# Development and imaging validation of fluorescent lipid nanoparticles for RNA delivery applicable to personalized *in vitro* oncology models

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## Abstract #1867

Tebubio offers Contract Research Services (CRS) in RNA-based therapeutic discovery, *in vitro* modeling, biomarker mapping, and data analysis. Leveraging these platforms, we have developed innovative lipid nanoparticles (LNPs) designed to monitor the RNA delivery. This advancement features a fluorescent lipid, synthesized by Echelon Inc., which enables imaging-based tracking of the LNPs. Using this fluorescent lipid, we formulated LNPs encapsulating fluorescent RNA. We validated the distribution of these LNPs within mammalian cells and confirmed RNA expression in these cells through imaging techniques.

This technology is applicable to *in vitro* oncology models, including patient-derived spheroids and tumoroids, which allow for a personalized approach to therapeutic testing. Ultimately, Tebubio's platform provides end-to-end support, from RNA optimization and custom LNP formulation to efficacy validation *in vitro*, enabling personalized medicine models for RNA-based therapeutics.

## INTRODUCTION

RNA vaccines have shown their full potential in the Covid-19 pandemic. The application of these technologies for the treatment of cancers represents a significant hope, but clinical effectiveness remains to be demonstrated. For this purpose, researchers require high quality production and encapsulation of vaccine RNA, and accurate and cost-saving test tools that are adapted to oncology research.

To answer to this growing need for RNA, improvements in manufacturing throughput and turnaround time are required. Tebubio CRS laboratory offers RNA production and encapsulation services providing µg amounts of custom encapsulated RNA to be used for screening purposes in preclinical research (vaccine, therapeutic agent, personalized medicine, etc) for the pharmaceutical industries, biotechs and academics.

Here, we used fluorescent lipids to monitor eGFP mRNA in a 3D model of CRC to monitor its incorporation and eGFP expression allowing us to select specific formulation recipe targeting CRC spheroids for subsequent RNA-based treatment.

## Material and methods

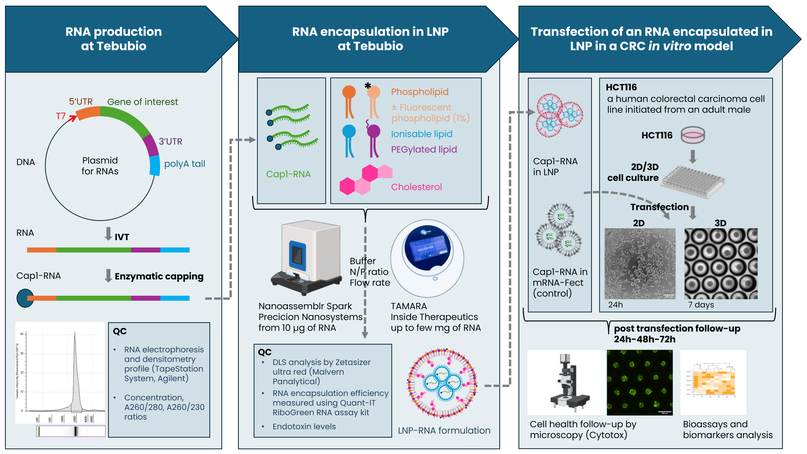


**Figure 1.** Study workflow from RNA production to readout after incubation on 2D or 3D culture of HCT116 including formulation in LNP and quality controls.

### RNA production at Tebubio

RNAs (here eGFP) are synthetized using Tebubio's proprietary plasmid DNA template with UTRs and poly-A tail optimized for efficient transcription and translation in mammalian cells. The T7-FlashScribe transcription and capping kits (Cellscript, Ref. C-ASF3507 and C-SCCS2250) were used for the RNA synthesis. RNAs were purified using Mag-Bind® TotalPure NGS magnetic beads (Omega Biotek M1378).

### RNA encapsulation in LNP at Tebubio

LNPs were prepared using the Spark Nanoassemblr (Precision Nanosystems, Cytiva). Fluorescently labelled LNPs were prepared by incorporating 0.1 % (mol %) of Cy3-1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine to the formulation (Echelon BioSciences, custom product). Different ionisable lipids were used: the classical ALC-0315 and SM-102 (Echelon Biosciences), CP-LC-0741 (Certest) as well as IMC21.5c (LipidBrick, Polyplus/Sartorius).

