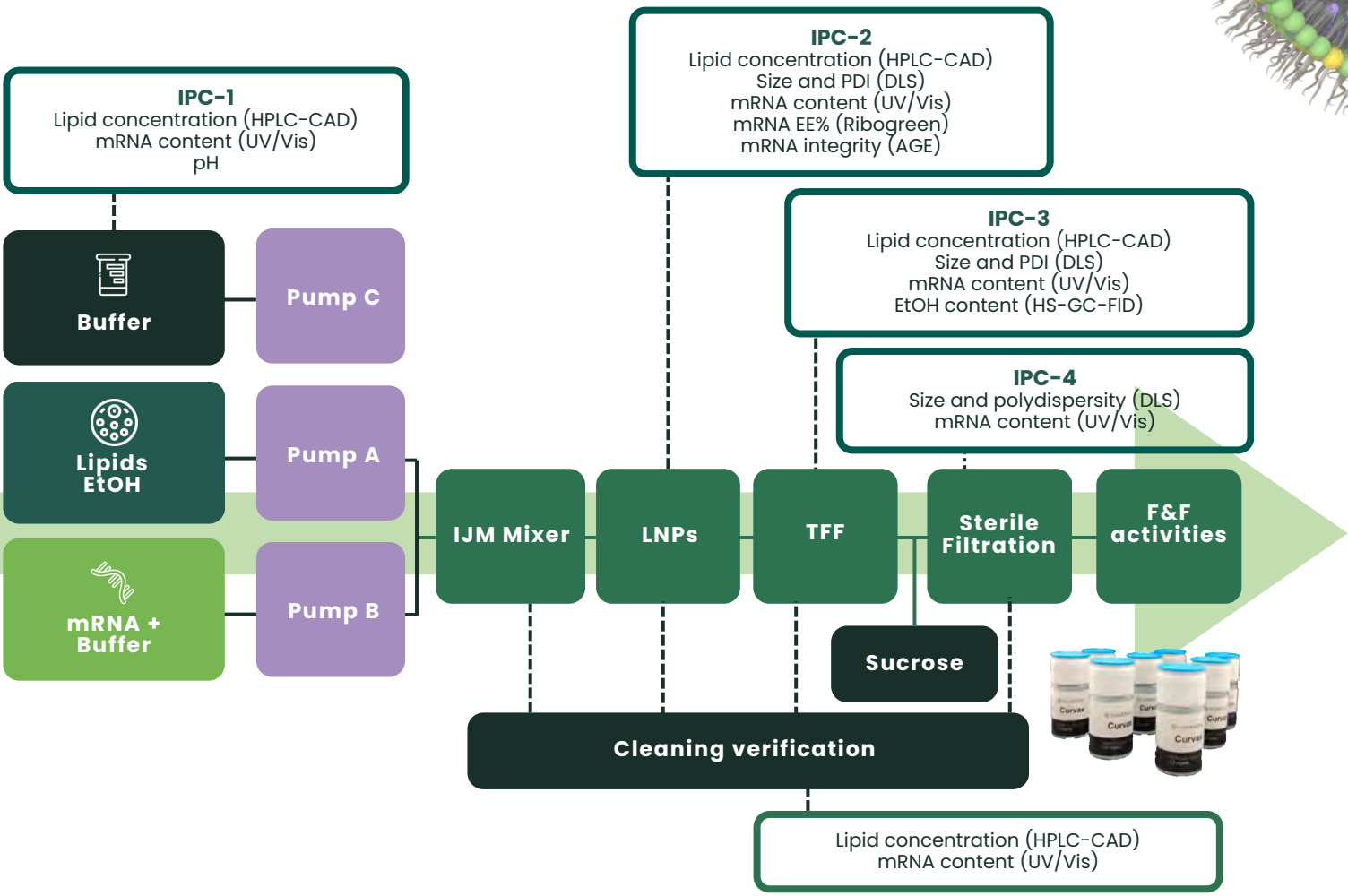
More than a CDMO

Analytical methods application note

Lipid Nanoparticle Formulation Characterization: Bridging the Gap from R&D to GMP Scale through Robust Analytical Excellence

