Determination of nucleic acid encapsulation efficiency in Lipid Nanoparticles using fluorometry

CCMC will prepare and attach the official title page.

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Foreword

This CEN Workshop Agreement (CWA XXXX:YYYY) has been developed in accordance with the CEN-CENELEC Guide 29 "CEN/CENELEC Workshop Agreements – A rapid way to standardization" and with the relevant provisions of CEN/CENELEC Internal Regulations - Part 2. It was approved by the Workshop CEN "Determination of nucleic acid encapsulation efficiency in Lipid Nanoparticles using fluorometry", the secretariat of which is held by "UNE" consisting of representatives of interested parties on 2025-06-04, the constitution of which was supported by CEN following the public call for participation made on 2025-04-16. However, this CEN Workshop Agreement does not necessarily include all relevant stakeholders.

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Introduction

In recent years, lipid nanoparticles (LNPs) have emerged as a powerful platform for the delivery of nucleic acids, gaining increasing relevance in both academic research and clinical applications. Their successful use in mRNA-based vaccines and gene therapies has accelerated the need for reliable characterization tools that ensure the safety, efficacy, and reproducibility of LNP-based formulations. Among the key parameters influencing the performance of these systems, encapsulation efficiency plays a critical role, as it directly affects protection from enzymatic degradation, intracellular delivery, and therapeutic potency.

Currently, several analytical techniques are employed to estimate the encapsulation efficiency of RNA and DNA within LNPs. These include gel electrophoresis, UV absorbance, and dynamic light scattering (DLS). While commonly used, these methods suffer from important limitations such as low sensitivity, interference from free nucleic acids, and inability to distinguish encapsulated from non-encapsulated species with sufficient accuracy.

In this context, fluorescence-based assays offer a promising alternative due to their high sensitivity and specificity. Fluorometric quantification methods enable a clear distinction between free and encapsulated nucleic acids by employing selective intercalating dyes or fluorescent probes that do not penetrate lipid membranes. Despite the increasing adoption of fluorometry in research laboratories, no standardized method currently exists for its application in the context of LNP nucleic acid encapsulation.

The aim of this CEN Workshop Agreement (CWA) is therefore to define a robust and reproducible fluorometric protocol for the determination of RNA and DNA encapsulation efficiency in lipid nanoparticles. The scope of the document is strictly analytical and does not extend to LNP formulation design, *in vivo* performance, or therapeutic evaluation.

This CWA was developed within the framework of the ETERNAL European Project, which identified the lack of standardization in this area as a major barrier to the development of reproducible and scalable delivery systems. The resulting protocol is intended to support research laboratories, pharmaceutical developers, and biotechnology companies in the harmonization of analytical practices, enabling more efficient and comparable product development pipelines.

1 Scope

This document specifies a standardized analytical protocol for the quantification of RNA and DNA encapsulation efficiency in lipid nanoparticles (LNPs) using a fluorometric method. The method is based on the use of fluorescence dyes that selectively interact with free nucleic acids, enabling a clear distinction between encapsulated and non-encapsulated species. To determine the total nucleic acid content, the LNPs are lysed using a detergent-based treatment, allowing complete release of the encapsulated material for accurate measurement.

The protocol described herein is designed to offer a rapid, sensitive, and reproducible approach for the non-destructive quantification of nucleic acid encapsulation efficiency. It addresses the limitations of traditional techniques such as gel electrophoresis and UV spectroscopy, which may lack sensitivity and specificity.

This document is applicable to the characterization of LNP formulations used for nucleic acid delivery, particularly in research and early development phases. It is intended for use by academic laboratories, biotechnology companies, and pharmaceutical developers working on LNP-based systems for therapeutic applications.

The scope of this document is limited to the analytical determination of encapsulation efficiency by fluorometry. It does not cover aspects related to LNP formulation, physicochemical characterization, in vivo performance, or therapeutic efficacy.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at http://www.iso.org/obp/

— IEC Electropedia: available at http://www.electropedia.org/

3.1
encapsulation efficiency
the percentage of nucleic acid (RNA or DNA) that is successfully enclosed within lipid nanoparticles (LNPs) relative to the total amount of nucleic acid initially used in the formulation

3.2
free nucleic acid
nucleic acid (RNA or DNA) that remains unencapsulated and is freely available in the formulation medium

3.3
fluorometric assay
an analytical technique that measures the intensity of fluorescence emitted by a dye that binds specifically to nucleic acids, used to quantify free or total nucleic acids

3.4

lipid nanoparticles (LNPs)

nanoscale colloidal carriers composed of lipids, designed to encapsulate and deliver therapeutic nucleic acids such as mRNA, siRNA, or plasmid DNA

3.5

selective dye

a fluorescent molecule that binds specifically to nucleic acids but cannot penetrate the lipid bilayer of LNPs, allowing differentiation between encapsulated and free nucleic acids

3.6

lysis agent

a reagent that disrupts the lipid bilayer of the nanoparticles, allowing the release of encapsulated nucleic acids for quantification

3.7

working solutioin

a reagent based in TE buffer (Tris-EDTA, Tris-HCl 10 mM pH 7.5 + EDTA 1nM) that contain the fluorescent dye that bind to DNA or RNA

4   Principle of the method

The method described in this document is based on the use of a fluorescent dye that selectively binds to nucleic acids but cannot permeate intact lipid bilayers (Figure 1). This property enables the selective detection of free (non-encapsulated) nucleic acids in lipid nanoparticle (LNP) formulations.
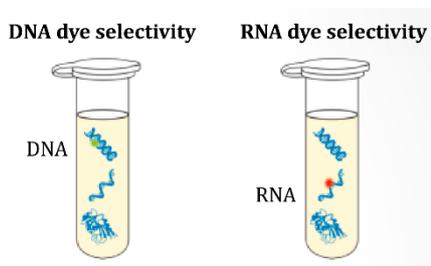
Figure 1 — Fluorescent dyes selectively bind to DNA or RNA — Dyes only emit signal when bound to the target

The total nucleic acid content is determined in a second step by disrupting the LNPs using a lysis agent, which allows the dye to access all nucleic acids in the sample. The encapsulation efficiency is calculated as the percentage of nucleic acids that were not detected in the free nucleic acid measurement, relative to the total:

$$\% \, EE = \frac{Total\, NA - Free\, NA}{Total\, NA} \cdot 100\,\%$$

This two-step fluorometric assay provides a simple and fast approach to accurately quantify the encapsulation efficiency of RNA and DNA in LNPs.

