

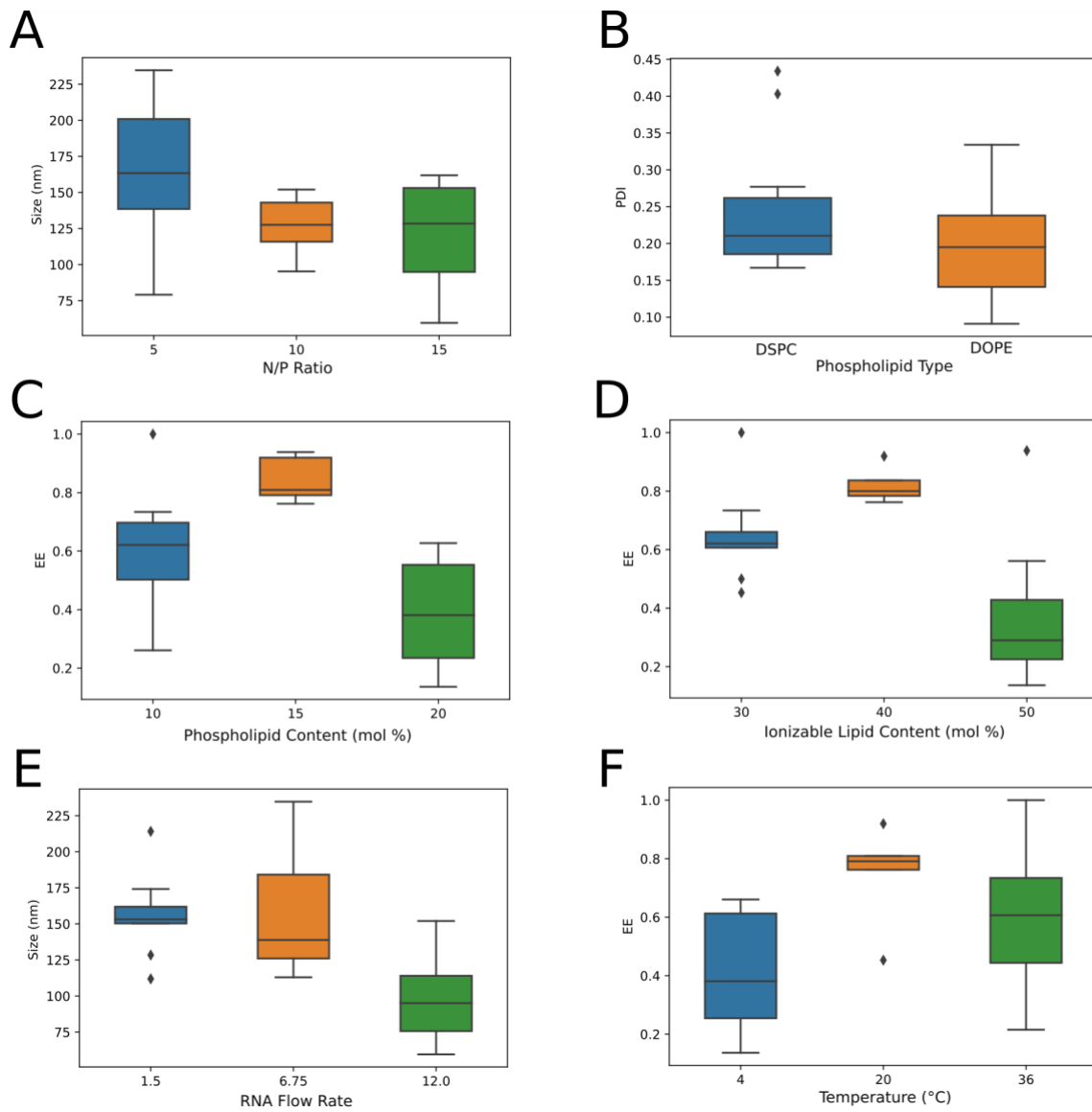
Supplementary Information: Optimization of Lipid Nanoparticles for saRNA Expression and Cellular Activation Using a Design-of-Experiment Approach