Abstract

Currently, lipid nanoparticles (LNPs) stand as the predominant delivery system for emerging mRNA-based therapeutic solutions. LNPs can be swiftly produced, consistently replicated, and exhibit remarkable scalability. Nevertheless, their sensitivity to the manufacturing and purification procedures remains considerably elevated, and optimized analytical methods are essential to ensure a clinically accepted LNP drug product. This application note aims to provide an overview of the most relevant techniques developed for In-Process Control (IPC) and final product analysis, necessary for the successful release of a GMP-compliant LNP drug product.



The remarkable achievements of the SARS-CoV-2 vaccines, BNT162b2 ("Comirnaty" by BioNTech/Pfizer) and mRNA-1273 ("Spikevax" by Moderna) (1), have spurred pharmaceutical companies to emphasize the utilization of lipid nanoparticles (LNPs) for delivering nucleic acids. Consequently, well-optimized and robust analytical methods are necessary to monitor key parameters of LNPs throughout the process and facilitate the production of the GMP product until its release.

Analytical methods application note

Bridging the Gap from R&D to GMP

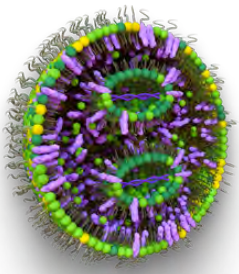
Precision in Formulation:
An In-depth Exploration of Critical Analytical Techniques

As illustrated above, the production of Lipid Nanoparticles (LNPs) unfolds in four distinct steps: Knauer formulation, Tangential Flow Filtration (TFF) purification, sterilizing filtration, and F&F activities. In the rigorous adherence to Good Manufacturing Practice (GMP) standards, our approach involves a meticulous array of analytical techniques, crucial for delving into the composition and properties of the formulations.

At Curapath, we leverage specific techniques chosen for their robustness in the developed and optimized methods. Dynamic Light Scattering (DLS) takes the lead, providing invaluable insights into particle size distribution (d.nm) and polydispersity (PDI), ensuring formulation homogeneity from initial formulation through F&F activities.

A versatile tool in our arsenal is High- Performance Liquid Chromatography with Charged Aerosol Detection (HPLC-CAD), facilitating precise quantification of key lipid components: DMG-PEG, cholesterol, SM-102, and DSPC concentrations.
The mRNA content is meticulously determined through a UV assay using advanced instrumentation such as the Stunner system, employing tiny amounts of formulation.

For assessing the percentage of RNA encapsulation as well as orthogonally in mRNA content, Fluorescence Spectroscopy plays a pivotal role, providing nuanced insights into formulation efficiency. Lipid purity is scrutinized using HPLC-CAD and mRNA integrity by automated gel electrophoresis (AGE, TapeStation), and residual ethanol levels are measured with Head Space Gas Chromatography with Flame Ionization Detector (HS-GC-FID). This holistic approach ensures a thorough and accurate characterization of LNP formulations at every stage, setting the stage for advancements in RNA delivery systems.



Size & PDI

mRNA Content

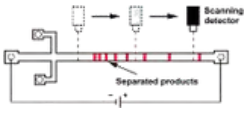
DLS & UV/Vis

Lipid Purity

Lipid Concentration

HPLC-CAD

mRNA Integrity



Automated Electrophoresis

Analytical methods application note

Bridging the Gap from R&D to GMP

LNP Purification:

Maximizing Efficiency with Monitoring Strategies in TFF Processes

TFF purification stands out as a robust method for lipid nanoparticle LNPs purification, offering several advantages. By leveraging TFF, LNPs can undergo efficient separation from impurities, achieving high yields and purity levels. This technique allows for precise control over particle size and distribution, crucial for maintaining product consistency. Additionally, TFF enables continuous processing, reducing downtime and increasing productivity. Its scalability makes it suitable for both small-scale research and large-scale production, providing versatility in LNP purification strategies.

