Supporting Information

Enhanced immunogenicity induced by mRNA vaccines with various lipid nanoparticles as carriers for SARS-CoV-2 infection

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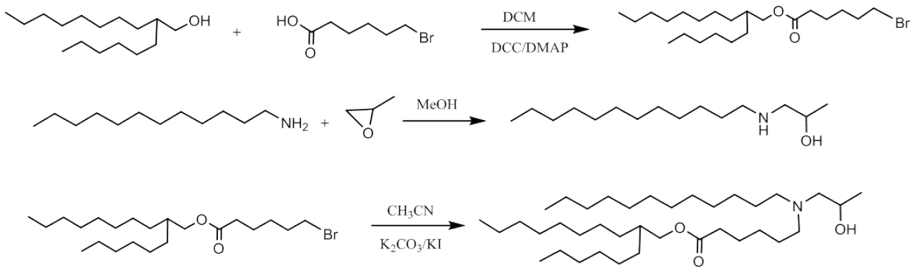
1 Synthesis of ionizable lipids

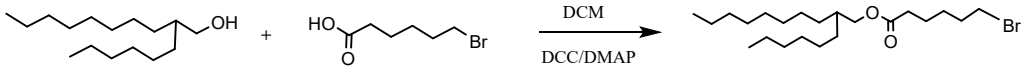
1.1 Synthesis of 2-hexyldecyl 6-(dodecyl(2-hydroxypropyl)amino)hexanoate (H01)

Compound **H01** was synthesized by a three-step reaction as shown in scheme 1.



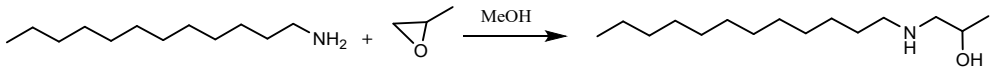
Scheme 1

1.1.1Synthesis of 2- hexyldecyl 6-bromohexanoate



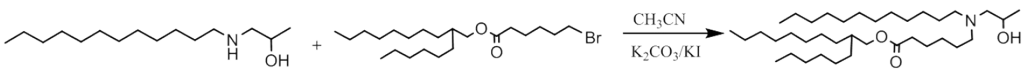
DCC (7.50 g, 36.4 mmol) was added to the dichloromethane (DCM) solution containing 6-bromohexanoic acid (5.76 g, 29.8 mmol) at 25 °C and the solution was stirred for 20 mins at room temperature. Then, 2-hexyldecan-1-ol (6.0 g, 24.8 mmol) and DMAP (170 mg) were added in the above solution overnight at room temperature. The crude product was obtained under reduced pressure and purified by silica column chromatography (methanol/dichloromethane = 1/20) to give colorless oil. (8.5g, 20.33 mmol, 77.3% yield). ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.97 (d, J = 5.8 Hz, 2H), 3.40 (t, J = 6.8 Hz, 2H), 2.33 (t, J = 7.4 Hz, 2H), 1.92 – 1.82 (m, 2H), 1.64 (dp, J = 15.4, 6.4, 5.2 Hz, 3H), 1.48 (p, J = 7.6, 7.1 Hz, 2H), 0.88 (t, J = 6.9 Hz, 6H).

1.1.2 Synthesis of 1-(dodecylamino)propan-2-ol



Dodecan-1-amine (5.76 g, 29.8 mmol) and 2-methyloxirane (7.50 g, 36.4 mmol) were added to methanol (100 mL) and the solution was stirred under ambient condition for 24 h. The reaction mixture was dried under decompression condition to remove the excess 2-methyloxirane. The product was obtained as colorless oil (8.5g, 20.33 mmol, 77.3% yield) and confirmed by LCMS $[M + H]^+$ 244.26, found 244.2.

1.1.3 Synthesis of 2-hexyldecyl 6-(dodecyl(2-hydroxypropyl)amino)hexanoate) (H01)

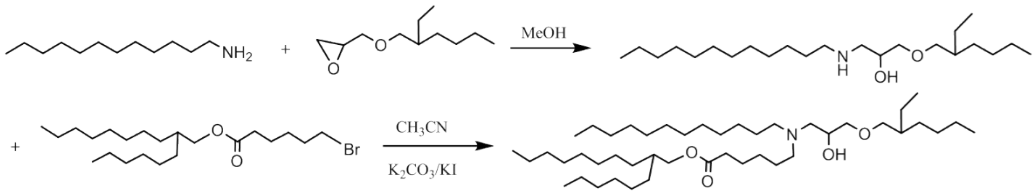


H01

2-Hexyldecyl 6-bromohexanoate (0.836g, 2 mmol), K_2CO_3 (447mg, 6.0 mmol) and KI (48.6 mg, 0.6 mmol), 1-(dodecylamino)propan-2-ol (0.442 g, 1mmol) were dissolved in $CNCH_3$ (15 mL). After the mixture solution was stirred at 90 °C for 5 hours, the solution was filtered and dried under decompression condition. The obtained residues were redissolved in dichloromethane (50 mL) and washed with brine (2 x 40 mL). The combined organic layer was concentrated to give crude product which was further purified by silica column chromatography (methanol/dichloromethane = 1/20). Compound **H01** was light yellow oil (0.32 g, 3.6 mmol, 39.0%) and confirmed by LC–TOF $[M + H]^+$ 582.57, found 582.58 and ^1H NMR. ^1H NMR (600 MHz, $CDCl_3$) δ 3.97 (d, *J* = 5.8 Hz, 2H), 3.72 (dd, *J* = 14.0, 7.0 Hz, 1H), 2.59 – 2.24 (m, 8H), 1.97 (s, 1H), 1.43 (d, *J* = 14.5 Hz, 6H), 1.31 – 1.25 (m, 44H), 1.12 (d, *J* = 6.0 Hz, 3H), 0.88 (t, *J* = 7.0 Hz, 9H).

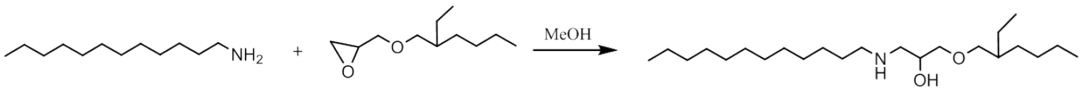
1.2 Synthesis of 2-hexyldecyl 6-(dodecyl(2-hydroxypropyl)amino)hexanoate (H02)

Compound **H02** was synthesized by a two-step reaction as shown in scheme **2**.