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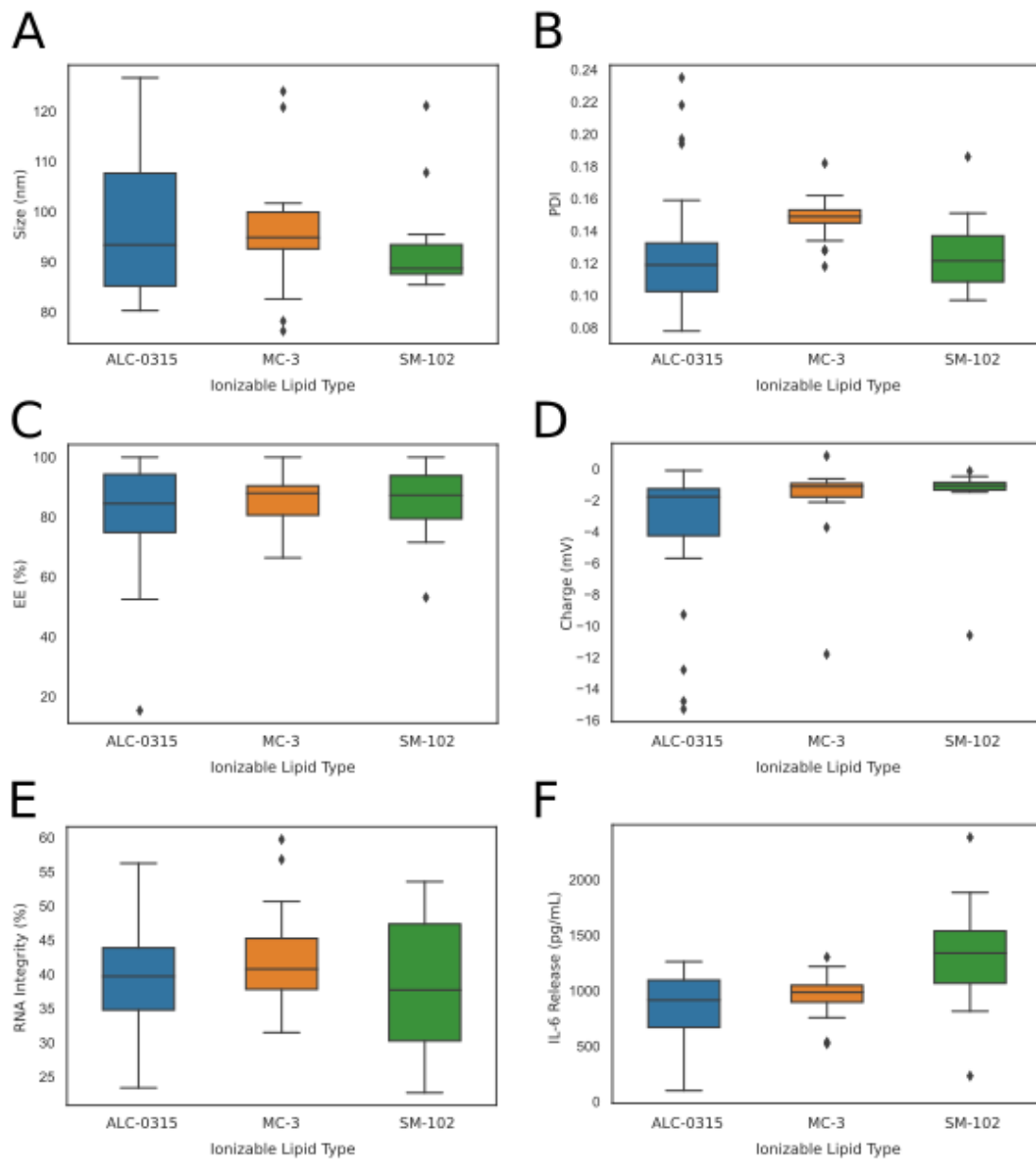
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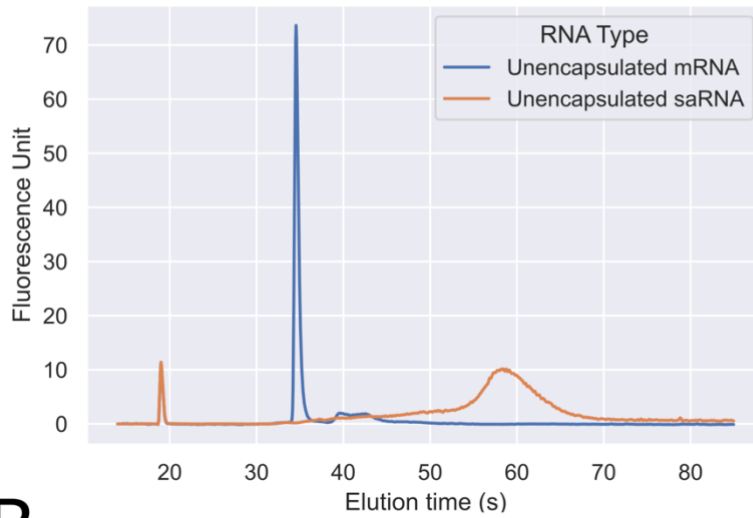
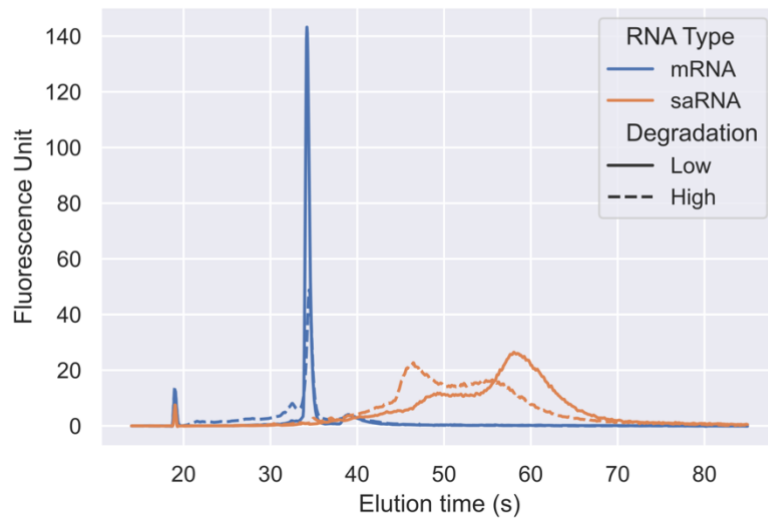
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Supplementary Figure 1: Multiple pairwise analysis for variable selection. Boxplot (A-F) from Definite Screening Design of experiments (Iteration A). Only statistically significant interactions between experimental inputs and outputs (see Figure 2C in main text) are displayed.