Monitoring the size throughout the process of formulating Lipid Nanoparticles (LNPs) provides crucial insights into the critical quality attributes of the resulting nanoparticles (2). In the example described of GMP production, we closely tracked the size changes at every stage. This observation aimed to identify any potential impact on LNPs and assess the overall success of mRNA delivery. In Figure 2, DLS size data from the key stages of the process are presented, illustrating optimal control over the overall stability of the formulation.

One aspect to consider during the purification process is the effective volume of buffer needed to efficiently remove the organic solvent, in this case, ethanol, used to formulate the LNPs with Knauer equipment, which needs to be removed in the final product. Curapath's monitoring strategy involves performing several In-Process Controls (IPCs) during purification (**Figure 1**), taking aliquots to be submitted to HS-GC-FID. This approach allows for the monitorization of ethanol during the purification process, providing prompt feedback regarding the termination of the TFF process.

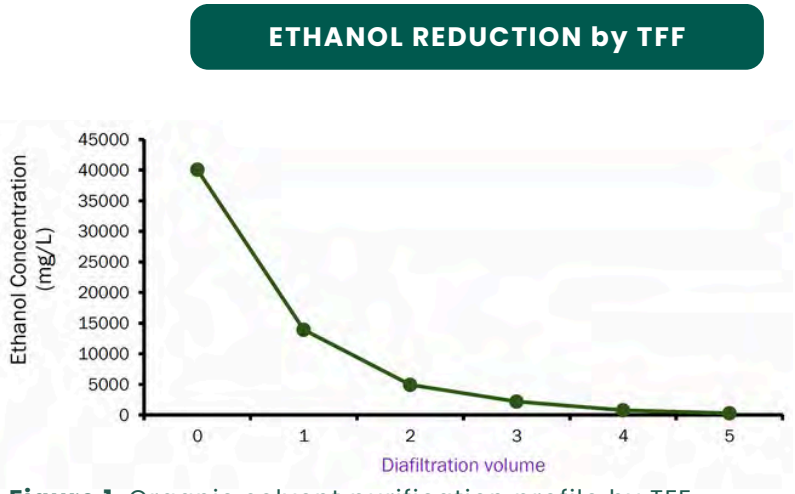


Figure 1. Organic solvent purification profile by TFF.

DLS Insight: Illuminating Size Across Every Step of the Process

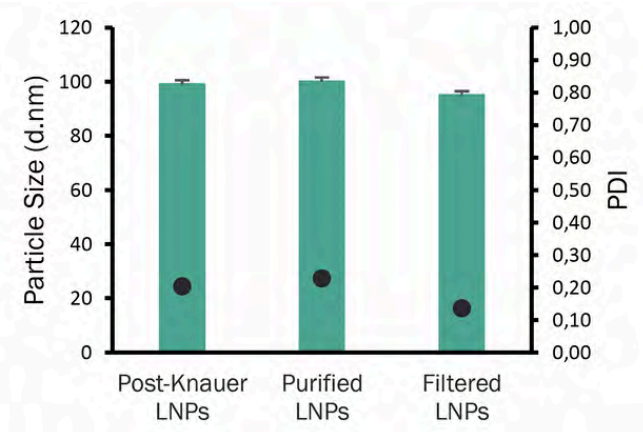


Figure 2. Hydrodynamic diameter and PDI of LNPs formulated during scale up to 1L GMP batch scale by DLS.

Monitoring the size throughout the process of formulating Lipid Nanoparticles (LNPs) provides crucial insights into the critical quality attributes of the resulting nanoparticles (2). In the example described of GMP production, we closely tracked the size changes at every stage. This observation aimed to identify any potential impact on LNPs and assess the overall success of mRNA delivery. In **Figure 2**, DLS size data from the key stages of the process are presented, illustrating optimal control over the overall stability of the formulation.