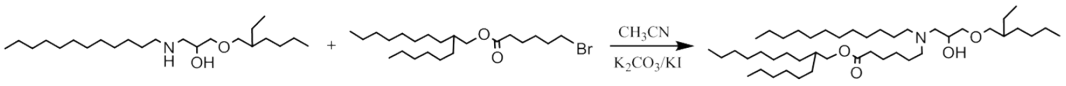
Scheme 2

1.2.1 Synthesis of 1-(dodecylamino)-3-((2-ethylhexyl)oxy)propan-2-ol



Dodecan-1-amine (5.76 g, 29.8 mmol) and 2-(((2-ethylhexyl)oxy)methyl)oxirane (7.50 g, 36.4 mmol) were added to methanol (100 mL) and the solution was stirred under ambient condition for 24 h. The reaction mixture was dried under decompression condition to remove the excess 2-methyloxirane. The crude product was further purified by silica column chromatography (methanol/dichloromethane = 1/20). The product **C1** was light yellow oil (0.32 g, 3.6 mmol, 39.0%) and confirmed by LCMS $[M + H]^+$ 372.38, found 372.2.

1.2.2 Synthesis of 2-hexyldecyl 6-(dodecyl(3-((2-ethylhexyl)oxy)-2-hydroxypropyl)amino)hexanoate (H02)



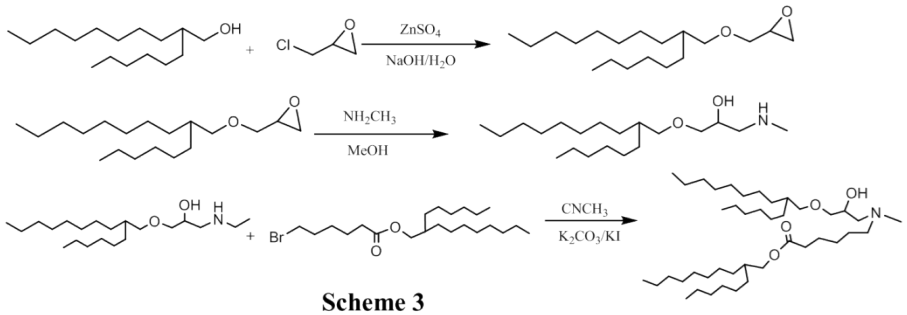
H02

2-Hexyldecyl 6-bromohexanoate (0.836g, 2 mmol), K_2CO_3 (447mg, 6.0 mmol) and KI (48.6 mg, 0.6 mmol), 1-(dodecylamino)propan-2-ol (0.442 g, 1mmol) were dissolved in $CNCH_3$ (15 mL). After the mixture solution was stirred at 90 °C for 5 hours, the solution was filtered and dried under decompression condition. The obtained residues were redissolved in dichloromethane (50 mL) and washed with brine (2 x 40 mL). The

combined organic layer was concentrated to give crude product which was further purified by silica column chromatography (methanol/dichloromethane = 1/20). Compound **H02** was light yellow oil (0.32 g, 3.6 mmol, 39.0%) and confirmed by LC–TOF $[M + H]^+$ 710.69, found 710.70 and ^{1}H NMR. ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.97 (d, *J* = 5.8 Hz, 2H), 3.74 (d, *J* = 6.9 Hz, 1H), 3.46 – 3.27 (m, 4H), 2.71 – 2.18 (m, 7H), 1.60 (d, *J* = 27.2 Hz, 14H), 1.37 – 1.12 (m, 44H), 0.95 – 0.65 (m, 15H).

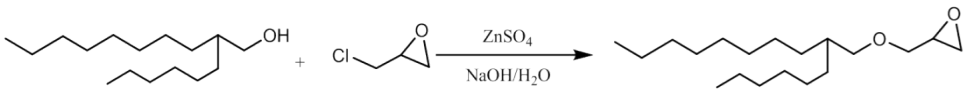
1.3 Synthesis of 2-hexyldecyl 6-(ethyl(3-((2-hexyldecyl)oxy)-2-hydroxypropyl)amino)hexanoate (H03)

Compound **H03** was synthesized by a three-step reaction as shown in scheme **3**.



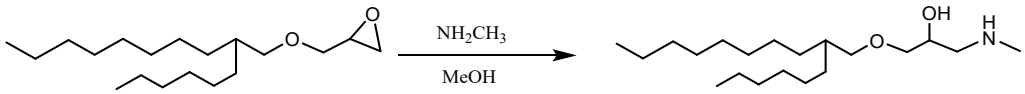
Scheme 3

1.3.1 Synthesis of 2-(((2-hexyldecyl)oxy)methyl)oxirane



To a flask was added into 2-hexyldecan-1-ol (2.42g, 10 mmol), Zinc chloride (80 mg) and a catalytic amount of sulfuric acid. The mixture was added epichlorohydrin (1.84 g, 20 mmol) dropwise within 30 mins, followed by a reaction at 120 °C for 2 hours. After the mixture solution was cooled to room temperature, the solid residues were removed by filtration and the excess epichlorohydrin was removed under decompression condition. Next, the above solution was mixed with sodium hydroxide aqueous (20%) and was stirred at 40 °C for 4h. Finally, the water phase was removed and the organic phase was dried under reduced pressure to give the product (1.87g, 6.2 mmol, 74.8% yield) which was confirmed by ^{1}H NMR and LCMS. LCMS: $[M + Na]^+$ 321.29, found 321.1. ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.96 (p, J = 5.5 Hz, 1H), 3.78 – 3.22 (m, 6H), 1.56 (p, J = 5.1, 4.0 Hz, 1H), 0.88 (t, J = 6.9 Hz, 6H).

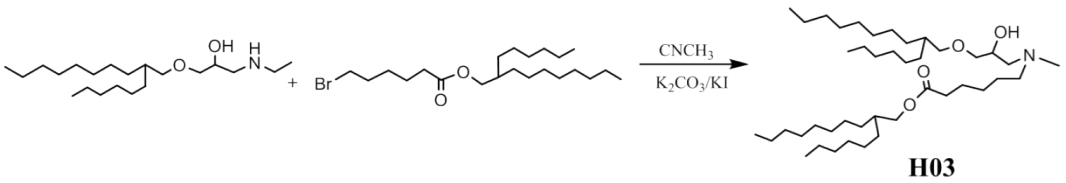
1.3.2 Synthesis of 1-((2-hexyldecyl)oxy)-3-(methylamino)propan-2-ol



To a flask was added into Methnol (15 mL), Ethylamine (0.60 g, 13.3 mmol) and 2-(((2-hexyldecyl)oxy)methyl)oxirane (1.0 g, 3.34 mmol). The mixture solution was stirred overnight at room temperature. The reaction solution was concentrated and further purified by silica column chromatography (methanol/dichloromethane=1/20). The obtained product was light yellow oil (0.64 g, 12.4 mmol, 55.6%) and confirmed

by LC-TOF $[M + H]^+$ 330.33, found 330.32.