### Transfection of an RNA encapsulated in LNP in a CRC *in vitro* model

For 2D studies, cells were plated into 96-well plates and transfected with mRNA either LNP or mRNA-Fect (RJH Biosciences, Ref. 80-40) 24h post plating and followed under the microscope for 72h.

For 3D studies, cells were plated into 96-well ultralow-attachment plates at densities of 40 cells/well or microwell and grow for 7 days to reach ~400 µm diameter. Spheroids were then transfected with mRNA in either LNP or mRNA Fect reagent or incubated in drug-doped (5-fluorouracile (5-FU)) media for treatment controls and followed under the microscope for 72h. Incucyte® Cytotox Dye (Sartorius, 4632/33) was used to assess the proportion of apoptotic cells per spheroid.

## Fluorescent LNPs encapsulating eGFP mRNA exhibit similar features to standard LNPs

To better track the fate of the LNPs, we incorporated a fluorescent dye (Cy3) which was conjugated to a structural lipid (DPPE). This would allow us to correlate protein expression with LNP interaction with cellular membranes.

We first checked the physico-chemical properties of the LNPs produced with or without fluorescent lipids. The particles were both very similar in terms of size (z-average ~ 70-75 nm) and polydispersity (PI<0.2), suggesting that the addition of fluorescent lipids did not impact the physico-chemical properties of the LNPs. Similarly, encapsulation efficiency, as assessed by Quant-iT RiboGreen (R11490), led to similar results (~80 % for both) suggesting that addition of a fluorophore did not impact mRNA encapsulation.

Given these results, we prepared several formulations encompassing different ionizable lipids and structural lipids.

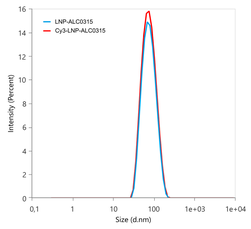


**Figure 2.** Size distribution by density obtained by DLS on LNP with (Cy3-LNP-ALC0315) or without incorporation of Cy3-labelled lipid (LNP-ALC-0315)

| Formulation | LNP-ALC0315 | Cy3-LNP-ALC0315 |
|---|---|---|
| z-average (nm) | 71.1 +/- 0.9 | 74.8 +/- 1.4 |
| Polydispersity index | 0.15 +/- 0.004 | 0.13 +/- 0.006 |

**Table 1.** DLS analysis of LNP with (Cy3-LNP-ALC0315) or without (LNP-ALC0315) Cy3-labelled lipids shows little to no effects on particle properties.

## eGFP mRNA encapsulated in fluorescent LNP is successfully delivered and expressed in HCT116 cultured in 2D

In order to screen different LNPs formulations for their efficiency to transfect HCT116 cell models, we prepared 7 different LNPs containing a fluorescent ionizable lipid (**Table 2**). Then, we performed tranfection of 2D HCT116 cells using 200 ng of mRNA. Protein expression was assessed by following eGFP expression at 24 and 48h (**Figure 3**).

- Using fluorescent LNPs allowed us to track our LNPs and their fate in the culture up to 3 days post transfection
- Three formulations allowed the efficient transfection and high expression of eGFP (F4, F6, F7)
- Some formulations (F1, F3) displayed high Cy3 fluorescent signal, suggesting LNP fusion to cellular membranes, but without correlation of protein expression.

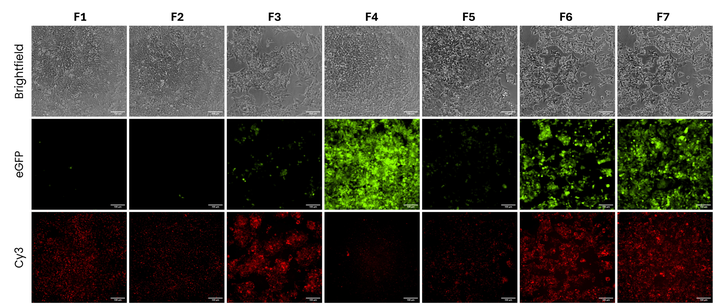


**Figure 3.** Transfection efficiency of 7 different LNP formulations in HCTT116 culture models. Cells were transfected with 200 ng of eGFP-mRNA encapsulated in LNPs. Acquisitions were performed 48 hrs post-transfection to monitor protein expression (eGFP), cell survival (Brightfield) and LNP distribution (Cy3).