Analytical methods application note

Bridging the Gap from R&D to GMP

Strategies for Monitoring mRNA Content and Integrity during LNP Formulation Process & stability studies

Encapsulation efficiency (EE%) of mRNA-loaded LNPs was assessed using the Quant-iT RiboGreen assay, ensuring rigorous monitoring of mRNA encapsulation throughout the formulation process. The assay employs a fluorescent dye that selectively binds to mRNA molecules in the formulation, allowing for precise quantification of mRNA content in samples and enabling robust assessment of encapsulation efficiency in mRNA loaded LNPs. Various aliquots were sampled at critical stages, including post-TFF purification, following addition of the cryo preservative; sucrose, and after sterilization filtration using a 0.22 µm filter. The EE%, Free mRNA (ng/µL), and Total mRNA were quantified for each step, as summarized in **Figure 3**. This evaluation strategy aims to guarantee optimal mRNA encapsulation and sustained stability of the LNPs, thereby minimizing the risk of nucleic acid loss during formulation and subsequent handling.

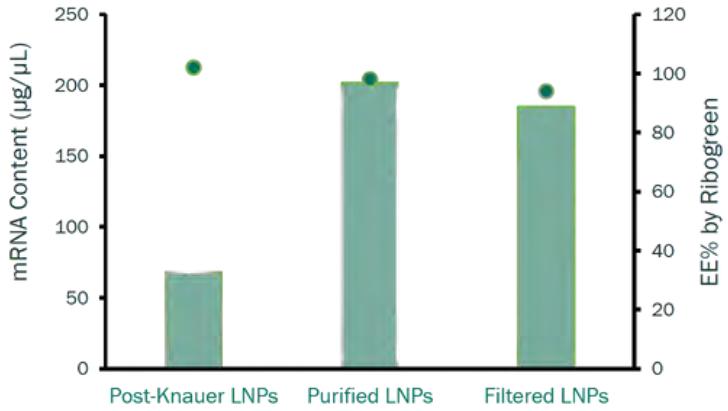


Figure 3. EE%, Free mRNA (ng/µL), and Total mRNA determined by Quant-iT RiboGreen assay.

Maintaining mRNA integrity within lipid nanoparticle (LNP) formulations is crucial in order to achieve the successful efficacy results (3). Throughout LNP production, mRNA integrity has been monitored before LNP manufacturing and after its preparation. This analysis was conducted using an automatic electrophoresis instrument, TapeStation. The equipment operates in a simplified workflow with a fully automated sample processing, with a reduced sample consumption with as little as to 1 to 2 µL per sample. In

Figure 4, data from the mRNA drug substance and mRNA LNP encapsulated provided analogue integrity profiles. It is noteworthy that mRNA integrity has been demonstrated that remains unaltered during the LNP encapsulation process.

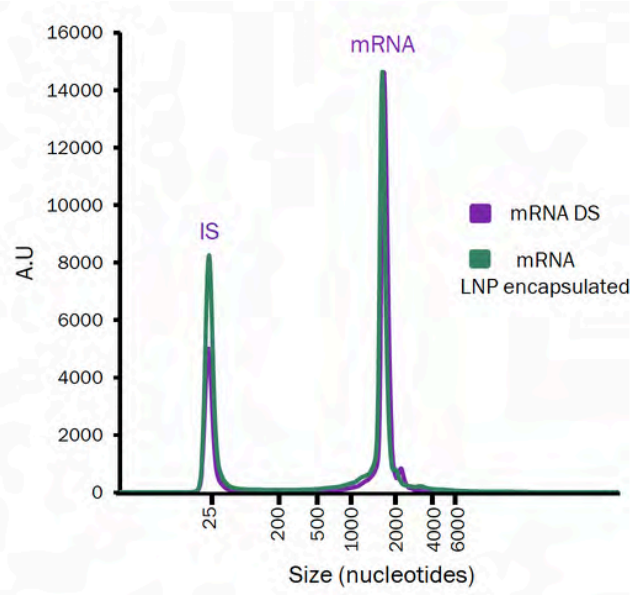


Figure 4. mRNA electropherogram of mRNA DS and encapsulated mRNA-LNPs. Marker of 25 nucleotides is employed as internal standard (IS).