1.3.3 Synthesis of 2-hexyldecyl 6-((3-((2-hexyldecyl)oxy)-2-hydroxypropyl)(methyl)amino)hexanoate (H03)

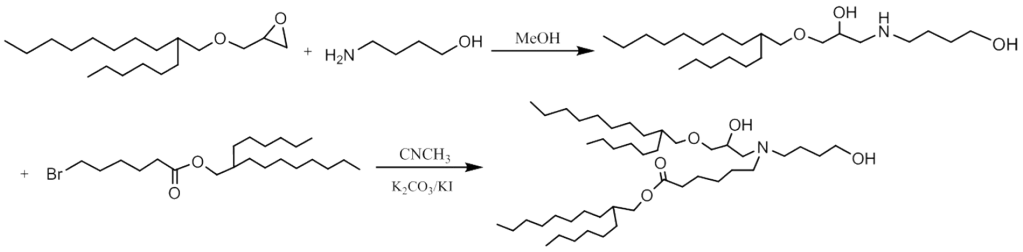


H03

2-Hexyldecyl 6-bromohexanoate (0.836g, 2 mmol), K_2CO_3 (447mg, 6.0 mmol) and KI (48.6 mg, 0.6 mmol), 1-((2-hexyldecyl)oxy)-3-(methylamino)propan-2-ol (0.442 g, 1mmol) were dissolved in $CNCH_3$ (15 mL). After the mixture solution was stirred at 90 °C for 5 hours, the solution was filtered and dried under decompression condition. The obtained residues were redissolved in dichloromethane (50 mL) and washed with brine (2 x 40 mL). The combined organic layer was concentrated to give crude product which was further purified by silica column chromatography (methanol/dichloromethane = 1/20). Compound **H03** was light yellow oil (0.32 g, 3.6 mmol, 39.0%) and confirmed by LC–TOF $[M + H]^+$ 668.65, found 668.66 and ^{1}H NMR. ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.95 (dd, *J* = 21.8, 6.3 Hz, 2H), 3.86 (dd, *J* = 9.3, 4.3 Hz, 1H), 3.45 – 3.25 (m, 4H), 2.57 – 2.26 (m, 9H), 1.52 (ddd, *J* = 53.8, 38.6, 22.4 Hz, 4H), 1.43 – 1.03 (m, 50H), 0.98 – 0.77 (m, 12H).

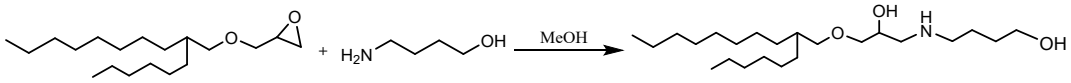
1.4 Synthesis of 2-hexyldecyl 6-((3-((2-hexyldecyl)oxy)-2-hydroxypropyl)(4-hydroxybutyl)amino)hexanoate (H04)

Compound **H04** was synthesized by a two-step reaction as shown in scheme 4.



Scheme 4

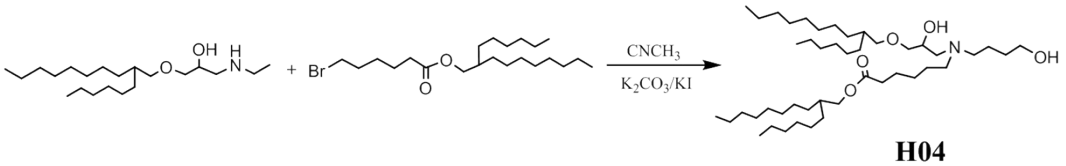
1.4.1 Synthesis of 4-((3-((2-hexyldecyl)oxy)-2-hydroxypropyl)amino)butan-1-ol



To a flask was added into methnol (15 mL), 4-aminobutan-1-ol (0.60 g, 13.3 mmol) and 2-(((2-hexyldecyl)oxy)methyl)oxirane (1.0 g, 3.34 mmol). The mixture solution was stirred overnight at room temperature. The reaction solution was concentrated and further purified by silica column chromatography (methanol/dichloromethane=1/20).

The obtained product was light yellow oil (0.64 g, 12.4 mmol, 55.6%) and confirmed by LC–TOF $[M + H]^+$ 388.37, found 388.37.

1.4.2 Synthesis of 2-hexyldecyl 6-((3-((2-hexyldecyl)oxy)-2-hydroxypropyl)(4-hydroxybutyl)amino)hexanoate (H04)

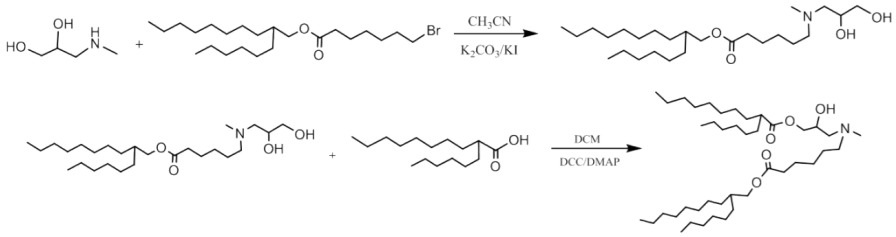


H04

2-Hexyldecyl 6-bromohexanoate (0.836g, 2 mmol), K_2CO_3 (447mg, 6.0 mmol) and KI (48.6 mg, 0.6 mmol), 4-((3-((2-hexyldecyl)oxy)-2-hydroxypropyl)amino)butan-1-ol (0.442 g, 1mmol) were dissolved in $CNCH_3$ (15 mL). After the mixture solution was stirred at 90 °C for 5 hours, the solution was filtered and dried under decompression condition. The obtained residues were redissolved in dichloromethane (50 mL) and washed with brine (2 x 40 mL). The combined organic layer was concentrated to give crude product which was further purified by silica column chromatography (methanol/dichloromethane = 1/20). Compound **H04** was light yellow oil (0.32 g, 3.6 mmol, 39.0%) and confirmed by LC–TOF $[M + H]^+$ 726.69, found 726.70 and ^{1}H NMR. ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.97 (d, J = 5.8 Hz, 2H), 3.89 (dd, J = 8.5, 3.5 Hz, 1H), 3.67 – 3.54 (m, 2H), 3.43 – 3.26 (m, 4H), 2.63 – 2.36 (m, 6H), 2.31 (t, J = 7.5 Hz, 2H), 1.63 – 1.52 (m, 6H), 1.56 – 1.05 (m, 53H), 0.88 (t, J = 7.0 Hz, 12H).