5   Reagent and materials

5.1   Reagents

The following reagents shall be used for the fluorometric determination of encapsulation efficiency:

- **Fluorescent dye**

A dye that selectively binds to RNA or DNA, depending on the assay used. The dye does not penetrate intact lipid bilayers, allowing selective detection of free nucleic acids in non-lysed samples. Alternative fluorochromes that bind specifically to ssDNA or RNA could also be used.

The dye is mixed with the TE buffer to prepare the working solution used for both standards and sample measurements.

- **Buffer solution: TE buffer**

A TE buffer solution (TE: Tris-EDTA, Tris-HCl 10 mM pH 7.5 + EDTA 1 mM) is used for dilution of the fluorescent dye to prepare the working solution. It provides the correct ionic conditions for fluorescence stability and dye–nucleic acid interaction.

- **Nucleic acid standards**

Two standard solutions of known concentration. These are used to generate a calibration curve directly within the fluorometer software.

- **Lysis agent**

A 2 % Triton X-100 solution in PBS, prepared separately, is used to disrupt the LNP membrane prior to quantification of total nucleic acids. The LNP sample is mixed 1:1 with this solution and incubated at 37 °C for 10 minutes before analysis.

NOTE 1   All reagents should be handled in RNAse-/DNAse-free conditions.

NOTE 2   It is possible to use a commercial kit, e.g. Qubit™ nucleic acid quantification kits (Qubit™ ssDNA Assay Kit, Qubit™ RNA HS Assay Kit). In this case, all reagents should be used according to the manufacturer's instructions.

5.2   Materials

- Fluorometer, (Qubit™ 4 or similar).

- Assay tubes or Thin-wall, clear 0.5 mL PCR tubes.

- Micropipettes and sterile pipette tips.

- Nuclease-free tubes and reagents.

- Vortex mixer.

- Dry incubator or heating block (set to 37 °C).

- Timer.

5.2   Sample requirements

- LNP formulations should be diluted appropriately to remain within the dynamic range of the assay.

- Samples must be handled under RNAse- and DNAse-free conditions.

6   Procedure

The method consists of two main steps:

- **Step A:** Quantification of free (non-encapsulated) nucleic acids.

- **Step B:** Quantification of total nucleic acids after LNP disruption with Triton X-100.

The encapsulation efficiency is calculated from the difference between these two measurements.
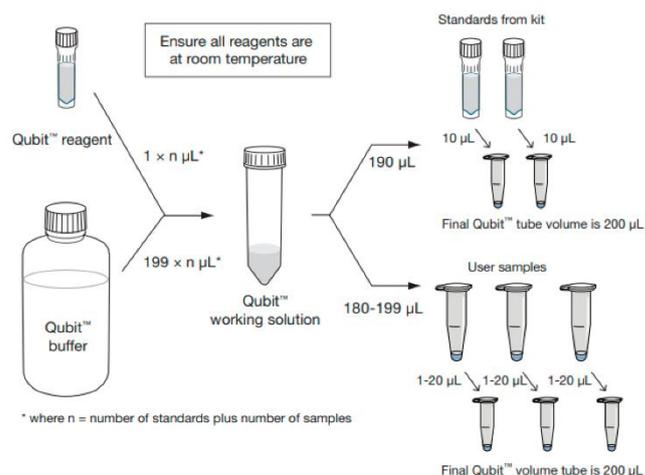
Figure 2 — Schematic representation of the preparation of the different solutions and samples, using the Qubit™ kit — Qubit™ buffer is TE: Tris-EDTA, Tirs-HCl 10 mM pH 7.5 + EDTA 1 mM

6.1   Preparation of the working solution

1. Prepare the working solution by mixing the reagent, containing the fluorochrome and TE buffer typically in a 1:200 dilution (e.g. 199 µL buffer + 1 µL dye per sample).
2. Mix thoroughly and protect from light. Prepare sufficient volume for all samples and standards.

NOTE   Qubit™ 1X dsDNA assays are supplied with a ready-to-use working solution, and do not require preparation.

6.2   Calibration of the fluorometer

1. Label two assay tubes as "Standard 1: 0 ng/ µL" and "Standard 2: 20 ng/ µL ssDNA".
2. Add 190 µL of the working solution to each tube.
3. Add 10 µL of each nucleic acid standard to the corresponding tube.
4. Mix by vortexing for 2–3 seconds.
5. Incubate at room temperature for 2 minutes.
6. Insert the tubes into the fluorometer and follow the device instructions to create the standard curve.

6.3 Measurement of free nucleic acids

1. Add 1 to 20 µL of the undisturbed LNP sample to an assay tube.

2. Add the required volume of working solution that contains the specific fluorochrome to reach a final volume of 200 µL (e.g., 2 µL sample + 198 µL working solution).

3. Mix gently and incubate for 2 minutes at room temperature, protected from light.

4. Measure the fluorescence using the fluorometer and record the nucleic acid concentration.

NOTE 1   The actual sample volume should be selected based on the expected concentration and within the detection range of the assay.

NOTE 2   It is recommended to perform all measurements in duplicates or triplicates and include appropriate negative controls.

6.4 Measurement of total nucleic acids after lysis

1. Prepare a 2 % Triton X-100 solution in PBS.

2. Mix the LNP sample 1:1 with the Triton solution (e.g., 10 µL sample + 10 µL Triton solution).

3. Incubate at 37 °C for 10 minutes to allow complete lysis and release of encapsulated nucleic acids.

4. Add 1 to 20 µL of the lysed sample to a assay tube (preferably small volumes since the concentration will be higher).

5. Add the working solution to reach a final volume of 200 µL.

6. Mix gently and incubate for 2 minutes at room temperature.

7. Measure fluorescence using the fluorometer and record the total nucleic acid concentration.

NOTE 1   The actual sample volume should be selected based on the expected concentration and within the detection range of the assay.

NOTE 2   It is recommended to perform all measurements in duplicates or triplicates and include appropriate negative controls.

7   Calculation of encapsulation efficiency

Encapsulation efficiency is calculated using the following formula:

$$\% \, EE = \frac{Total\,NA - Free\,NA}{Total\,NA} \cdot 100\,\%$$

Where:

Total NA   = total nucleic acid concentration (after lysis)

Free NA   = concentration of non-encapsulated nucleic acids (before lysis)

8   Validation of the method

This section describes the validation parameters for the fluorometric determination of RNA/DNA encapsulation efficiency in lipid nanoparticles (LNPs).

