Application Note

Encapsulation and Transfection of RNA Using LipidLaunch™ SM-102 Lipid Nanoparticles (Loadable)

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Key Features

- Cayman's **LipidLaunch™ SM-102 LNP Kit (Loadable)** is comprised of lyophilized SM-102-based lipid nanoparticles (LNPs) prepared without cargo.
- Allows researchers to encapsulate RNA cargo of choice for LNP-mediated transfection without microfluidic mixing devices.
- Enable RNA delivery to difficult-to-transfect cell lines and primary cultures.
- LipidLaunch™ SM-102 LNPs (Loadable) facilitate effective RNA transfection with minimal cytotoxicity compared to traditional transfection reagents.

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Introduction

Nanoparticle delivery systems such as lipid nanoparticles (LNPs) have been at the forefront of recent advances in delivery of bioactives for diagnosing and treating disease.[1] LNPs are typically composed of four types of lipids: a cationic or ionizable lipid, a helper phospholipid, a PEGylated lipid, and cholesterol. Release of LNP cargo into target cells is strongly influenced by the ionizable lipid component. Ionizable cationic lipids are protonated in the acidic environment of endosomes, resulting in membrane disruption and release of cargo into the cell.[2] SM-102 is a synthetic amino lipid that has been used in the Moderna COVID-19 vaccine and is among the best characterized ionizable lipids for LNP formulation.[3]

In conventionally loaded LNPs, particles are simultaneously formed and loaded with nucleic acid cargo by controlled mixing of an ethanolic lipid mixture with an aqueous cargo-containing component. This is possible without specialized equipment using manual or injection-based mixing methods. However, these methods typically result in poorly controlled particle diameter with a larger particle size distribution, which may compromise the efficacy of cargo delivery and release.[4]

Commercially available microfluidic injection methods overcome these limitations, enabling careful and reproducible control of flow rates, and resulting in highly consistent LNP diameter and narrow size distribution. The relatively high instrument cost may, however, limit access to these methods for many investigators, resulting in reliance on imperfect manual methods.

The LipidLaunch™ SM-102 LNP Kit (Loadable) is comprised of empty SM-102-containing LNPs, which can encapsulate nucleic acid cargo for subsequent delivery to target cells or tissues. Following LNP rehydration, the LipidLaunch™ SM-102 LNPs (Loadable) may be mixed with RNA cargo of choice and subsequently delivered to cells (**Figure 1**).

In this study, the performance of LipidLaunch™ SM-102 LNPs (Loadable) was characterized in the presence of three different types of RNA cargo. Particle size parameters, encapsulation efficiency, and transfection efficiency were assessed in four different cell lines. The impact of transfection on cell viability with LipidLaunch™ SM-102 LNPs (Loadable) was compared with two leading competitor transfection reagents.

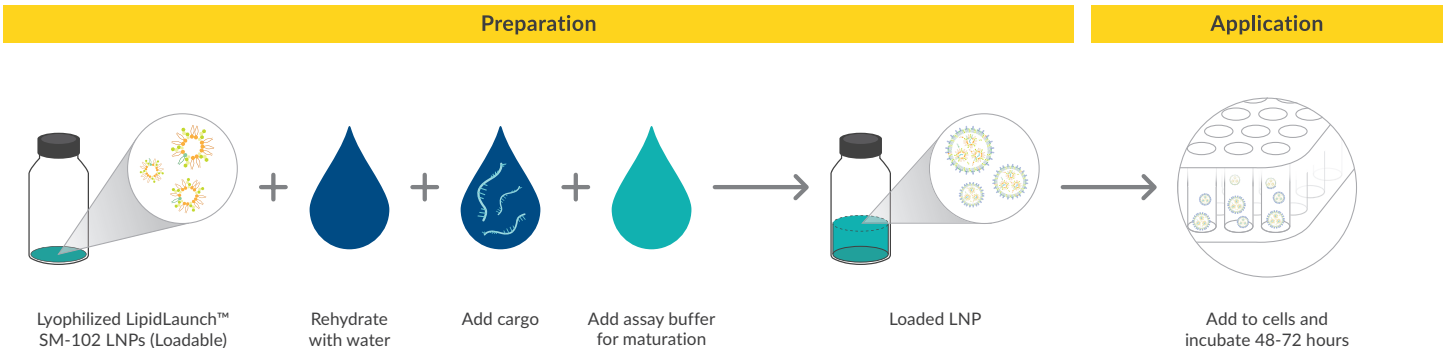


Figure 1. Cargo encapsulation with LipidLaunch™ SM-102 LNPs (Loadable). LipidLaunch™ SM-102 LNPs (Loadable) are rehydrated with nuclease-free water. The cargo is introduced, and assay buffer is added to promote cargo encapsulation. Loaded LNPs may then be added directly to cell culture media.