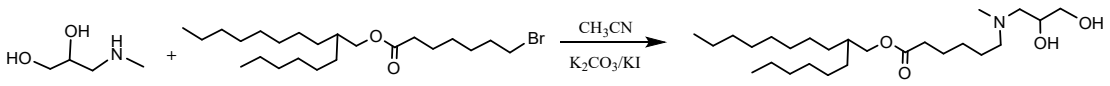
1.5 Synthesis of 3-((6-((2-hexylundecyl)oxy)-6-oxohexyl)(methyl)amino)-2-hydroxypropyl 2-hexyldecanoate (H05)

Compound **H05** was synthesized by a two-step reaction as shown in scheme 5.



Scheme 5

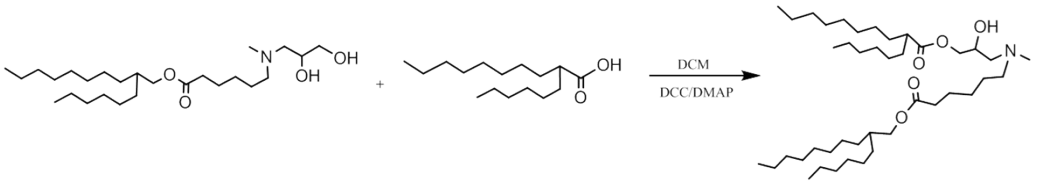
1.5.1 Synthesis of 2-hexyldecyl 6-((2,3-dihydroxypropyl)(methyl)amino)hexanoate



2-Hexyldecyl 6-bromohexanoate (0.836g, 2 mmol), K_2CO_3 (447mg, 6.0 mmol) and KI (48.6 mg, 0.6 mmol), (1-(ethylamino)-3-((2- hexyldecyl)oxy)propan-2-ol (0.442 g, 1mmol) were dissolved in $CNCH_3$ (15 mL). After the mixture solution was stirred at 90 °C for 5 hours, the solution was filtered and dried under decompression condition. The obtained residues were redissolved in dichloromethane (50 mL) and washed with brine (2 x 40 mL). The combined organic layer was concentrated to give crude product which was further purified by silica column chromatography (methanol/dichloromethane =

1/20). The product was light yellow oil (0.32 g, 3.6 mmol, 39.0%) and confirmed by LC–TOF$[M + H]^+$ 444.40, found 444.40.

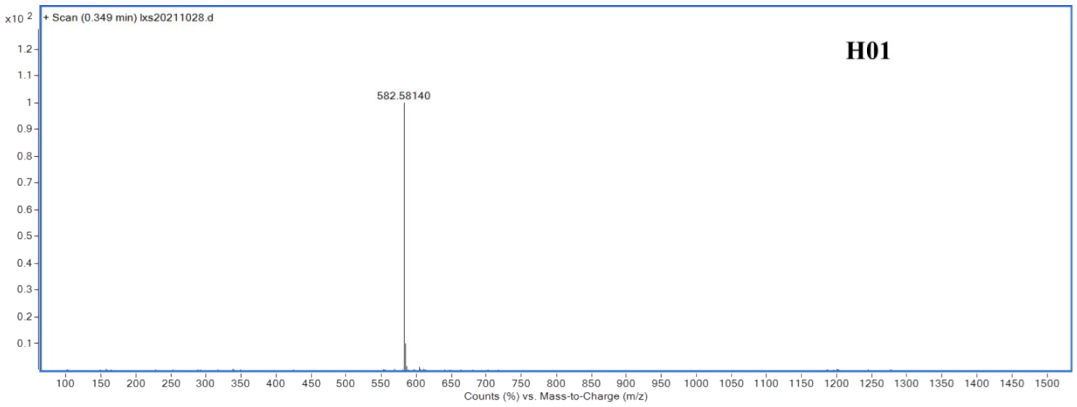
1.5.2 Synthesis of 3-((6-((2-hexyldecyl)oxy)-6-oxohexyl)(methyl)amino)-2-hydroxypropyl 2-hexyldecanoate (H05)