8.1 Specificity and selectivity

Specificity refers to the ability of the analytical method to unequivocally detect the analyte—in this case, RNA or DNA—without interference from formulation excipients, solvents, or lysis reagents.

To evaluate the specificity of the fluorometric assay, a series of blank and spiked samples were prepared to assess potential interference from the formulation components. The following experimental conditions were applied:

- Each test sample was prepared by mixing 10 μL of the corresponding material with 190 μL of the working solution, mixing in vortex mixer for 2–3 seconds, and incubating for 2 minutes at room temperature.

- Fluorescence was measured immediately after incubation.

The following samples were tested with the corresponding results obtained (Table 1):

Table 1 — Specificity and selectivity results

Sample description	Concentration (ng/μL)	Result and Acceptance criteria	Conform
2 % Triton X-100 in PBS (w/v)	0.038 ± 0.002	<0.10	Conform
2 % Tween 20 in PBS (w/v)	0.046 ± 0.002		Conform
Mix of lipids in ethanol	0.041 ± 0.005		Conform
Mix of 2 % Triton X-100 in PBS and lipids in ethanol in a 1:1 ratio	0.057 ± 0.003		Conform
DNA at 10 ng/μL	10.05 ± 0.356	$p = 0.079$ (Not Significant)	Conform
Mix of 2 % Triton X-100 in PBS and DNA (10 ng/μL) in a 1:1 ratio	8.96 ± 0.721		Conform
Mix of DNA (10 ng/μL) and lipids in ethanol in a 3:1 ratio	10.52 ± 0.108	$p = 0.090$ (Not Significant)	Conform
Mix of DNA (10 ng/μL) and lipids in ethanol in a 3:1 ratio and then mixed with 2 % Triton X-100 in PBS in a 1:1 ratio	10.46 ± 0.885	$p = 0.493$ (Not Significant)	Conform
RNA at 10 ng/μL	9.49 ± 0.253	$p = 0.085$ (Not Significant)	Conform
Mix of 2 % Triton X-100 in PBS and RNA (10 ng/μL) in a 1:1 ratio	10.61 ± 0.666		Conform
Mix of RNA (10 ng/μL) and lipids in ethanol in a 3:1 ratio	9.34 ± 0.212	$p = 0.477$ (Not Significant)	Conform
Mix of RNA (10 ng/μL) and lipids in ethanol in a 3:1 ratio and then mixed with 2 % Triton X-100 in PBS in a 1:1 ratio	10.68 ± 0.630	$p = 0.066$ (Not Significant)	Conform

Conclusion:

The results confirmed that the method is specific for nucleic acid detection. None of the formulation components (Triton X-100 or lipid mixture in ethanol) interfered with the fluorescence signal. All blank samples produced fluorescence values below 0.10 ng/µL, which is considered negligible and does not affect the quantification of the nucleic acid concentration. Furthermore, the fluorescence intensity of analyte-containing samples remained consistent across all tested conditions, demonstrating the robustness of the assay in the presence of potential formulation excipients.

8.2 Linearity

Linearity refers to the ability of an analytical method to obtain test results that are directly proportional to the analyte concentration over a defined range.

To evaluate the linearity of the fluorometric assay, a series of DNA and DNA-LNP dilutions were prepared and analyzed. The standard reference used was Qubit™ ssDNA Standard 2 (20 ng/µL), and the method was validated across a dynamic range of 0.01 ng/µL to 240 ng/µL.

8.2.1 DNA

8.2.1.1 Sample preparation

For both free DNA and DNA encapsulated in LNPs, a set of serial dilutions were prepared using nuclease-free water, as described in Tables X and Y (see Annex A). Each sample was further diluted in Qubit™ working solution to a final volume of 200 µL per tube and measured in triplicate.

NOTE   The measured concentration in the Qubit™ tube (ng/µL) reflects the effective working range of the assay and is used to build the calibration curve.

8.2.1.2 Results

8.2.1.2.1 Standard

The fluorescence signal showed a linear correlation with the DNA concentration across the tested range.

Table 2 — Comparison of theoretical and experimental concentrations for each DNA standard sample

Sample	Theoretical concentration (ng/µl)	Experimental concentration (ng/µL)
M1	20.0	19.8
M2	15.0	14.5
M3	10.0	9.8
M4	5.0	4.9
M5	2.0	2.1
M6	0.2	0.072
M7	0.0	*
* Out of range		

Correlation coefficient	0.9997
y-intercept	0.014
Slope	1.017
Equation y = ax + b	y = 1.017x + 0.014

Figure 3 — Linear regression performance: calibration curve and regression parameters for standard DNA

Correlation coefficient = 0.9997 (specification higher or equal to 0.9). Complies.

8.2.1.2.2 DNA

The fluorescence signal showed a linear correlation with the DNA concentration across the tested range.

Table 3 — Comparison of theoretical and experimental concentrations for each DNA sample

Sample	Theoretical concentration (ng/µl)	Experimental concentration (ng/µL)
M1	281.7	*
M2	234.8	220.0
M3	117.4	120.0
M4	58.7	47.6
M5	29.3	28.4
M6	11.7	11.9
M7	1.2	0.974
M8	0.0	*
* Out of range		

Correlation coefficient	0.9958
y-intercept	0.4876
Slope	1.0496
Equation y = ax + b	y = 1.0496x + 0.4876

Figure 4 — Linear regression performance: calibration curve and regression parameters for DNA samples

Correlation coefficient = 0.9958 (specification higher or equal to 0.9). Complies.

8.2.1.2.3 DNA-LNPs

The fluorescence signal showed a linear correlation with the DNA concentration across the tested range.

Table 4 — Comparison of theoretical and experimental concentrations for each DNA-LNP sample

Sample	Theoretical concentration (ng/µl)	Experimental concentration (ng/µL)
M1	20.0	16.4
M2	15.0	12.4
M3	10.0	7.6
M4	5.0	4.2
M5	0.2	0.25
M6	0.0	0.068

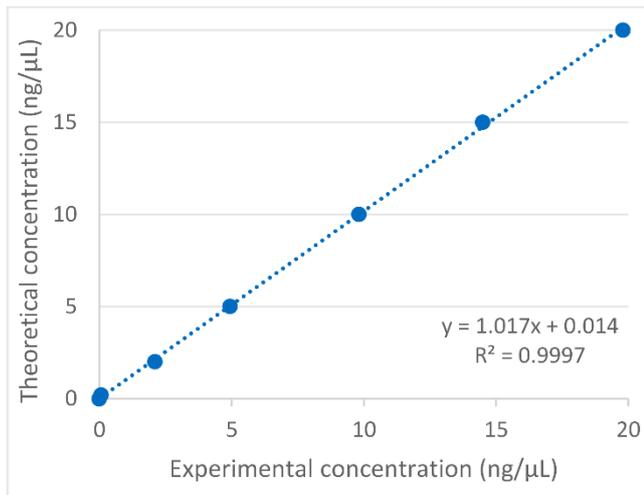|	
Correlation coefficient	0.9992
y-intercept	0
Slope	1.2273
Equation y = ax + b	y = 1.2273x

Figure 5 — Linear regression performance: calibration curve and regression parameters for DNA-LNP samples

Correlation coefficient = 0.9992 (specification higher or equal to 0.9). Complies.