Supplementary Figure 2: Multiple pairwise analysis for the effect of the Ionizable lipid type on critical quality attributes. Boxplot (A-F) from Box-Behnken Design of experiments (Iteration B).

A**B**

Supplementary Figure 3: RNA electropherogram raw data (Bioanalyzer 100) from 6 typical samples. Electropherograms from unencapsulated RNA species (**A**) and LNP-encapsulated RNA (**B**) are displayed. The assessed RNA Integrity values from saRNA samples are : 65.7 % (unencapsulated), (59,7 % (low degradation) and 23.4% (high degradation). The assessed values from mRNA samples are: 76.4% (unencapsulated), 71.1 % (low degradation) and 38.6 % (high degradation).

Explanatory coded variable for Supplementary Table 1-6:

X1 : Ionizable Lipid Content

X2 : pH

X3 : Phospholipid content

X4 : Ionizable lipid pKa

Supplementary Table 1: Response surface methodology results for LNP size (nm) based on polynomial Ordinary Least Square (OLS) regression

Variable	Parameter Estimate	Standard Error	t-value	p-value
Intercept	103.2706	2.817	36.661	0.000*
Main effect				
X1	1.0945	1.739	0.630	0.542
X2	-9.0928	1.739	- 5.230	0.000*
X3	2.7043	1.739	1.555	0.148
X4	-4.6421	1.408	-3.296	0.007*
Interaction effect				
X1*X2	5.4333	2.519	2.157	0.054
X1*X3	2.0842	2.519	0.827	0.426
X1*X4	-3.1026	1.905	-1.629	0.132
X2*X3	1.5675	2.519	0.622	0.547
X2*X4	-2.2317	1.905	-1.172	0.26
X3*X4	-1.2573	1.905	-0.660	0.523
Quadratic effect				
X1	-6.4066	2.412	2.656	0.022*
X2	-1.2791	2.412	-0.530	0.606
X3	-7.3360	2.412	-3.041	0.011*
X4	-1.2573	1.905	-0.660	0.523