Methods

Cell Culture

Cells were seeded 24 hours prior to treatments at 4,000 cells per well (96-well plates) or 20,000 cells per well (24-well plates) for all tested cell lines. A549 cells were cultured in F12K media supplemented with 10% FBS and 1% penicillin-streptomycin, SH-SY5Y cells were cultured in DMEM/F12 containing 10% FBS and 1% penicillin-streptomycin, and RAW 264.7 cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin.

Primary differentiated osteoblast cultures were prepared by isolating mesenchymal stem cells from rat long bones and culturing for three weeks in complete medium supplemented with 10 nM dexamethasone and 50 µM ascorbic acid. On day 21, media were supplemented with 10 mM β-glycerophosphate to induce mineralization. Transfection experiments were initiated on day 23.

LipidLaunch™ SM-102 LNP (Loadable) Encapsulation and Transfection

For all transfection experiments, one vial of LipidLaunch™ SM-102 LNPs (Loadable) was rehydrated with 200 µl nuclease-free water. Luciferase or mCherry mRNA cargo (ApexBio), or COX-2 siRNA (Ambion) was initially diluted in nuclease-free water prior to mixing with reconstituted LNPs in a 4:1 (LNP:RNA) volume ratio with gentle mixing. This mix was then further diluted 4-fold in assay buffer to promote encapsulation. Loaded LNPs were subsequently diluted in the appropriate complete medium at 10% the final well volume. Cells were incubated for 48-72 hours prior to analysis. For control (no LNP) conditions, cargo was diluted in PBS and added to media at equivalent concentrations. Scrambled siRNA (Ambion) was prepared in the same manner as experimental cargoes above as negative controls for siRNA experiments.

Particle Analysis

Average particle size (z average diameter) of LipidLaunch™ SM-102 LNPs (Loadable) encapsulating cargo was determined using a Stunner DLS instrument (Unchained Labs). RNA encapsulation efficiency was quantified *via* Quant-iT™ RiboGreen RNA Assay Kit (Invitrogen) as previously described.[5] The amount of encapsulated RNA was determined by subtracting the free unencapsulated concentration from the total concentration. Encapsulation efficiency (EE%) was then calculated as follows:

$$EE\% = (\text{Encapsulated RNA}/\text{Total RNA}) \times 100$$

Imaging Analysis

72 hours after cell treatments, culture medium was replaced with a phenol red-free medium containing 16 µM Hoechst 33342 (Cayman Chemical) dye to stain nuclei. 20X images were acquired using a Cytation™ 5 cell imaging multi-mode plate reader (Agilent) in brightfield mode with Ex390/Em442 and Ex586/Em647 filter sets to image Hoechst and mCherry fluorescence signals, respectively. To determine transfection efficiency, cell regions of interest were generated in the DAPI channel using Gen5 software, and the mCherry fluorescence intensity was calculated for each cell. The percentage of cells with positive mCherry fluorescence was subsequently determined to generate transfection efficiency. Error bars indicate the standard deviation.

Western Blotting

A549 cells were transfected in 24-well plates as described above and incubated for 72 hours prior to rinsing wells with PBS followed by cell lysis in M-PER buffer (Thermo Fisher). Protein was quantified *via* NanoDrop (Thermo Fisher) prior to sample preparation in 4X Laemmli buffer supplemented with β-mercaptoethanol. Samples were separated *via* SDS-PAGE, transferred onto PVDF membranes, then probed with monoclonal antibodies specific to COX-2 (Cayman Chemical) and GAPDH (Abcam) as a loading control.

Luciferase Assay

Luciferase activity was determined using a luciferase reporter assay (Cayman Chemical) according to manufacturer's instructions 72 hours following transfection. Culture media was removed, and luciferin assay buffer was added to the wells prior to immediate luminescence measurement using a Cytation™ 5 cell imaging multi-mode plate reader. Luminescence signal was monitored, and relative luminescence values were determined by averaging the signal for each well over the first 5 minutes.

Competitor Reagent Comparison

Competitor mRNA-specific or general lipofection reagents were prepared according to manufacturer's instructions. Cells were treated at mCherry mRNA concentrations ranging from 50 to 200 ng per well. Control wells were treated with complete culture medium only. Cell viability was determined after 72 hours *via* Calcein AM (Cayman Chemical) staining. 10 µM Calcein AM and Hoechst 33342 were added to media, and cells were incubated for 30 minutes prior to imaging at 4X magnification using a Cytation™ 5 cell imaging multi-mode plate reader with GFP (Calcein AM) and DAPI (Hoechst) filter sets. Viable cell counts were determined by generating cell regions of interest in the DAPI channel then counting the number of Calcein AM-positive cells using Gen5 cellular analysis software.