Analytical methods application note

Bridging the Gap from R&D to GMP

Lipid Profiling and purity in LNP Production:
Enhancing Precision with HPLC-CAD Analysis

Beyond mRNA analysis, we meticulously assess the lipid concentration, a crucial element in our LNPs crafted at Curapath. The here presented formulation included SM-102, DSPC, cholesterol, and DMG-PEG (2000) lipid. Employing advanced HPLC technology with a CAD detector (4), we unravel the lipid profile with precision. This cutting-edge analysis traces the journey of lipids through chromatography, where their concentration is revealed in the signal intensity, denoted as Lipid Concentration (LC%). Content for lipid components is scrutinized at pivotal stages of LNPs production, as illustrated in **Figure 5**.
From the initial lipid phase formulation to the TFF purification process, we ensure the perfect balance of components. LC% assessments persist even after the addition of cryopreserving sucrose and the final sterilization step through a 0.22 µm filter.
Moreover, HPLC-CAD analysis can give valuable information on lipid purity in final formulation, allowing for the quantification of impurities or degradation products, even at low concentrations or in complex matrices. Purity % (w/w) for the current production was evaluated, resulting in 98% (w/w).

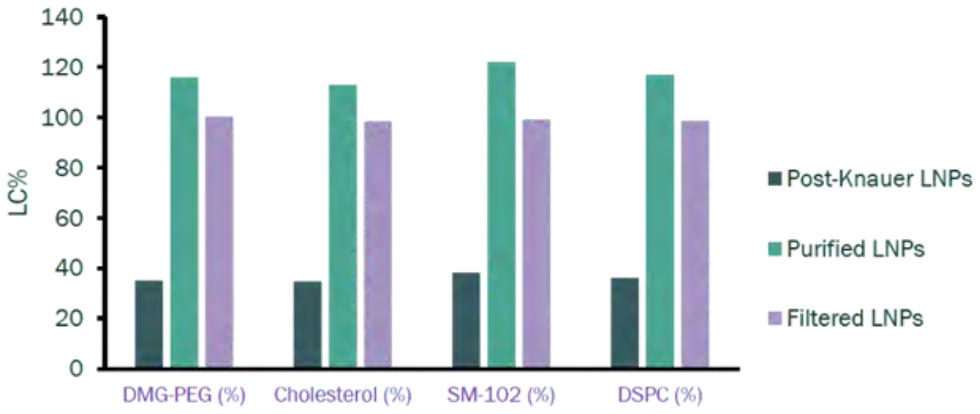
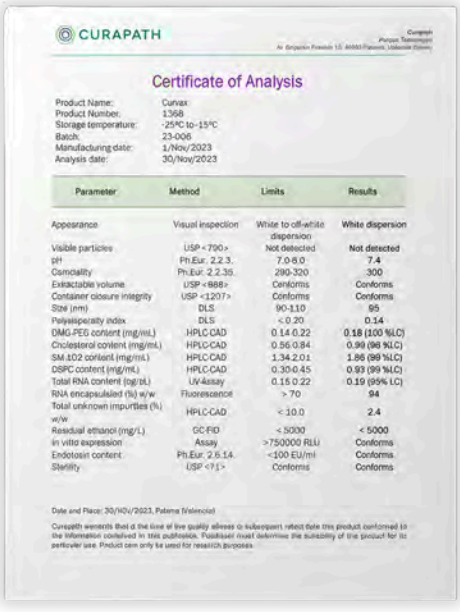