R-Squared= 0.862 ; Prob (F-statistic): 0.00586

Supplementary Table 2: Response surface methodology results for Encapsulation Efficiency (%) based on polynomial Ordinary Least Square (OLS) regression

Variable	Parameter Estimate	Standard Error	t-value	p-value
Intercept	89.1689	2.796	31.889	0.000
Main effect				
X1	-5.7937	1.726	-3.357	0.006
X2	-0.0899	1.726	-0.052	0.959
X3	-2.4813	1.726	-1.438	0.178
X4	0.7275	1.398	0.520	0.613
Interaction effect				
X1*X2	-13.7758	2.501	-5.508	0.000
X1*X3	-3.9967	2.501	-1.598	0.138
X1*X4	-0.0102	1.891	-0.005	0.996
X2*X3	-3.0242	2.501	-1.209	0.252
X2*X4	5.7040	1.891	3.017	0.012
X3*X4	1.6229	1.891	0.858	0.409
Quadratic effect				
X1	-3.6893	2.395	-1.541	0.152
X2	-5.3160	2.395	-2.220	0.048
X3	0.5803	2.395	0.242	0.813
X4	0.0336	2.475	0.014	0.989

R-Squared= 0.879

Supplementary Table 3: Response surface methodology results for RNA Integrity (% full length RNA) based on polynomial Ordinary Least Square (OLS) regression

Variable	Parameter Estimate	Standard Error	t-value	p-value
Intercept	45.8062	2.861	16.013	0.000
Main effects				
X1	-0.9593	1.766	-0.543	0.598
X2	-3.8496	1.766	-2.180	0.052
X3	2.3008	1.766	1.303	0.219
X4	-1.1650	1.430	-0.815	0.433
Interaction effects				
X1*X2	2.9929	2.559	1.170	0.267
X1*X3	2.1292	2.559	0.832	0.423
X1*X4	-2.1008	1.934	-1.086	0.301
X2*X3	3.3162	2.559	1.296	0.221
X2*X4	0.2006	1.934	0.104	0.919
X3*X4	-0.6469	1.934	-0.334	0.744
Quadratic effects				
X1	2.4018	2.450	0.980	0.348
X2	-6.8972	2.450	-2.816	0.017
X3	-4.2024	2.450	-1.716	0.114
X4	-3.0632	2.532	-1.210	0.252

R-Squared=0.758

Supplementary Table 4: Response surface methodology results for “Full length RNA per particle” based on polynomial Ordinary Least Square (OLS) regression

Variable	Parameter Estimate	Standard Error	t-value	p-value
Intercept	56.0833	7.056	7.948	0.000
Main effects				
X1	-0.1529	4.355	-0.035	0.973
X2	-14.0226	4.355	-3.220	0.008
X3	-0.8585	4.355	-0.197	0.847
X4	-3.7913	3.528	-1.075	0.306
Interaction effects				
X1*X2	2.8640	6.311	0.454	0.659
X1*X3	-9.7236	6.311	-1.541	0.152
X1*X4	-4.5379	4.771	-0.951	0.362
X2*X3	-3.0584	6.311	-0.485	0.637
X2*X4	-0.9230	4.771	-0.193	0.850
X3*X4	-6.6659	4.771	-1.397	0.190
Quadratic effects				
X1	1.8821	6.042	0.311	0.761
X2	-10.3653	6.042	-1.715	0.114
X3	-4.0833	6.042	-0.676	0.513
X4	-14.5125	6.245	-2.324	0.040

R-Squared=0.744

Supplementary Table 5: Response surface methodology results for IL-6 cytokine release (pg/mL) based on polynomial Ordinary Least Square (OLS) regression