Results

Characterization of mRNA-loaded LipidLaunch™ SM-102 LNPs (Loadable)

LipidLaunch™ SM-102 LNPs (Loadable) were tested for their capacity to encapsulate and transfect mCherry mRNA into A549 lung epithelial cells. Following mRNA encapsulation, quantification of mRNA in the loaded LNPs indicated >80% of mCherry mRNA was encapsulated into the LNPs (**Table 1**), and LNPs exhibited an average particle size of ~115 nm, which is within the typical reported range.[6] A549 cells were then treated with mCherry-loaded LNPs for 72 hours prior to fluorescence imaging (**Figure 2**). 97% of cells demonstrated positive mCherry fluorescence.

Table 1. Particle characteristics of mCherry mRNA-loaded LNPs.

	Z Average Diameter (nm)	RNA Encapsulation Efficiency (%)	Transfection Efficiency (%)
LipidLaunch™ SM-102 LNPs (Loadable)	115 ± 5.1	82 ± 0.9	97 ± 1.1

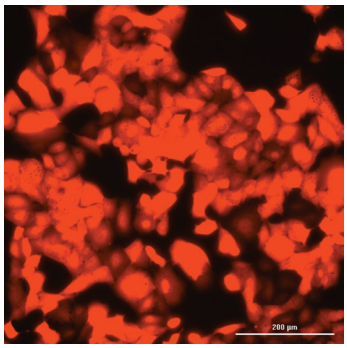
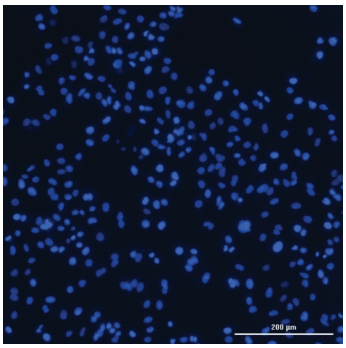
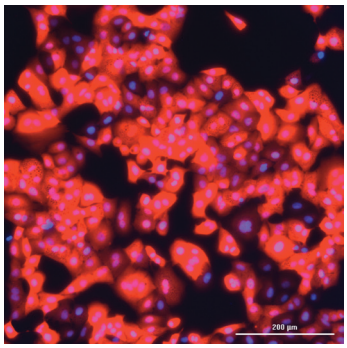
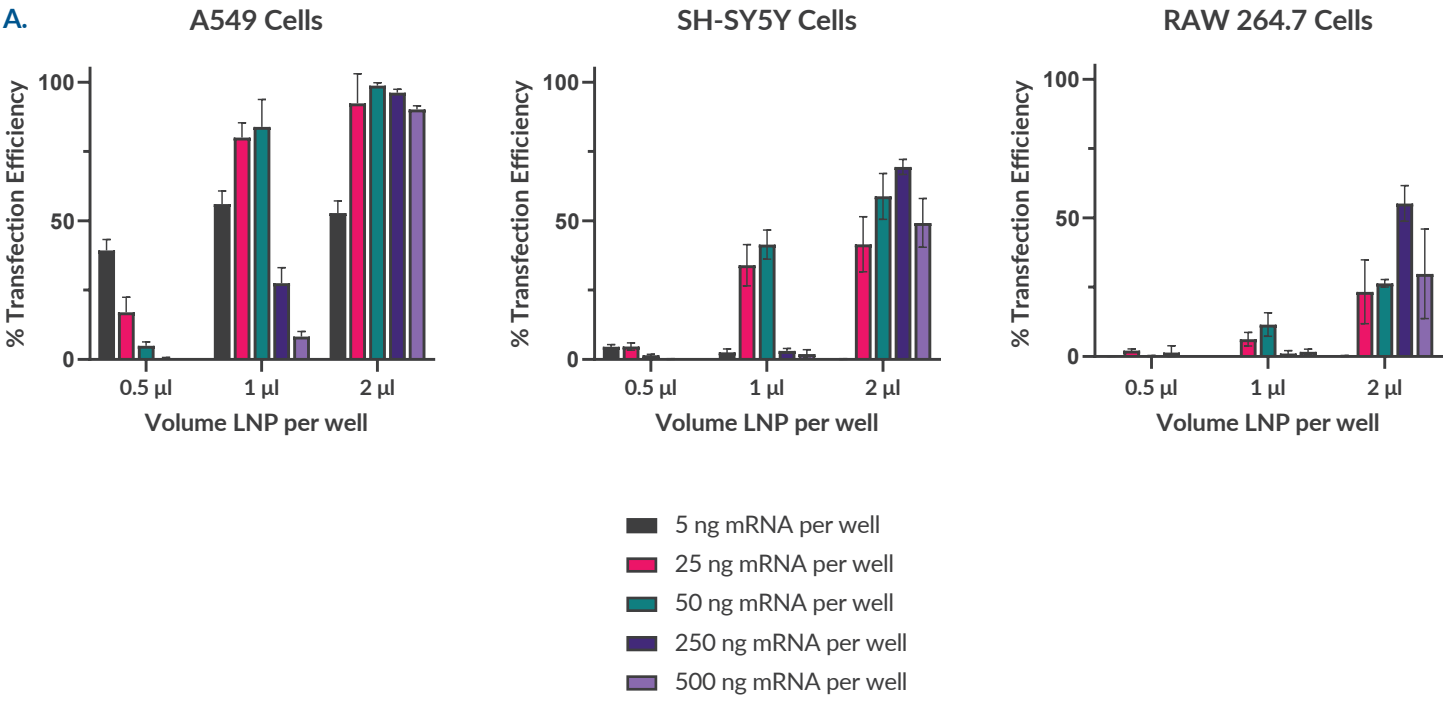
	mCherry	Hoechst	mCherry/Hoechst
A549 Cells			