8.2.2   RNA

A series of RNA and RNA-LNP dilutions were prepared and analyzed using the RNA HS assay kit. The standard reference used was Qubit™ RNA Standard 2 (100 ng/µL), and the method was validated across a dynamic range of 0.2 ng/µL to 200 ng/µL.

8.2.2.1   Sample preparation

For both free RNA and RNA encapsulated in LNPs, a set of serial dilutions were prepared using nuclease-free water. Each sample was further diluted in TE: Tris-EDTA, Tirs-HCl 10 mM pH 7.5 + EDTA 1 mM containing the fluorochrome (Qubit™ working solution) to a final volume of 200 µL per tube and measured in triplicate.

NOTE   The measured concentration in the tube (ng/µL) reflects the effective working range of the assay and is used to build the calibration curve.

8.2.2.2   Results

8.2.2.2.1   Standard

The fluorescence signal showed a linear correlation with the RNA concentration across the tested range.

Table 5 — Comparison of theoretical and experimental concentrations for each RNA standard

Sample	Theoretical concentration (ng/µl)	Experimental concentration (ng/µL)
M1	100.0	97.0
M2	50.0	59.0
M3	25.0	31.8
M4	12.5	16.7
M5	6.3	9.4
M6	3.1	5.0
M7	1.6	*
* Out of range		

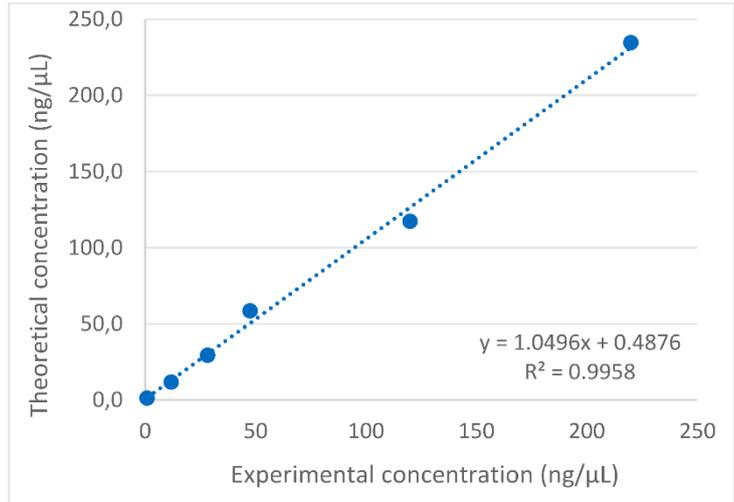|	
Correlation coefficient	0.9887
y-intercept	5.2097
Slope	0.9529
Equation y = ax + b	y = 0.9529x + 5.2097

Figure 6 — Linear regression performance: calibration curve and regression parameters for standard RNA

Correlation coefficient = 0.9887 (specification higher or equal to 0.9). **Complies**.

8.2.2.2.2 RNA

The fluorescence signal showed a linear correlation with the RNA concentration across the tested range.

Table 6 — Comparison of theoretical and experimental concentrations for each RNA sample

Sample	Theoretical concentration (ng/µl)	Experimental concentration (ng/µL)
M1	202.6	118.0
M2	67.5	43.7
M3	22.5	15.7
M4	7.5	5.2
M5	2.5	1.8
M6	0.8	0.6
M7	0.3	*
* Out of range		

Correlation coefficient	0.9985
y-intercept	1.4683
Slope	0.5806
Equation y = ax + b	y = 0.5806x + 1.4683
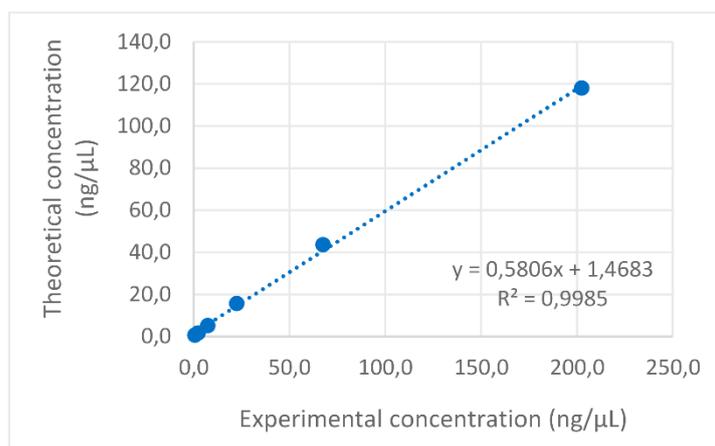
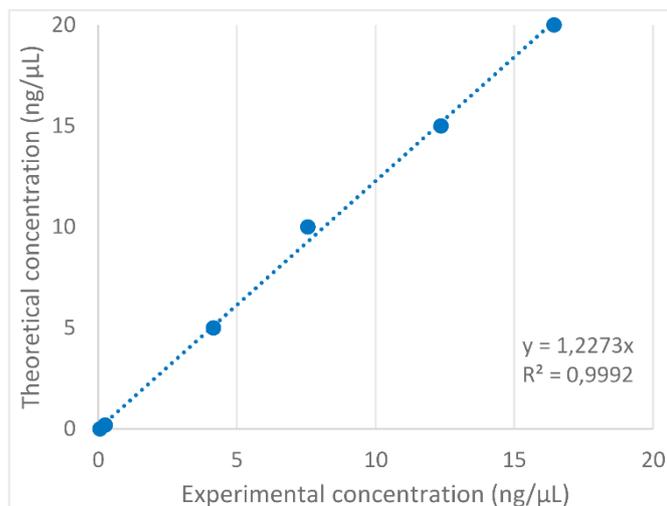**Figure 7 — Linear regression performance: calibration curve and regression parameters for RNA samples**

Correlation coefficient = 0.9985 (specification higher or equal to 0.9). **Complies**.