Figure 5. LC% for SM-102, DSPC, cholesterol, and DMG-PEG (2000) by HPLC-CAD.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Certificate of Analysis

Product Name: Curvax
Product Number: 1368
Storage temperature: -25°C to -15°C
Batch: 23-006
Manufacturing date: 1/Nov/2023
Analysis date: 30/Nov/2023

Parameter	Method	Limits	Results
Appearance	Visual inspection	White to off-white dispersion	White dispersion
Visible particles	USP <790>	Not detected	Not detected
pH	Ph.Eur. 2.2.3.	7.0-8.0	7.4
Osmolality	Ph.Eur. 2.2.35.	280-320	300
Extractable volume	USP <788>	Conforms	Conforms
Container closure integrity	USP <1207>	Conforms	Conforms
Size (nm)	DLS	90-110	95
Polydispacity index	DLS	< 0.20	0.14
DMG-PEG content (mg/mL)	HPLC-CAD	0.14-0.22	0.18 (100 %LC)
Cholesterol content (mg/mL)	HPLC-CAD	0.56-0.84	0.99 (96 %LC)
SM-102 content (mg/mL)	HPLC-CAD	1.34-2.01	1.86 (99 %LC)
DSPC content (mg/mL)	HPLC-CAD	0.30-0.45	0.93 (99 %LC)
Total RNA content (ng/µL)	UV-assay	0.15-0.22	0.19 (95% LC)
RNA encapsulated (%) w/w	Fluorescence	> 70	94
Total unknown impurities (%) w/w	HPLC-CAD	< 10.0	2.4
Residual ethanol (mg/L)	GC-FID	< 5000	< 5000
In vitro expression	Assay	>750000 RLU	Conforms
Endotoxin content	Ph.Eur. 2.6.14.	<100 EU/ml	Conforms
Sterility	USP <71>	Conforms	Conforms

Date and Place: 30/NOV/2023, Paterna (Valencia)

Curapath warrants that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. Purchaser must determine the suitability of the product for its particular use. Product can only be used for research purposes.

As described along this technical note, the LNP product manufactured at Curapath successfully met all the release specifications and monitored IPCs. At Curapath, we possess robust expertise and confidence in utilizing innovative analytical techniques for the comprehensive characterization of cutting-edge nanosystems. Our team is adept at supporting customers throughout the entire development process, from the early stages to clinical trials, enabling a deep understanding of the contents within the final administration vial.