Figure 2. mCherry transfection with LipidLaunch™ SM-102 LNPs (Loadable). A549 cells were transfected with LipidLaunch™ SM-102 LNPs (Loadable) loaded with mCherry mRNA. 72 hours later, fluorescence imaging was performed, and transfection efficiency was determined (Table 1). Four wells per condition were measured.

LNP/mRNA Cargo Titration in Different Cell Lines

mCherry mRNA was encapsulated into LipidLaunch™ SM-102 LNPs (Loadable) at various concentrations prior to transfection in three different cell lines, including typically difficult-to-transfect SH-SY5Y and RAW 264.7 cells (**Figure 3**). All three cell lines exhibited positive mCherry fluorescence at various mRNA/LNP ratios, with as little as 0.5 µl LNP and 5 ng mRNA per 100 µl media.

A.



B.

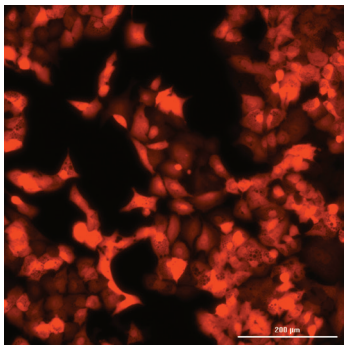
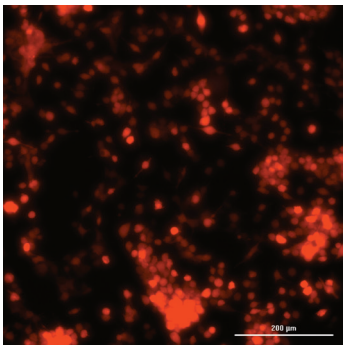
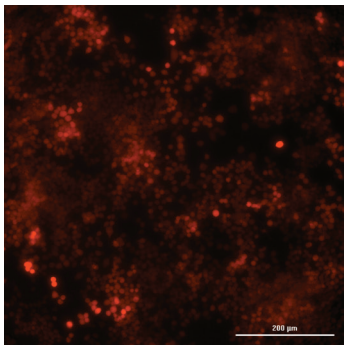
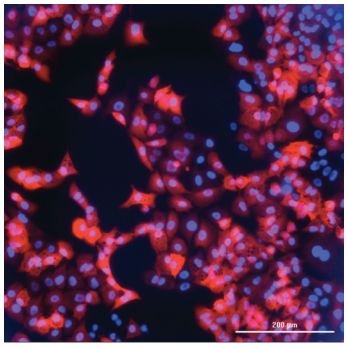
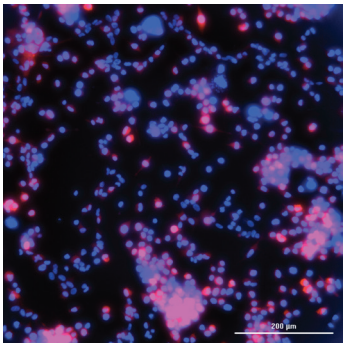
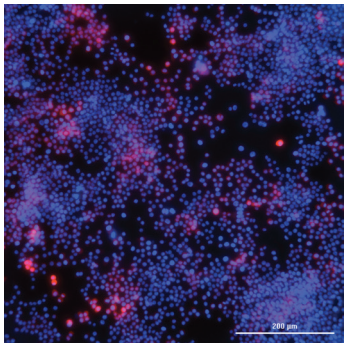
	A549 Cells	SH-SY5Y Cells	RAW 264.7 Cells
mCherry			
mCherry/Hoechst			