8.2.2.2.3   RNA-LNPs

The fluorescence signal showed a linear correlation with the RNA concentration across the tested range.

Table 7 — Comparison of theoretical and experimental concentrations for each RNA-LNP sample

Sample	Theoretical concentration (ng/µl)	Experimental concentration (ng/µL)
M1	40.0	34.4
M2	20.0	10.5
M3	10.0	4.2
M4	5.0	2.0
M5	2.5	0.8
M6	1.3	*
* Out of range		

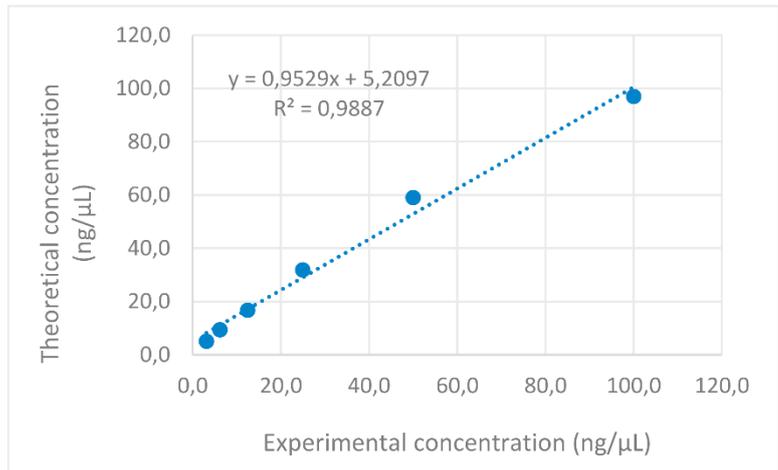|	
Correlation coefficient	0.9654
y-intercept	0.8975
Slope	3.5212
Equation y = ax + b	y = 0.8975x − 3.5212
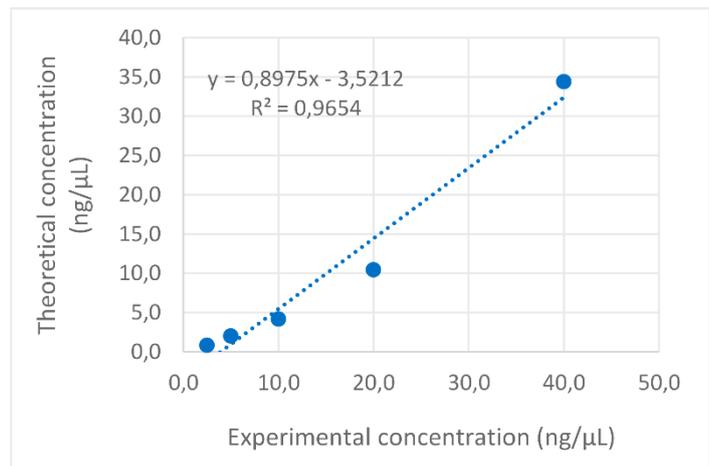
Figure 8 — Linear regression performance: calibration curve and regression parameters for RNA-LNP samples

Correlation coefficient = 0.9654 (specification higher or equal to 0.9). **Complies**.

8.3 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. It was performed with DNA and DNA-LNP sample and with RNA and RNA-LNP sample. The following parameters were analyzed:

— **Instrumental precision**

Instrumental repeatability expresses variability due to instrument.

— **Method repeatability**

Repeatability expresses the precision under the same operating conditions over a short interval of time.

— **Intermediate precision**

Intermediate precision expresses the precision with-in laboratories variations: different days, different analysts.

8.3.1 Instrumental precision

8.3.1.1 DNA

Instrumental precision was assessed performing 10 determinations of a solution of 20 ng/μL prepared from the same solution (Table 8).

Table 8 — Instrumental precision results for standard DNA

Replicates	STD 20 ng/μL
1	19.8
2	19.0
3	19.7
4	19.8
5	19.1
6	18.9
7	18.9
8	19.5
9	19.7
10	18.3
Mean	19.3
SD	0.5
%RSD	**2.6 %**

Acceptance criteria: Complies. RSD %= 2.62, this value is lower than the specification of 10 %.

8.3.1.2 RNA

Instrumental precision was assessed performing 10 determinations of a solution of 10 ng/μL prepared from the same solution (Table 9).

**Table 9 — Instrumental precision results
for standard RNA**

Replicates	STD 10 ng/µL
1	9.5
2	9.5
3	9.2
4	9.2
5	9.5
6	10.0
7	10.0
8	10.0
9	9.6
10	9.2
Mean	9.6
SD	0.3
%RSD	**3.4 %**

Acceptance criteria: **Complies**. RSD %= 3.4, this value is lower than the specification of 10 %.

8.3.2   Method repeatability

8.3.2.1   DNA

The method repeatability was assessed using the three replicates of the following dilutions: 5, 10 and 20 ng/µL of DNA and RNA (Table 10).

Table 10 — Method repeatability results for DNA

Sample	Theoretical concentration (ng/µL)	Experimental concentration (ng/µL)	Recovery (%)		
M5.1.1	5.0	4.9	98.8	Mean	97.7
M5.1.2	5.0	4.7	94.4	SD	2.9
M5.1.3	5.0	5.0	100.0	%RSD	3.0 %
M10.1.1	10.0	9.8	98.2	Mean	94.9
M10.1.2	10.0	10.0	100.0	SD	7.3
M10.1.3	10.0	8.7	86.6	%RSD	7.7 %
M20.1.1	20.0	19.8	99.0	Mean	97.3
M20.1.2	20.0	19.0	95.0	SD	2.1
M20.1.3	20.0	19.6	98.0	%RSD	2.1 %
			TOTAL	Mean	96.7
				SD	2.8
				%RSD	**2.9 %**

Acceptance criteria: Complies

— RSD for every concentration level and total should be lower than 15 %.

8.3.2.2 RNA

The method repeatability was assessed using the three replicates of the following dilutions: 5, 10 and 20 ng/µL of DNA and RNA (Table 11).

Table 11 — Method repeatability results for RNA

Sample	Theoretical concentration (ng/µL)	Experimental concentration (ng/µL)	Recovery (%)		
M5.1.1	5.0	4.9	97.2	Mean	98.7
M5.1.2	5.0	5.1	101.2	SD	2.2
M5.1.3	5.0	4.9	97.6	%RSD	2.2 %
M10.1.1	10.0	9.6	96.4	Mean	95.4
M10.1.2	10.0	9.6	95.8	SD	1.2
M10.1.3	10.0	9.4	94.0	%RSD	1.3 %
M20.1.1	20.0	19.0	95.0	Mean	94.0
M20.1.2	20.0	19.0	95.0	SD	1.7
M20.1.3	20.0	18.4	92.0	%RSD	1.8 %
			TOTAL	Mean	96.0
				SD	0.5
				%RSD	**0.5 %**

Acceptance criteria: **Complies**

— RSD for every concentration level and total should be lower than 15 %.