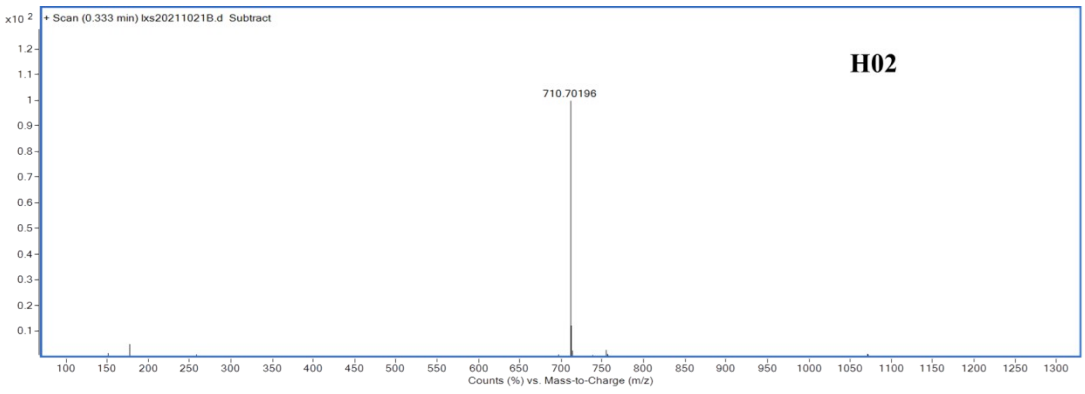


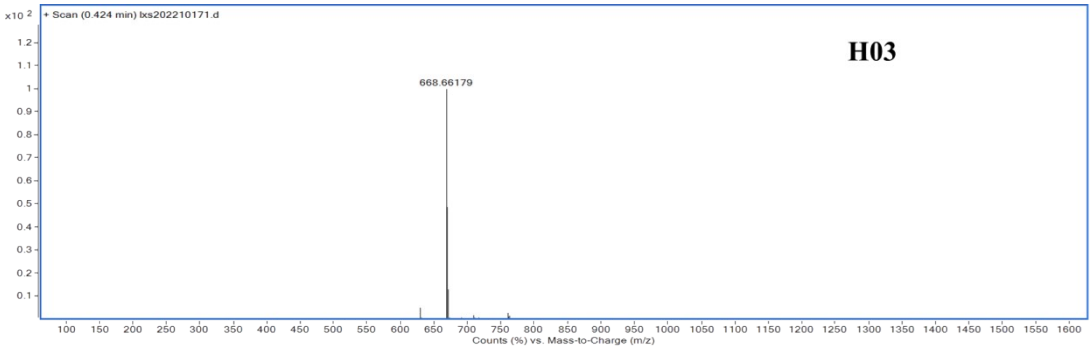
H05

DCC (7.50 g, 36.4 mmol) was added to the dichloromethane (DCM) solution containing 2-hexyldecanoic acid (5.76 g, 29.8 mmol) at 25 °C and the solution was stirred for 20 mins at room temperature. Then, 2-hexyldecyl 6-((2,3-dihydroxypropyl)(methyl)amino)hexanoate (6.0 g, 24.8 mmol) and DMAP (170 mg) were added in the above solution overnight at room temperature. The crude product was obtained under reduced pressure and purified by silica column chromatography (methanol/dichloromethane = 1/20). Compound **H05** was obtained as colorless oil. (8.5g, 20.33 mmol, 77.3%yield) and confirmed by LC–TOF $[M + H]^+$ 682.63, found 682.63 and ^{1}H NMR. ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.19 – 4.02 (m, 2H), 3.97 (d, J = 5.8 Hz, 2H), 3.74 (ddd, J = 21.1, 12.5, 6.8 Hz, 1H), 2.66 – 2.15 (m, 9H), 1.61 – 1.09 (m, 54H), 0.88 (qd, J = 6.6, 3.1 Hz, 12H).

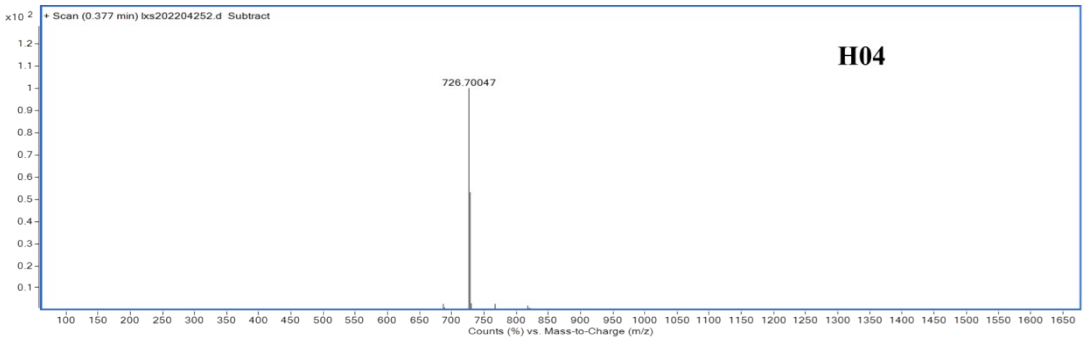
2. Mass spectra of five ionizable lipids


x10 2
+ Scan (0.349 min) lxs20211028.d
H01
582.58140
Counts (%) vs. Mass-to-Charge (m/z)


x10 2
+ Scan (0.333 min) lxs20211021B.d Subtract
H02
710.70196
Counts (%) vs. Mass-to-Charge (m/z)


x10 2
+ Scan (0.424 min) lxs202210171.d
H03
668.66179
Counts (%) vs. Mass-to-Charge (m/z)

225


x10 2
+ Scan (0.377 min) lxs202204252.d Subtract
H04
726.70047
Counts (%) vs. Mass-to-Charge (m/z)

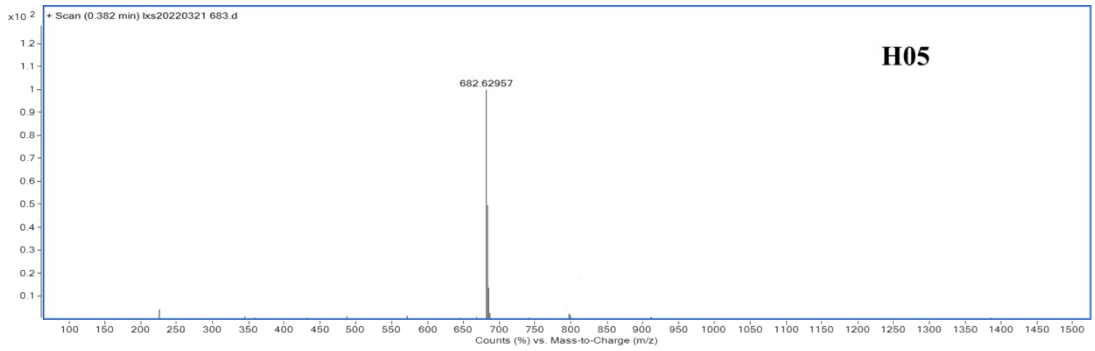
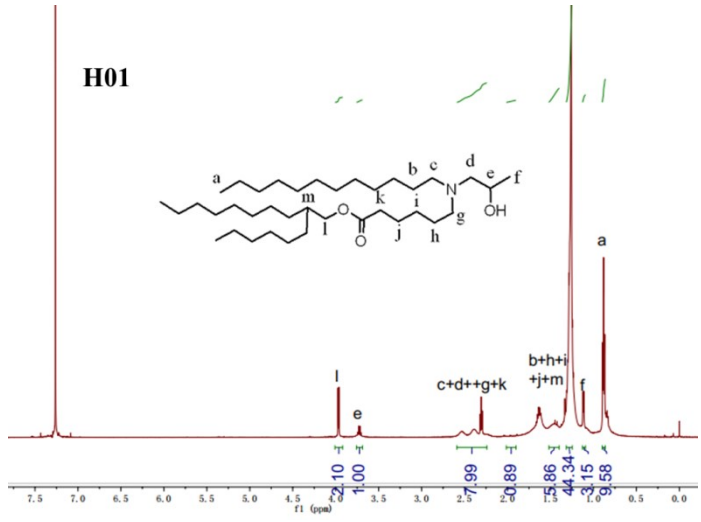
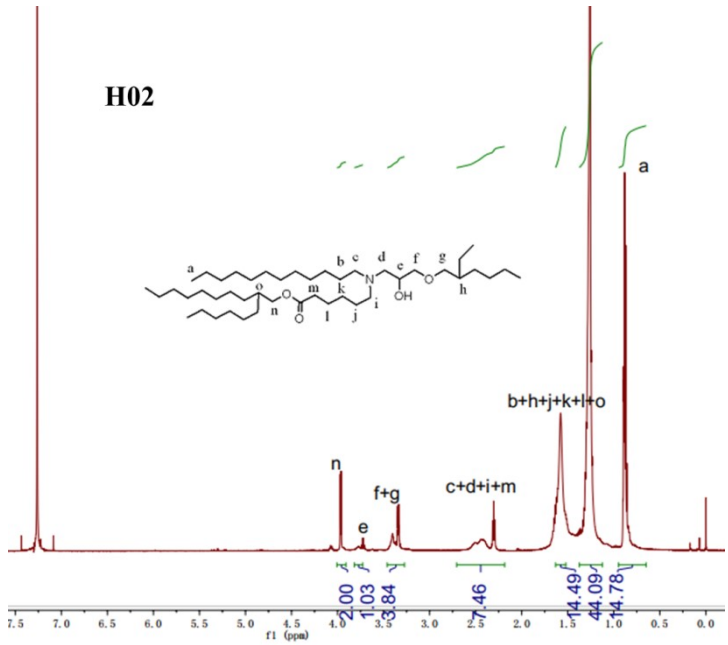