Figure 3. LNP/mRNA titration in different cell lines. 0.5-2 µl LNP per 100 µl media were loaded with 5-500 ng mCherry mRNA prior to transfection of A549, SH-SY5Y, and RAW 264.7 cells in 96-well plates. 72 hours later, fluorescence imaging was performed, and transfection efficiency was determined (**Panel A**). Representative images are shown from the top-performing transfection condition for each cell line (**Panel B**; A549: 2 µl LNP/50 ng mRNA; SH-SY5Y: 2 µl LNP/250 ng mRNA; RAW 264.7: 2 µl LNP/250 ng mRNA per well). Three wells per condition were measured.

The effect on cytotoxicity of LipidLaunch™ SM-102 LNPs (Loadable) was assessed in two different cell lines in the presence of 500 ng/ml mRNA-loaded LNPs (**Figure 4**). A549 and SH-SY5Y cells were incubated for 72 hours without media changes. Both cell lines exhibited negligible cytotoxicity in the 0.5-2 µl LNP per 100 µl media range, with cytotoxicity becoming evident at 50 µl LNP/ml. Collectively, these data suggest that LipidLaunch™ SM-102 LNPs (Loadable) can be used to transfect cells up to 2% final media concentration, yielding high transfection efficiency and minimal cytotoxic effects.

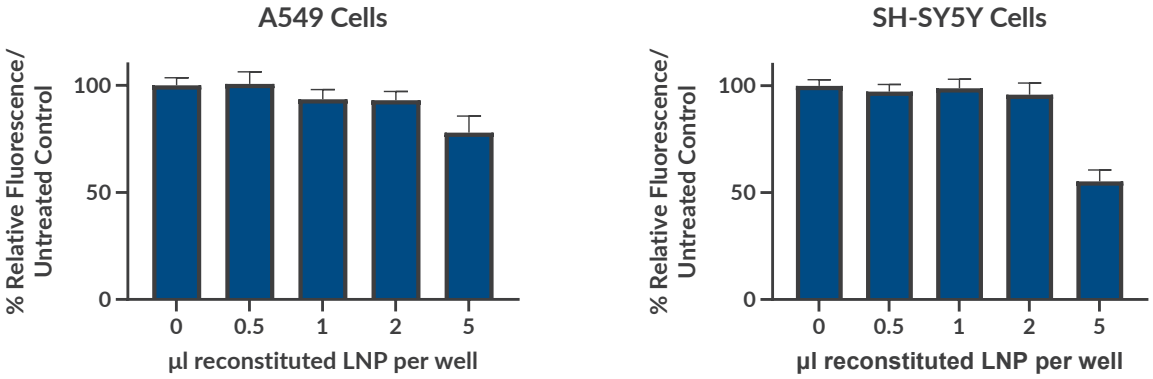


Figure 4. Cell viability in cells treated with LipidLaunch™ SM-102 LNPs (Loadable) encapsulating mCherry. A549 and SH-SY5Y cells were transfected with mCherry mRNA via LipidLaunch™ SM-102 LNPs (Loadable) with 0-5 µl LNP per 100 µl media and 50 ng mRNA per 100 µl media in 96-well plates. Cell viability was determined using the **Resazurin Cell Viability Assay Kit** (Cayman Chemical). Five wells per condition were measured.

Transfection with small and large RNAs using LipidLaunch™ SM-102 LNPs (Loadable)

A549 cells cultured in 24-well plates were transfected with scrambled siRNA or COX-2 siRNA using LipidLaunch™ SM-102 LNPs (Loadable). 48 hours later, effective knockdown of COX-2 was confirmed by Western blot (**Figure 5**).