Variable	Parameter Estimate	Standard Error	t-value	p-value
Intercept	1070.7424	128.432	8.337	0.000
Main effects				
X1	13.3994	79.270	0.169	0.869
X2	234.2870	79.270	2.956	0.013
X3	-14.7627	79.270	-0.186	0.856
X4	203.5250	64.216	3.169	0.009
Interaction effects				
X1*X2	64.9717	114.873	0.566	0.583
X1*X3	-6.0020	114.873	- 0.052	0.959
X1*X4	31.1357	86.836	0.359	0.727
X2*X3	38.0080	114.873	0.331	0.747
X2*X4	-57.0024	86.836	-0.656	0.525
X3*X4	3.1393	86.836	0.036	0.972
Quadratic effects				
X1	-13.0994	109.983	- 0.119	0.907
X2	-26.6679	109.983	- 0.242	0.813
X3	-310.8027	109.983	-2.826	0.016
X4	171.9190	113.670	1.512	0.159

R-Squared=0.808; Prob (F-statistic): 0.0264

Supplementary Table 6 : Response surface methodology results for protein expression (RLU) based on polynomial Ordinary Least Square (OLS) regression.

Variable	Parameter Estimate	Standard Error	t-value	p-value
Intercept	1.376e+07	1.74e+06	7.918	0.000
Main effects				
X1	3.267e+06	1.2e+06	2.723	0.034
X2	4.816e+06	1.2e+06	4.015	0.007
X3	1.557e+06	1.2e+06	1.298	0.242
X4	-1.982e+06	8.48e+05	-2.336	0.058
Interaction effects				
X1*X2	1.996e+06	1.52e+06	1.316	0.236
X1*X3	-1.523e+06	1.52e+06	-1.004	0.354
X1*X4	2.469e+06	1.2e+06	2.058	0.085
X2*X3	2.445e+05	1.52e+06	0.161	0.877
X2*X4	8.263e+05	1.54e+06	0.537	0.610
X3*X4	1.574e+06	1.2e+06	1.312	0.237
Quadratic effects				
X1	2.795e+06	1.54e+06	1.817	0.119
X2	-8.166e+06	1.54e+06	-5.309	0.002
X3	-1.97e+06	1.2e+06	-1.642	0.152

R-Squared=0.946 ; Prob (F-statistic): 0.00884

Supplementary Table 7: List of all formulation variations explored for Iteration A.

ID	N/P	Phospholipid Type	Phospholipid (mol %)	DMG-PEG-2000 (mol%)	Ionizable Lipid Type	Ionizable Lipid (mol %)	Total Flow Rate (mL/min)	Temperature (°C)	Aqueous Buffer pH	RNA Type
A-1	10	DOPE	10	0	MC3	30	2	4	3	saRNA
A-2	5	DSPC	15	0	SM-102	30	2	4	7	mRNA
A-3	15	DOPE	20	2.5	SM-102	30	2	4	7	mRNA
A-4	5	DSPC	20	1.25	MC3	50	2	4	3	mRNA
A-5	15	DSPC	10	2.5	SM-102	50	2	4	3	saRNA
A-6	5	DOPE	20	2.5	MC3	50	9	4	7	saRNA
A-7	15	DSPC	20	0	MC3	30	16	4	5	saRNA
A-8	5	DOPE	10	2.5	ALC-0315	30	16	4	3	mRNA
A-9	15	DSPC	10	2.5	MC3	40	16	4	7	mRNA
A-10	5	DOPE	10	0	SM-102	50	16	4	7	saRNA
A-11	15	DOPE	20	0	SM-102	50	16	4	3	mRNA
A-12	15	DOPE	10	0	MC3	50	2	20	7	mRNA
A-13	10	DOPE	15	1.25	ALC-0315	40	9	20	5	saRNA
A-14	10	DSPC	15	1.25	ALC-0315	40	9	20	5	mRNA
A-15	5	DSPC	20	2.5	SM-102	30	16	20	3	saRNA
A-16	5	DSPC	10	2.5	MC3	30	2	36	7	saRNA
A-17	15	DSPC	20	2.5	MC3	30	2	36	3	mRNA
A-18	5	DOPE	20	0	SM-102	40	2	36	3	saRNA
A-19	15	DSPC	20	0	ALC-0315	50	2	36	7	saRNA
A-20	5	DOPE	10	2.5	SM-102	50	2	36	5	mRNA
A-21	15	DSPC	10	0	SM-102	30	9	36	3	mRNA
A-22	5	DOPE	20	0	MC3	30	16	36	7	mRNA
A-23	15	DOPE	10	1.25	SM-102	30	16	36	7	saRNA
A-24	5	DSPC	10	0	MC3	50	16	36	3	saRNA
A-25	15	DOPE	15	2.5	MC3	50	16	36	3	saRNA
A-26	10	DSPC	20	2.5	SM-102	50	16	36	7	mRNA

Supplementary Table 8. List of all formulation variations explored for Iteration B.