+ Scan (0.382 min) lxs20220321 683.d
H05
682.62957
Counts (%) vs. Mass-to-Charge (m/z)

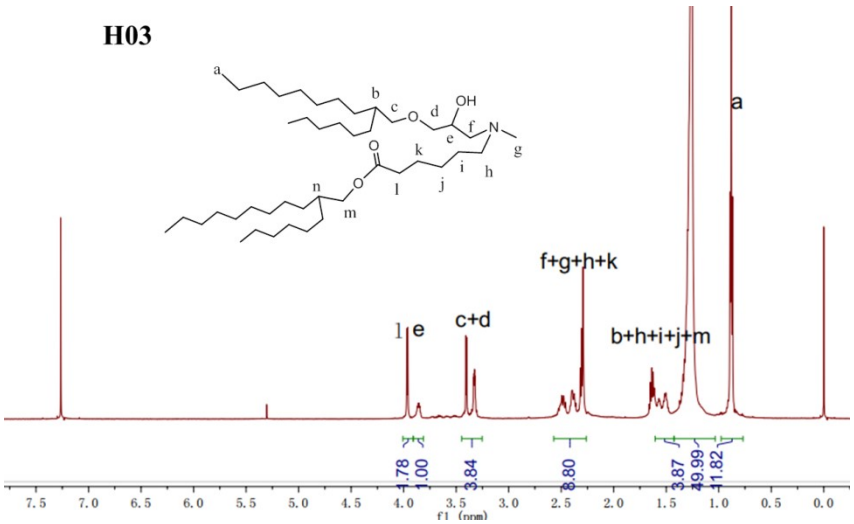
Figure S1

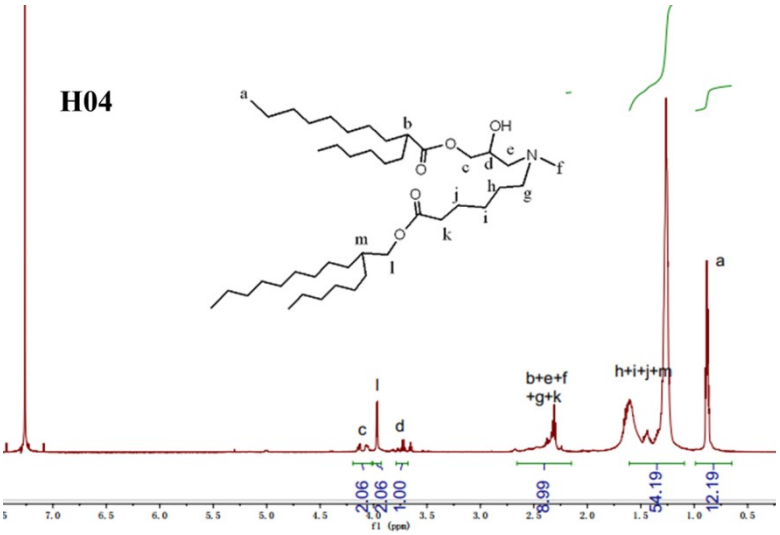
3. ^{1}H NMR spectra of five ionizable lipids



265






H04
a
b
OH
O
c
d
e
N
f
O
j
h
g
i
k
m
O
l
l
c
d
b+e+f
+g+k
h+i+j+m
a
2.06
2.06
1.00
8.99
54.19
12.19
f1 (ppm)

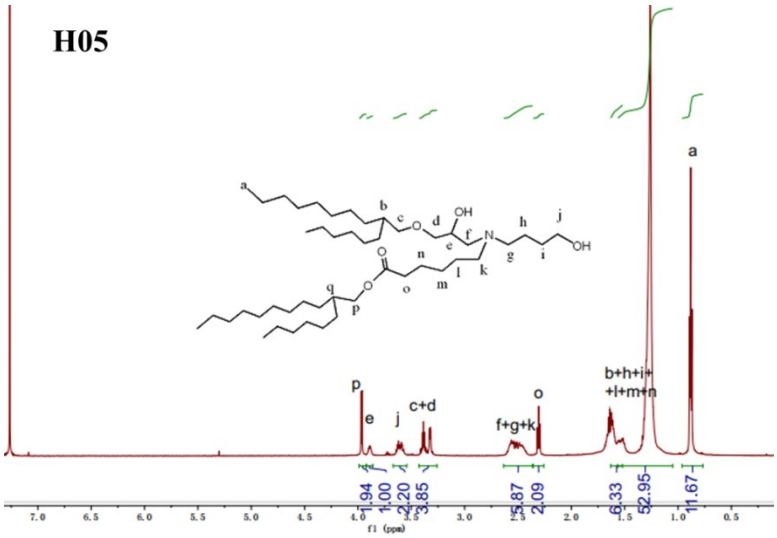

H05
a
b
c
O
d
OH
f
N
h
j
e
g
i
OH
O
n
l
k
q
O
o
m
p
a
p
e
j
c+d
o
f+g+k
b+h+i+
+l+m+n
1.94
1.00
2.20
3.85
5.87
2.09
6.33
52.95
11.67
f1 (ppm)