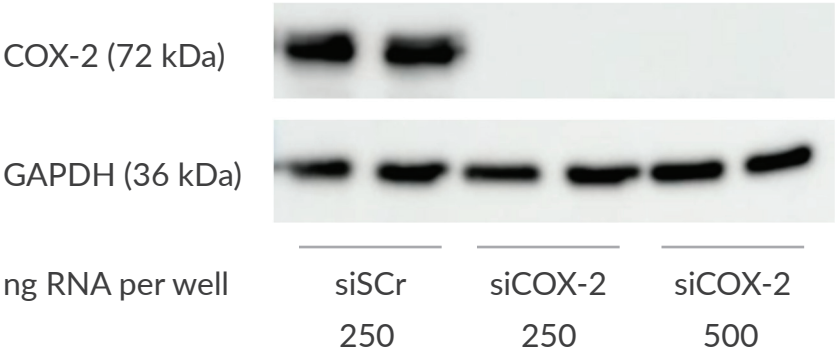


Figure 5. COX-2 knockdown in A549 cells with LipidLaunch™ SM-102 LNPs (Loadable). A549 cells were transfected with COX-2 siRNA via LipidLaunch™ SM-102 LNPs (Loadable) (250-500 ng siRNA per well). Knockdown was confirmed by Western blot using anti-COX-2 and GAPDH (loading control) antibodies.

The capacity of LNPs to encapsulate RNA cargo may depend on both the concentration and size of the transcript.[7] The mCherry mRNA tested in prior experiments was 996 nucleotides in length. Luciferase mRNA, at approximately double the size (1,921 nucleotides), was subsequently tested with LipidLaunch™ SM-102 LNPs (Loadable). A549 cells cultured in 96-well plates were transfected with various concentrations of luciferase mRNA using 0-4 µl LipidLaunch™ SM-102 LNPs (Loadable). Luminescence was evident in all LNP-treated conditions, confirming encapsulation and transfection of larger mRNA transcript sizes (**Figure 6**).

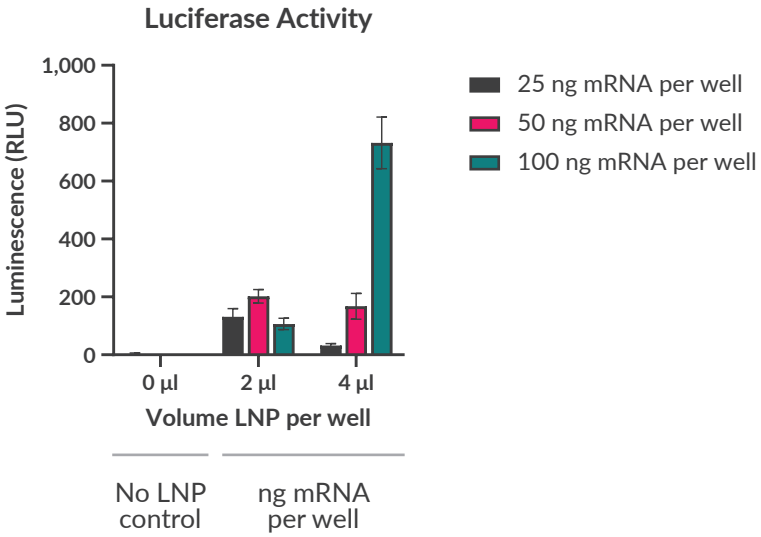


Figure 6. Luciferase transfection of A549 cells with LipidLaunch™ SM-102 LNPs (Loadable). A549 cells were transfected with luciferase various concentrations of mRNA via LipidLaunch™ SM-102 LNPs (Loadable) (25-100 ng mRNA per well). Luciferase activity was determined 72 hours later using a cell-based luciferase assay. Five wells per condition were measured.

Primary Cell Transfection with LipidLaunch™ SM-102 LNPs (Loadable)

Primary mammalian cells are typically less amenable to transfection than other cell types.[8] Primary differentiated osteoblasts were transfected with mCherry mRNA *via* LipidLaunch™ SM-102 LNPs (Loadable) in the same manner as previously tested cell lines. 48 hours following transfection, fluorescence imaging demonstrated widespread mCherry fluorescence in LNP-transfected wells (**Figure 7**).

Of particular note, the dense mass of cells present in the lower panels, reflecting formation of bone-like nodules, exhibited positive fluorescence beyond the focal plane, indicating LipidLaunch™ SM-102 LNPs (Loadable) may penetrate multiple layers of cells and extracellular matrix to successfully deliver RNA cargo.[9]