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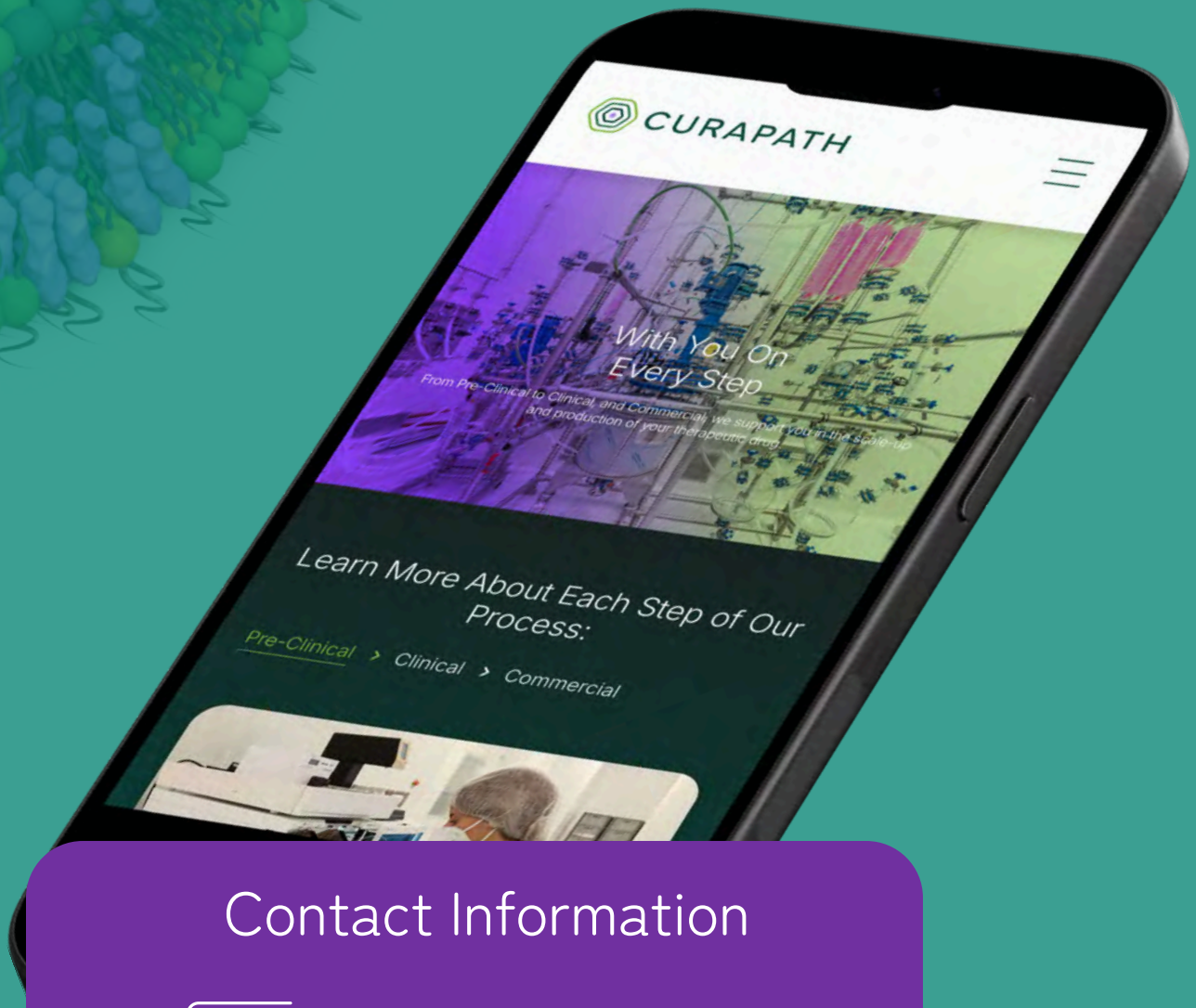

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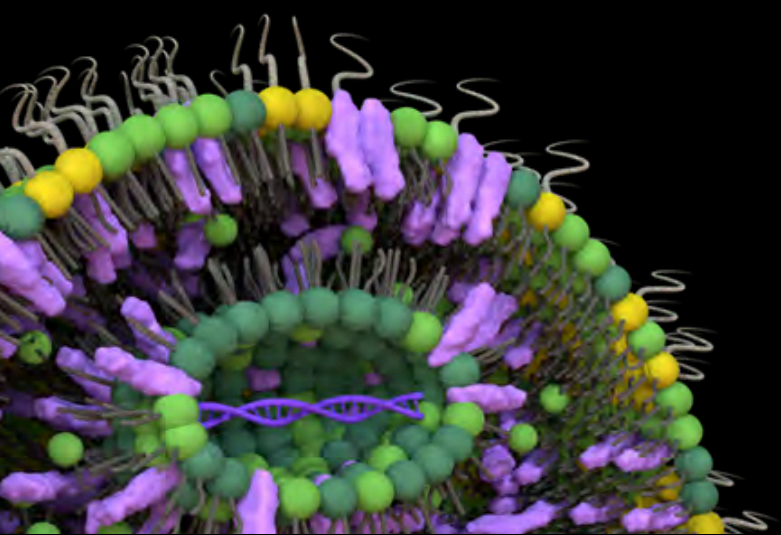

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