Figure S2

4. Percentages of eGFP positive HEK293T cells measured by flow cytometry

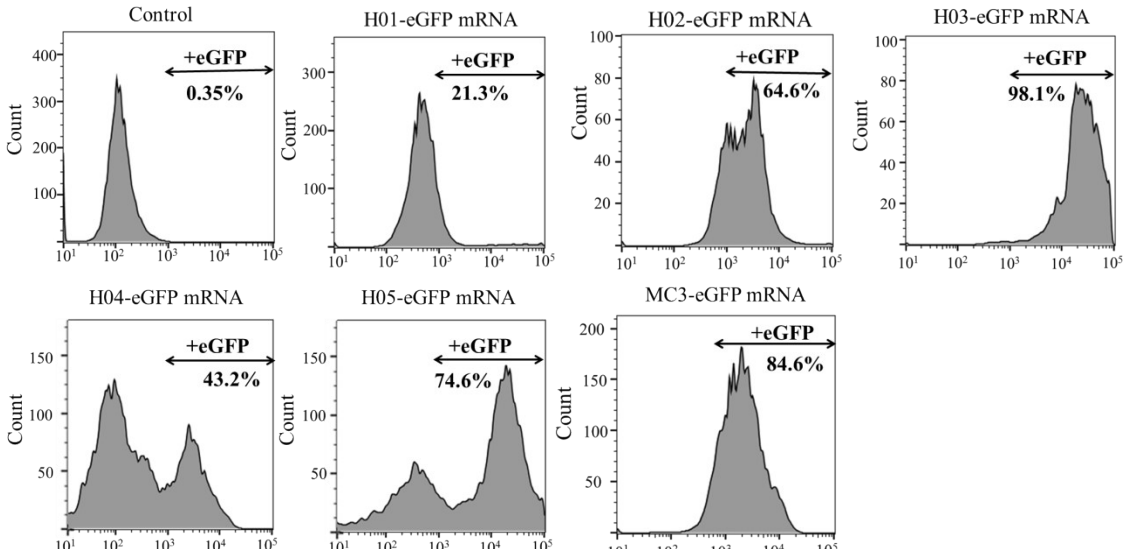


Figure S3

5. Images of lysosomal escape assay *in vitro* of H01, H02, H04 and H05-mRNA

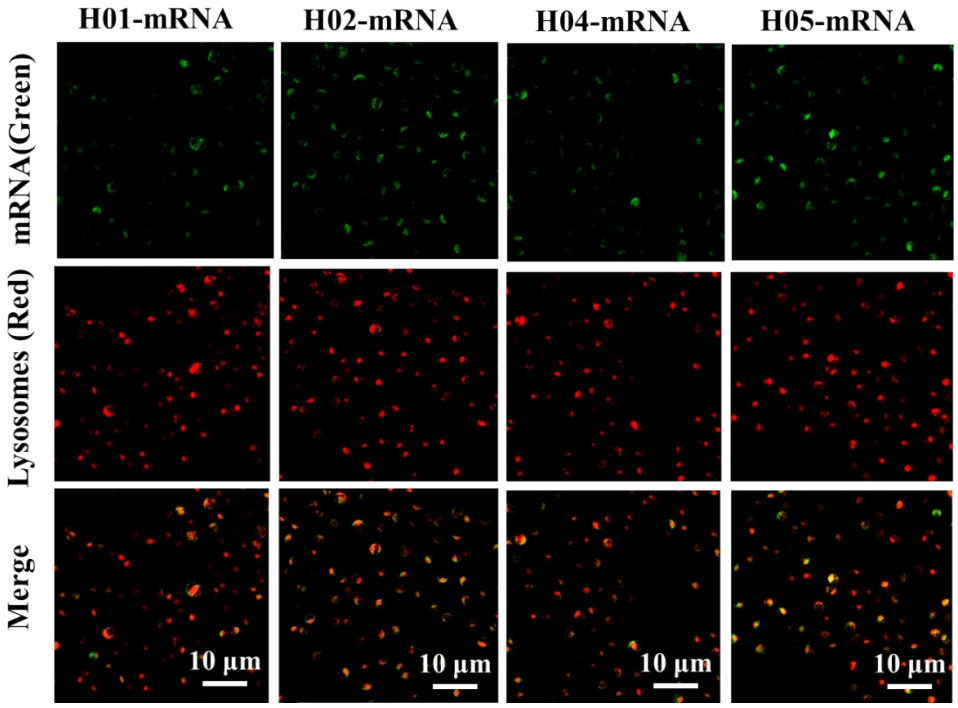


Figure S4

6. SARS-CoV-2 (B.1.617.2) RBD-specific CD4+ (a) and CD8+ (b) Tem cells in splenocytes were detected by flow cytometry

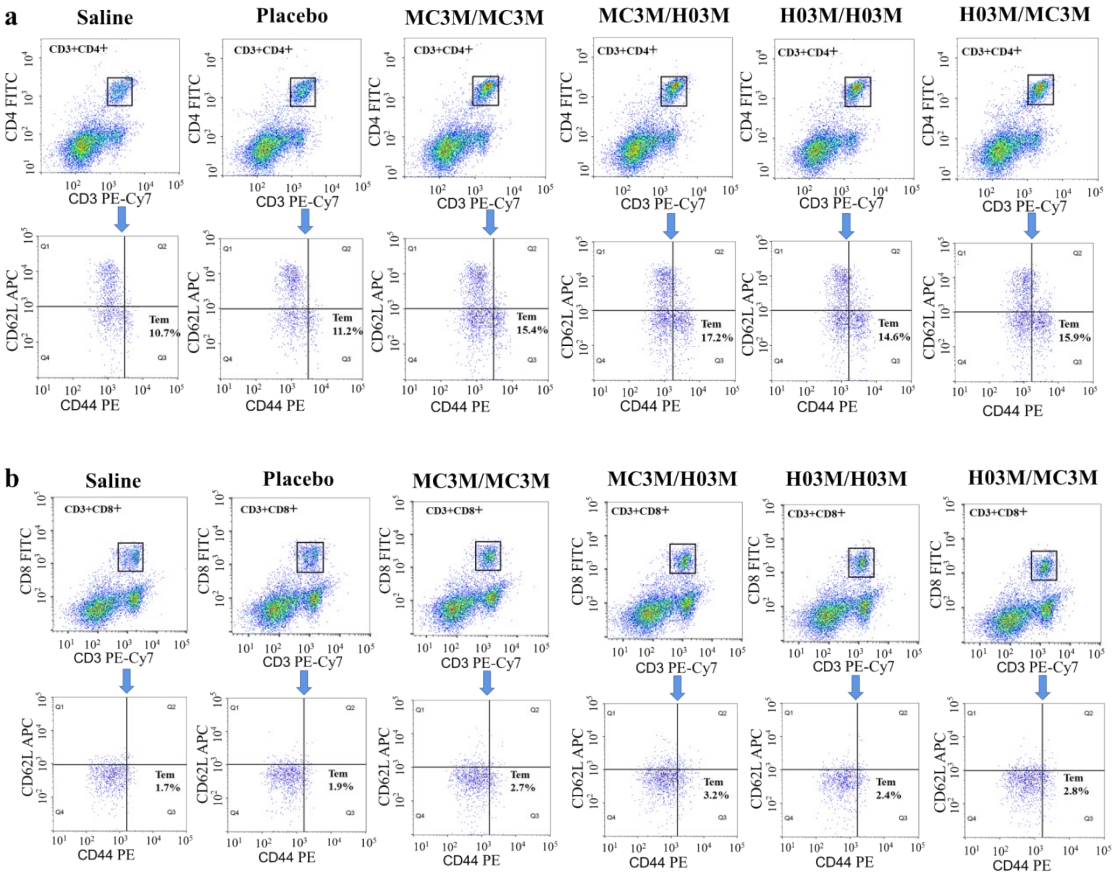


Figure S5

7. Storage stability of H03-Fluc mRNA and MC3-Fluc mRNA formulations stored at 25 °C, 4 °C and -20 °C

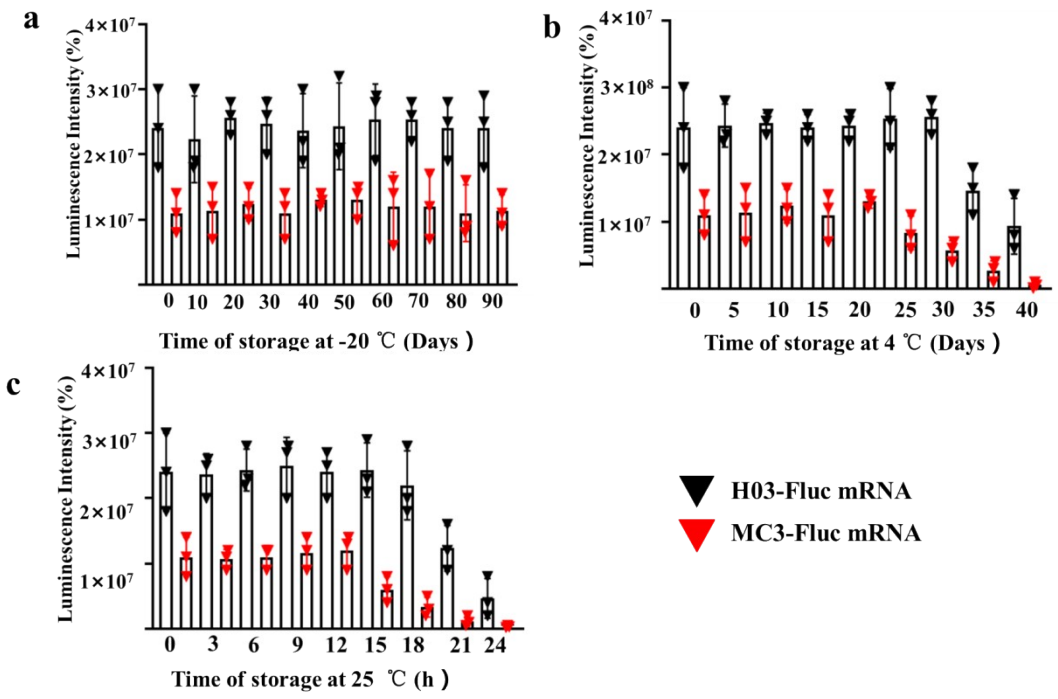


Figure S6

8. Amino acid sequence alignment of the SARS-CoV-2(B.1.617.2) RBD

Delta (B.1.617.2) RBD

RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSA
SFSTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVRQIAPGQTGKIADYNYKL
PDDFTGCVIAWNSNNLDSKVGGNYNYRYRLFRKSNLKPFERDISTEIYQAGSK
PCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHAPATVCGPKKS
TNLVKNKCVNF

Highlights represent amino acid mutation sites

Figure S7

9. The peptide pools of the RBD antigen

Serial No.	Sequence	Length (amino acid)
1	RVQPTESIVRFPNITNL	17
2	VFNATRFASVYAWNRKRI	18
3	SVYAWNRKRISNCVADY	17
4	KCYGVSPTKLNDLCFTNV	18
5	KLNDLCFTNVYADSFVIR	18
6	LFRKSNLKPFERDISTEI	18
7	PFERDISTEIYQA	13
8	VVLSFELLHAPATVCGPK	18
9	HAPATVCGPKKSTNLVK	17

Figure S8

10. Raw western bot data for Fig.3h

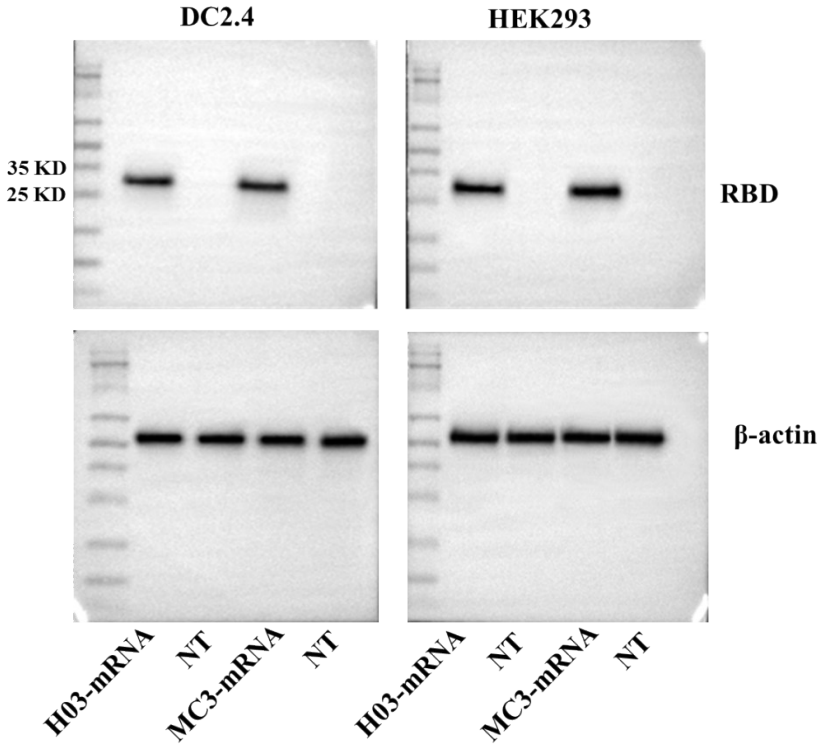


Figure S9

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