8.3.3 Intermediate precision

8.3.3.1 DNA

The intermediate precision was assessed using the three replicates of the following dilutions: 5, 10 and 20 ng/µL of DNA and RNA and performing two independent assays at day 1 and 2 by the same analyst that include three replicated of the following dilutions: 5, 10 and 20 ng/µL of DNA and RNA (Table 12).

Table 12 — Intermediate precision results for DNA

	Sample	Theoretical concentration (ng/µL)	Experimental concentration (ng/µL)	Recovery (%)	Mean	SD	%RSD
Day 1	M5.1.1	5.0	4.9	98.8			
	M5.1.2	5.0	4.7	94.4			
	M5.1.3	5.0	5.0	100.0	99.1	6.016	**6.1**
Day 2	M5.2.1	5.0	4.5	90.4			
	M5.2.2	5.0	5.3	106.6			
	M5.2.3	5.0	5.2	104.2			
Day 1	M10.1.1	10.0	9.8	98.2			
	M10.1.2	10.0	10.0	100.0			
	M10.1.3	10.0	8.7	86.6	94.0	7.870	**8.4**
Day 2	M10.2.1	10.0	8.2	81.6			
	M10.2.2	10.0	9.7	97.4			
	M10.2.3	10.0	10.0	100.0			
Day 1	M20.1.1	20.0	19.8	99.0			
	M20.1.2	20.0	19.0	95.0			
	M20.1.3	20.0	19.6	98.0	97.4	1.908	**2.0**
Day 2	M20.2.1	20.0	19.8	99.0			
	M20.2.2	20.0	19.0	95.0			
	M20.2.3	20.0	19.7	98.5			
				TOTAL	Mean	96.8	
					SD	3.05	
					%RSD	**3.15**	

Acceptance criteria: Complies.

Total % RSD (2 days same analyst and all concentration levels) is 3.15 % and it is lower than the specified value 20 %.

8.3.3.2 RNA

The intermediate precision was assessed using the three replicates of the following dilutions: 5, 10 and 20 ng/µL of DNA and RNA and performing two independent assays at day 1 and 2 by the same analyst that include three replicated of the following dilutions: 5, 10 and 20 ng/µL of DNA and RNA (Table 13).

Table 13 — Intermediate precision results for RNA

	Sample	Theoretical concentration (ng/µL)	Experimental concentration (ng/µL)	Recovery (%)	Mean	SD	%RSD
Day 1	M2.5.1.1	2.5	2.8	113.6			
	M2.5.1.2	2.5	2.8	113.6			
	M2.5.1.3	2.5	2.8	112.8	110.0	3.7	**3.4**
Day 2	M2.5.2.1	2.5	2.7	107.2			
	M2.5.2.2	2.5	2.7	107.2			
	M2.5.2.3	2.5	2.6	105.6			
Day 1	M5.1.1	5.0	5.0	99.6			
	M5.1.2	5.0	5.1	101.2			
	M5.1.3	5.0	5.0	100.4	99.5	1.8	**1.8**
Day 2	M5.2.1	5.0	4.9	97.2			
	M5.2.2	5.0	5.1	101.2			
	M5.2.3	5.0	4.9	97.6			
Day 1	M10.1.1	10.0	9.6	96.4			
	M10.1.2	10.0	9.6	95.8			
	M10.1.3	10.0	9.4	94.0	97.3	2.3	**2.4**
Day 2	M10.2.1	10.0	9.8	98.4			
	M10.2.2	10.0	9.9	99.4			
	M10.2.3	10.0	10.0	100.0			
					TOTAL	Mean	102.3
						SD	1.00
						%RSD	**1.0**

Acceptance criteria: **Complies**.

Total % RSD (2 days same analyst and all concentration levels) is 0.98 % and it is lower than the specified value 20 %.

8.4 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference, and the value found.

8.4.1 DNA

8.4.1.1 Procedure

The accuracy was assessed using 9 determinations of DNA covering the specified range for procedure from the dilutions: 5, 10 and 20 ng/µL of DNA (3 replicates/3 dilutions). It was performed with standard DNA and DNA-LNP.

8.4.1.2 Results

Standard DNA:

Table 14 — Accuracy results for standard DNA

Sample	Theoretical concentration (ng/µL)	Experimental concentration (ng/µL)	Recovery (%)
M5.1	5.0	4.9	98.8
M5.2	5.0	4.7	94.4
M5.3	5.0	5.0	100. 0
M10.1	10.0	9.8	98.2
M10.2	10.0	10.0	100.0
M10.3	10.0	8.7	86.6
M20.1	20.0	19.8	99.0
M20.2	20.0	19.0	95.0
M20.3	20.0	19.7	98.5

Acceptance criteria: Complies

All percent recovery is between the intervals 80.0 – 120.0 %.

DNA-LNPs:

Table 15 — Accuracy results for DNA-LNP samples

Sample	Theoretical concentration (ng/µL)	Experimental concentration (ng/µL)	Recovery (%)
M10.1	10.0	9.96	99.60
M10.2	10.0	9.84	98.40
M10.3	10.0	9.92	99.20
M15.1	15.0	15.0	100.0
M15.2	15.0	14.6	97.07
M15.3	15.0	14.4	96.27
M20.1	20.0	22.8	114.0
M20.2	20.0	22.2	111.0
M20.3	20.0	21.4	107.0

Acceptance criteria: Complies

All percent recovery is between the intervals 80.0 – 120.0 %.

8.4.2 RNA

8.4.2.1 Procedure

The accuracy was assessed using 9 determinations of RNA covering the specified range for procedure from the dilutions: 5, 10 and 20 ng/µL of RNA (3 replicates/3 dilutions). It was performed with standard RNA and RNA-LNP.

8.4.2.2 Results

Standard RNA:

Table 16 — Accuracy results for standard RNA

Sample	Theoretical concentration (ng/µL)	Experimental concentration (ng/µL)	Recovery (%)
M5.1	5.0	4.9	97.2
M5.2	5.0	5.1	101.2
M5.3	5.0	4.9	97,6
M10.1	10.0	9.6	96.4
M10.2	10.0	9.6	95.8
M10.3	10.0	9.4	94.0
M20.1	20.0	19.0	95.0
M20.2	20.0	19.0	95.0
M20.3	20.0	18.4	92.0

Acceptance criteria: **Complies**

All percent recovery is between the intervals 80.0 – 120.0 %.