ID	N/P	Phospholipid Type	Phospholipid (mol %)	DMG-PEG-2000 (mol%)	Ionizable Lipid Type	Ionizable Lipid (mol %)	Total Flow Rate (mL/min)	Temperature (°C)	Aqueous Buffer pH	RNA Type
B-1	10	DOPE	12.5	1.25	ALC-0315	40	16	20	4	saRNA
B-2	10	DOPE	17.5	1.25	ALC-0315	40	16	20	4	saRNA
B-3	10	DOPE	15	1.25	MC3	40	16	20	4	saRNA
B-4	10	DOPE	15	1.25	SM-102	40	16	20	4	saRNA
B-5	10	DOPE	15	1.25	ALC-0315	35	16	20	4	saRNA
B-6	10	DOPE	15	1.25	ALC-0315	45	16	20	4	saRNA
B-7	10	DOPE	12.5	1.25	MC3	40	16	20	5	saRNA
B-8	10	DOPE	17.5	1.25	MC3	40	16	20	5	saRNA
B-9	10	DOPE	12.5	1.25	SM-102	40	16	20	5	saRNA
B-10	10	DOPE	17.5	1.25	SM-102	40	16	20	5	saRNA
B-11	10	DOPE	12.5	1.25	ALC-0315	35	16	20	5	saRNA
B-12	10	DOPE	17.5	1.25	ALC-0315	35	16	20	5	saRNA
B-13	10	DOPE	12.5	1.25	ALC-0315	45	16	20	5	saRNA
B-14	10	DOPE	17.5	1.25	ALC-0315	45	16	20	5	saRNA
B-15	10	DOPE	15	1.25	MC3	35	16	20	5	saRNA
B-16	10	DOPE	15	1.25	SM-102	35	16	20	5	saRNA
B-17	10	DOPE	15	1.25	MC3	45	16	20	5	saRNA
B-18	10	DOPE	15	1.25	SM-102	45	16	20	5	saRNA
B-19	10	DOPE	15	1.25	ALC-0315	40	16	20	5	saRNA
B-20	10	DOPE	15	1.25	ALC-0315	40	16	20	5	saRNA
B-21	10	DOPE	12.5	1.25	ALC-0315	40	16	20	6	saRNA
B-22	10	DOPE	17.5	1.25	ALC-0315	40	16	20	6	saRNA
B-23	10	DOPE	15	1.25	MC3	40	16	20	6	saRNA
B-24	10	DOPE	15	1.25	SM-102	40	16	20	6	saRNA
B-25	10	DOPE	15	1.25	ALC-0315	35	16	20	6	saRNA
B-26	10	DOPE	15	1.25	ALC-0315	45	16	20	6	saRNA

Supplementary Table 9. List of all formulation variations used to validate the optimized LNP formulations.

ID	N/P	Phospholipid Type	Phospholipid (mol %)	DMG-PEG-2000 (mol%)	Ionizable Lipid Type	Ionizable Lipid (mol %)	Total Flow Rate (mL/min)	Temperature (°C)	Aqueous Buffer pH	RNA Type
1-M	10	DOPE	15.9	1.25	ALC-0315	45	16	20	4.53	mRNA
1-S	10	DOPE	15.9	1.25	ALC-0315	45	16	20	4.53	saRNA
2-M	10	DOPE	17.5	1.25	SM-102	45	16	20	6	mRNA
2-S	10	DOPE	17.5	1.25	SM-102	45	16	20	6	saRNA
3-M	10	DOPE	17.5	1.25	ALC-0315	35	16	20	5.25	mRNA
3-S	10	DOPE	17.5	1.25	ALC-0315	35	16	20	5.25	saRNA