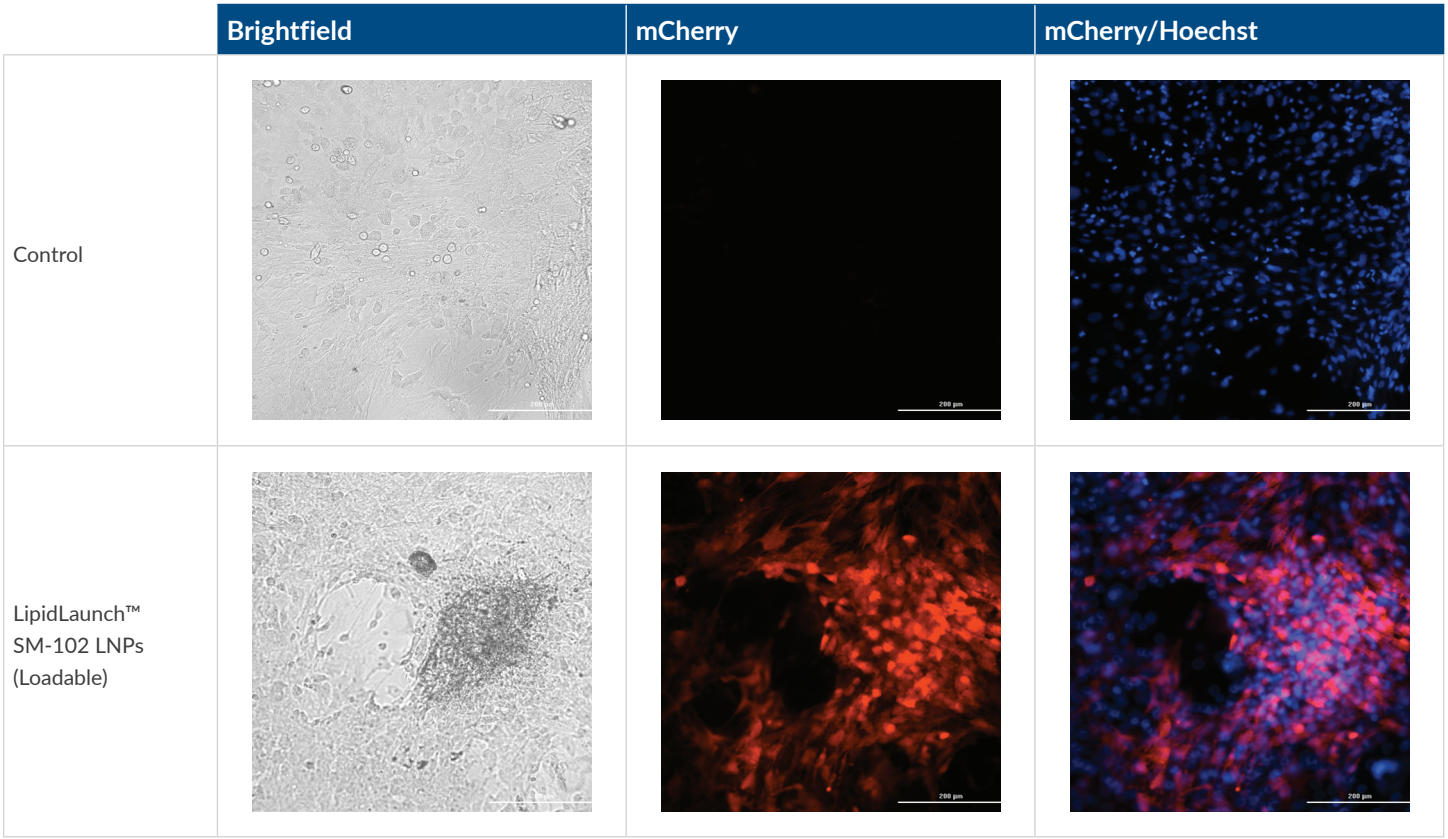


Figure 7. mCherry transfection of primary osteoblasts with LipidLaunch™ SM-102 LNPs (Loadable). Primary differentiated osteoblast cultures were transfected with 50 ng mCherry mRNA per 100 µl media *via* LipidLaunch™ SM-102 LNPs (Loadable). Cells were imaged 48 hours later to assess mCherry fluorescence. Representative images are shown for each condition. Bone-like nodules were also present in the control samples (data not shown).

Superior Cell Viability with LipidLaunch™ SM-102 LNPs (Loadable) when compared with traditional transfection reagents

Cell viability was compared in mCherry mRNA-transfected A549 cells with two competitor lipofection reagents according to manufacturer's instructions in the presence of 50-200 ng mRNA per 100 µl media (**Figure 8**). Cells were incubated for 72 hours following transfection without changing media. This time course was selected to model an experimental setup where transfection is performed on a Friday with cell analysis performed the following Monday. LipidLaunch™ SM-102 LNPs (Loadable) demonstrated superior cell viability across conditions compared with both competitor reagents.