RNA-LNPs:

Table 17 — Accuracy results for RNA-LNP samples

Sample	Theoretical concentration (ng/µL)	Experimental concentration (ng/µL)	Recovery (%)
M1.3.1	1.3	1.2	98.7
M1.3.2	1.3	1.2	98.3
M1.3.3	1.3	1.1	88.0
M2.1	2.0	2.0	97.6
M2.2	2.0	1.9	94.6
M2.3	2.0	1.9	93.2
M4.1	4.0	4.0	99.2
M4.2	4.0	3.9	96.5
M4.3	4.0	4.1	103.0

Acceptance criteria: **Complies**

All percent recovery is between the intervals 80.0 – 120.0 %.

8.5 Robustness

Robustness should show the reliability of an analysis with respect to deliberate variations in method parameters.

8.5.1 Procedure

Two types of robustness tests were performed:

1. The first evaluated the necessity of a heating step prior to sample analysis. A DNA sample was diluted 1:100 and analyzed under two conditions: with and without heating at 71 °C for 2 minutes. Nine determinations were carried out (three replicates per condition).

2. The second test evaluated the impact of different lysis agents and incubation times on encapsulation efficiency measurements. DNA and RNA-loaded lipid nanoparticles (LNPs) were incubated at 37 °C for 5, 10, and 15 minutes with three media:

   - Triton X-100 (2 %) in PBS (w/v).

   - Tween 20 (2 %) in PBS (w/v).

   - PBS (used as a negative control, without surfactant).

8.5.2 Results: Heating Test

Table 18 – Results of the heating test obtained for the DNA samples

Sample	Theoretical concentration (ng/µL)	Experimental concentration without heating (ng/µL)	Experimental concentration after heating (ng/µL)
1.1	11.18	12.0	13.9
1.2	11.18	12.7	13.1
1.3	11.18	13.3	13.1
2.1	11.18	14.2	12.8
2.2	11.18	14.5	12.1
2.3	11.18	13.7	12.5
3.1	11.18	12.9	13.0
3.2	11.18	12.9	12.3
3.3	11.18	12.9	11.4

An ANOVA was conducted to compare the results with and without heating (Table 19):

Table 19 — ANOVA analysis for the comparison of the results with and without heating

Type of variance	Sum of squares	Degree of freedom	Means of squares	F	Probability	Critical value of F
Inter-groups	1.334	1	1.334	2.369	0.143	4.494
Intra-groups	9.009	16	0.563			
Total	10.343	17				

Acceptance criteria: Complies.

Fexp= 2.37 > Ftables= 4.49 at a 95 % confidence level. The associated p-value was 0.143 > 0.05. Based on these results, no statistically significant difference was found between the groups, there's no need of heating the DNA-LNP samples before analyzing them.

8.5.3   Results: Robustness of lysis conditions

To evaluate the robustness of the method with respect to incubation time and lysis conditions, DNA and RNA-loaded lipid nanoparticles (LNPs) were incubated at 37 °C for 5, 10, and 15 minutes using three different media:

- Triton X-100 (2 %) in PBS (w/v).

- Tween 20 (2 %) in PBS (w/v).

- PBS (used as a negative control, without surfactant).

After incubation, encapsulation efficiency (% EE) was determined in triplicate. One-way ANOVA tests were performed to assess whether differences between timepoints and treatments were statistically significant.

8.5.3.1   DNA results

Table 20 — Results obtained for the robustness of the lysis conditions for DNA-LNP samples

Condition	Time (min)	Experimental concentration (ng/µL)			Mean ± SD	% EE	Mean ± SD
Triton X-100 2 % in PBS	5	47.2	50.6	52.0	49.9 ± 2.47	91.7	
	10	51.4	50.2	52.2	51.3 ± 1.01	91.9	91.7 ± 0.193
	15	46.2	50.2	50.4	48.9 ± 2.37	91.6	
Tween 20 2 % in PBS	5	44.2	48.8	49.8	47.6 ± 2.99	91.3	
	10	48.8	47.4	48.6	48.3 ± 0.757	91.4	91.2 ± 0.254
	15	45.6	45.4	46.0	45.7 ± 0.306	90.9	
PBS	5	4.44	5.86	5.96	5.42 ± 0.850	23.7	
	10	5.08	5.48	6.38	5.65 ± 0.666	26.8	23.6 ± 3.25
	15	4.62	5.84	5.10	5.19 ± 0.615	20.3	

a) Effect of incubation time within each treatment

For Triton X-100, Tween 20, and PBS, ANOVA showed no statistically significant differences between the %EE values obtained at 5, 10, and 15 minutes (p > 0.05 for all comparisons).

This confirms that the method is robust with respect to incubation time across the tested range, regardless of the surfactant used.

For Triton X-100 (2 %) in PBS (w/v):

Table 21 — ANOVA results for the effect of incubation time in DNA-LNPs within Triton X 100 (2%) treatment

Type of variance	Sum of squares	Degree of freedom	Means of squares	F	Probability	Critical value of F
Inter-groups	0.236	2	0.118	0.941	0.441	5.143
Intra-groups	0.755	6	0.126			
Total	0.992	8				

Acceptance criteria: Complies.

$F_{exp} = 0.94 < F_{tables} = 5.14$ at a 95 % confidence level. The associated p-value was 0.441 > 0.05.

Based on these results, no statistically significant difference was found between incubation times (5, 10, and 15 minutes) using Triton X-100, confirming the robustness of the method with respect to incubation duration under this condition.

For Tween 20 (2 %) in PBS (w/v):

Table 22 — ANOVA results for the effect of incubation time in DNA-LNPs within Tween 20 (2%) treatment

Type of variance	Sum of squares	Degree of freedom	Means of squares	F	Probability	Critical value of F
Inter-groups	0.375	2	0.188	1.653	0.268	5.143
Intra-groups	0.681	6	0.113			
Total	1.057	8				

Acceptance criteria: Complies.

$F_{exp} = 1.65 < F_{tables} = 5.14$ at a 95 % confidence level. The associated p-value was 0.269 > 0.05.

No statistically significant differences were found between incubation times using Tween 20. The method is robust over the tested incubation range with this lysis agent.