| Formulation | F1 | F2 | F3 | F4 | F5 | F6 | F7 |
|---|---|---|---|---|---|---|---|
| Particularities | LipidBrick® IM21,7c (Polyplus) + DODMA | LipidBrick® IM21,7c (Polyplus) | CP-LC-0741 (Certest) | ALC-0315 (ABP) | SM-102 (ABP) | ALC-0315 (Echelon) | SM-102 (Echelon) |
| Encapsulation efficiency (%) | 99,6 | 99,8 | 90,3 | 84,6 | 82,9 | 87,2 | 91,1 |
| z-average (nm) | 95,4 | 100,4 | 90,7 | 104,3 | 129,9 | 100,1 | 95,7 |
| Polydispersity index | 0,29 | 0,24 | 0,20 | 0,07 | 0,06 | 0,07 | 0,09 |

**Table 2.** Main characteristics of fluorescently-labelled LNPs produced without optimization using Nanoassemblr Spark instrument (Precision Nanosystems, Cytiva)

## Treatment with 5FU induces apoptosis in HCT116 spheroids in a concentration-dependent manner *in vitro*

5-Fluorouracil (5-FU) is a commonly used anticancer drug for colorectal cancer (CRC). We examined the sensitivity to 5-FU and survival of 7-days old tumor spheroids after 5FU exposition for three days.

Exposure to increasing doses of 5FU increases the apoptosis signal levels as shown in **Figure 4.** This indicates that we can replicate the effect of the 5FU drug in Tebubio's *in vitro* model for CRC.

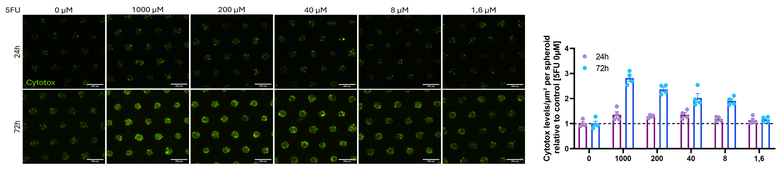


**Figure 4. (left)** Representative images of HCT116 spheroids exposed to different concentrations of 5FU at 24h or 72h post exposition. Cytotox (green) was used to quantify in **(right)** apoptosis levels in the spheroids relative to controls (5FU at 0 µM). n=4 wells with between 16 to 25 spheroids. Scale bar = 500 µm.

## eGFP encapsulated in ALC-0315 LNP is efficiently delivered and expressed in HCT116 spheroids

To check whether the efficiency of an anti-cancer mRNA/LNP-based treatment could be tested in our CRC *in vitro* model, we performed a proof-of-concept experiment where we encapsulated an eGFP mRNA in LNP **(F4)**, transfected the 7-days old tumor spheroids, and followed the expression of eGFP and cell survival for 3 days.

- 3 days after transfection, eGFP is expressed in a concentration dependent manner in the HCT116 spheroid **(Figure 5, eGFP)**
- No increase of cytotoxicity was observed in eGFP/LNP transfected spheroids **(Figure 5, Cytotox)**

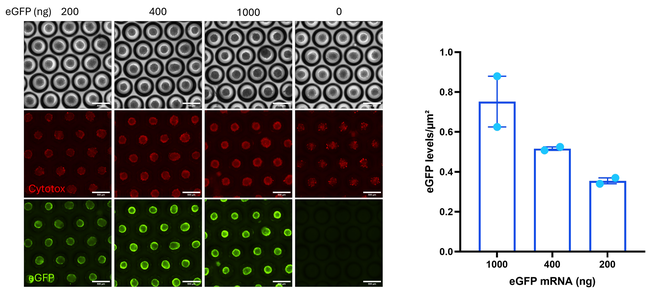


**Figure 5. (left)** Representative images of HCT116 spheroids transfected with different concentrations of eGFP (green) encapsulated in LNP at 72h post transfection. Cytotox (red) was used to assess apoptosis levels in the spheroids. **(right)** Levels of eGFP were quantified by Image J and reported in a graph. n=2 wells with between 20 to 24 spheroids. Scale bar = 500 µm.

## Conclusion & Perspectives

- Fluorescent lipids allowed us to track and follow the fate of the LNP in the cell culture overtime.
- Some transfections that showed high level of LNP attached to the cells but resulted in low eGFP expression could indicate defects in endocytosis, which will require further investigation.
- We successfully showed that transfecting an mRNA encapsulated in LNP could be used for anti-cancer treatment in a 3D *in vitro* model of CRC.
- New investigations will aim at testing whether expressing a target protein via the transfection of its mRNA encapsulated in LNP could be used as an anti-cancerous treatment in an *in vitro* model of CRC.