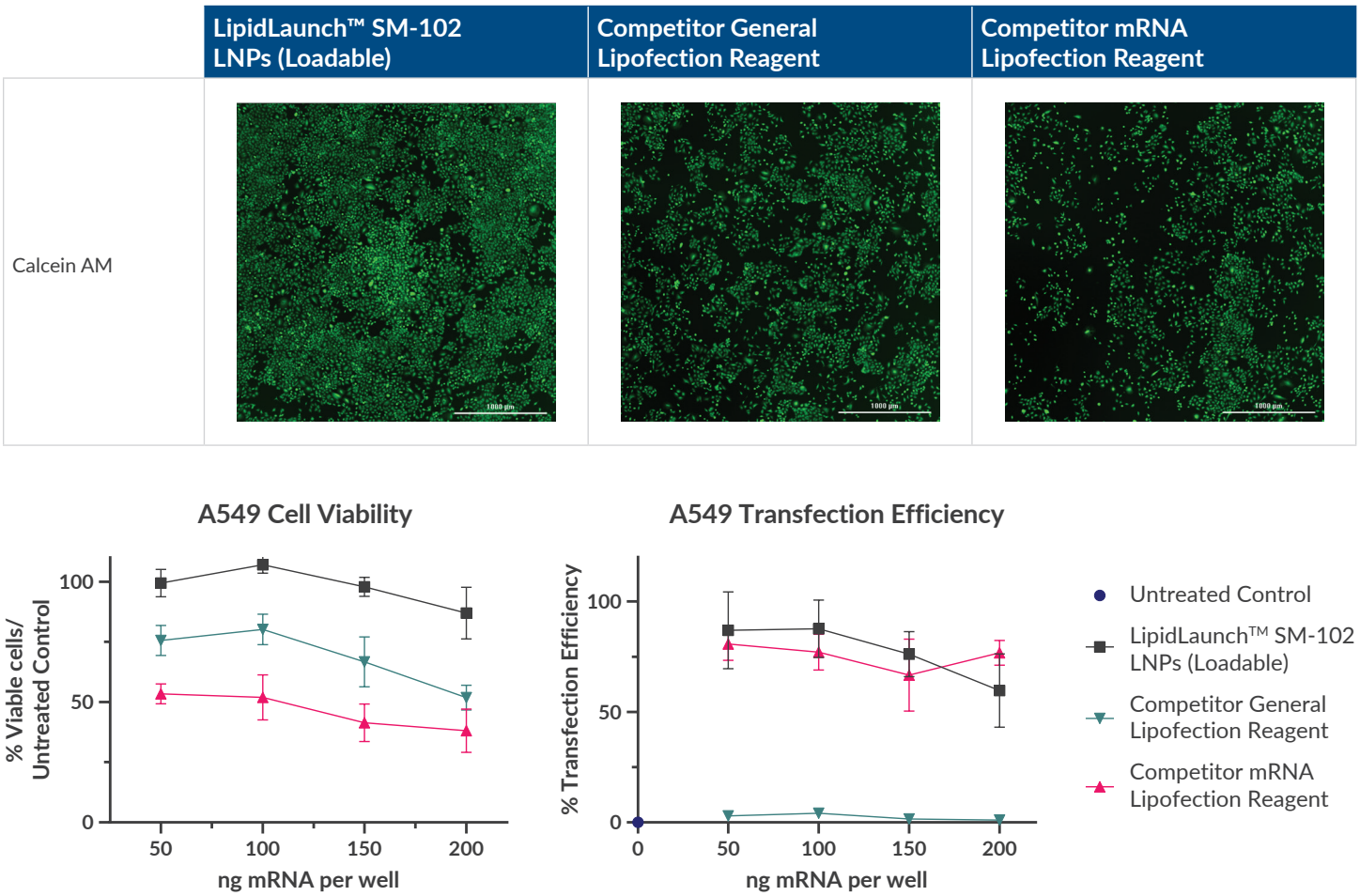


Figure 8. Comparison of LipidLaunch™ SM-102 LNPs (Loadable) with competitor lipofection reagents on cell viability and transfection efficiency. Calcein AM staining performed 72 hours following transfection with 50-200 ng mCherry mRNA per well. Representative 4X images from 200 ng per well condition for each reagent.

Conclusions

In this study, we demonstrated the application of Cayman's LipidLaunch™ SM-102 LNPs (Loadable) to transfect three different examples of RNA cargo. LipidLaunch™ SM-102 LNPs (Loadable) were able to deliver mRNA to typically difficult-to-transfect differentiated primary osteoblast cultures. LipidLaunch™ SM-102 LNPs (Loadable) exhibited good performance when transfecting mRNA into different cell lines across a range of LNP-cargo concentrations while minimizing cytotoxicity. When compared with competitor transfection reagents, LipidLaunch™ SM-102 LNPs (Loadable) exhibited superior cell viability, enabling increased recovery of RNA cargo-expressing cells.

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