For PBS:

Table 23 — ANOVA results for the effect of incubation time in DNA-LNPs within PBS treatment

Type of variance	Sum of squares	Degree of freedom	Means of squares	F	Probability	Critical value of F
Inter-groups	65.290	2	32.645	0.290	0.758	5.143
Intra-groups	674.326	6	112.388			
Total	739.616	8				

Acceptance criteria: Complies.

F_exp = 0.29 < F_critical = 5.14 at a 95 % confidence level. The associated p-value was 0.758 > 0.05.

Although encapsulation efficiencies were considerably lower with PBS (as expected), no significant difference was observed between the three timepoints. This confirms PBS does not effectively disrupt LNPs under any of the tested conditions and serves as a negative control.

b) Comparison between treatments

A one-way ANOVA comparing the mean values of the three treatments (Triton X-100, Tween 20, and PBS) revealed a statistically significant difference ($p = 1.23 \times 10^{-8}$).

This confirms that PBS is not effective in lysing LNPs, as expected.

Table 24 — ANOVA summary evaluating the impact of different treatments on DNA LNP encapsulation efficiency

Type of variance	Sum of squares	Degree of freedom	Means of squares	F	Probability	Critical value of F
Inter-groups	9212.196	2	4606.098	1295.870	1.232 E-08	5.143
Intra-groups	21.327	6	3.554			
Total	9233.522	8				

Fexp = 1295.87 > Fcritical = 5.14 at a 95 % confidence level. The associated p-value was 1.23×10^{-8} < 0.05.

Statistically significant differences were found between treatments. PBS-treated samples showed substantially lower encapsulation efficiency values, confirming its ineffectiveness as a lysis agent.

c) Direct comparison: Triton vs. Tween 20

Table 25 — Direct comparison of Triton X 100 and Tween 20 in the DNA LNP assay: ANOVA results

Type of variance	Sum of squares	Degree of freedom	Means of squares	F	Probability	Critical value of F
Inter-groups	0.427	1	0.427	9.143	0.039	7.709
Intra-groups	0.187	4	0.047			
Total	0.613	5				

Acceptance criteria: Does not comply statistically, but acceptable in practice.

Fexp = 9.14 > Ftables = 7.71 at a 95 % confidence level. The associated p-value was 0.039 < 0.05.

Although a statistically significant difference was observed, the absolute variation between treatments was less than 0.6 %, and both yielded high and consistent encapsulation efficiencies. The statistical significance is attributed to extremely low intra-group variability, rather than to a meaningful difference

in performance. Therefore, the difference is not considered practically relevant, and both agents are deemed equivalent for routine use.

Conclusion

While statistical significance was found between Triton and Tween 20, the absolute difference in %EE was negligible and within the method's variability range. Both surfactants can therefore be considered equivalent for practical purposes, confirming the method's robustness with respect to lysis agent selection.

Furthermore, no statistically significant differences were observed between incubation times (5, 10, and 15 minutes) for any of the tested lysis conditions. This indicates that the method is robust across a range of incubation durations. For practical purposes, 10 minutes is recommended as the standard incubation time, as it provides consistent results and aligns with protocol simplicity and reproducibility.

9   Limitations

Although the method has demonstrated acceptable performance in terms of specificity, linearity, precision, accuracy, and robustness, certain limitations should be considered:

- **Lower detection limit**: The method is not suitable for nucleic acid concentrations below 0.01 ng/µL, as signal-to-noise ratio becomes insufficient and results are no longer reliable.

- **Maximum concentration limit**: At concentrations above 240 ng/µL, the fluorescent signal may exceed the linear range of the fluorometer, leading to saturation and inaccurate readings. Samples in this range must be appropriately diluted.

- **Matrix interference**: Although formulation components such as Triton X-100 and ethanol-solubilized lipids were shown not to interfere at the tested concentrations, excessive lipid or detergent content may affect dye performance and fluorescence baseline.

- **Lysis efficiency**: Complete disruption of LNPs is dependent on the correct concentration and incubation time with Triton X-100. Variations in lipid composition or particle size could affect lysis and, therefore, total nucleic acid quantification.

- **Kit-specific reagents**: The method has been validated based on the use of proprietary reagents from Qubit™ DNA or RNA assay kits. Substitution with alternative dyes or buffers may alter assay behavior and must be revalidated accordingly.

- **Sample stability**: Once mixed with the Qubit™ working solution, fluorescence stability is time-limited (up to 30 minutes for ssDNA kit and up to 3 hours for RNA kit at room temperature). Measurements should be performed within these time frames.

Overall, the method proved to be suitable for the accurate and reliable determination of nucleic acid encapsulation efficiency in LNPs, fulfilling analytical validation criteria across all parameters tested.

Annex A
(informative)

Example of application of the method: determination of DNA encapsulation efficiency in LNPs

This annex provides a practical example of how to apply the fluorometric method described in this document to determine the encapsulation efficiency of a DNA-loaded LNP formulation.

A.1 Sample description

- Sample: DNA-loaded lipid nanoparticles (LNPs). The composition is 96.25 % (w/w) a lipid mixture in ethanol (ALC-0315:DSPC:Chol:ALC-0159 in the following molar ratios 46.3:9.4:42.7:1.6) and 3.75 % (w/w) DNA in citrate buffer pH 4.8.

- Nucleic acid: single-stranded DNA (ssDNA).

- Fluorometric assay: Qubit™ ssDNA Assay Kit, Qubit™ 4 Fluorometer.

- Dye incubation time: 2 min.

- Lysis conditions: 1:1 dilution with 2 % Triton X-100 in PBS, 10 min at 37 °C.

A.2 Experimental data

Follow the steps in 6. Procedure.

A.3 Experimental results

Table A.1 — Experimental results for the example of the determination of the encapsulation efficiency

Sample	Without Triton		With Triton	
	Free DNA concentration (ng/µL)	Mean	Total DNA concentration (ng/µL)	Mean
1.1	0.798		67.6	
1.2	0.760	0.771 ± 0.023	67.6	66.9 ± 1.155
1.3	0.756		65.6	

A.4 Encapsulation efficiency calculation

$$\% \, EE = \frac{Total \, NA \, - Free \, NA}{Total \, NA} \cdot 100 = \frac{66.9 - 0.771}{69.9} \cdot 100 = \mathbf{98.85} \, \%$$

A.5 Conclusion

The encapsulation efficiency of the tested DNA-LNP formulation was found to be 98.85 %, demonstrating the applicability of the method to quantify the nucleic acid loading efficiency in nanoparticulate systems.