PROTOCOL

LipidBrick® Library
Imidazolium-based lipids for LNP formulation

General Protocol

As an innovator in the field of nucleic acid delivery, Polyplus® has developed LipidBrick®, a new range of cationic lipids, dedicated to the formulation of lipid nanoparticles (LNPs). These active lipids protect nucleic acids such as mRNA, siRNA or DNA and deliver them to the cells. Based on an imidazolium polar head, LipidBrick® broadens the current applications spectrum in terms of potency and targeting by adding positive charges to LNPs, as the Zeta potential of LNPs is known to impact their biodistribution and nucleic acid expression.

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1. Introduction

Lipid nanoparticles (LNPs) are the non-viral delivery system of choice for RNA therapeutics. Their lipidic structure encapsulates and protects nucleic acids from degradation, prolongs their circulation in the body and facilitates their uptake into cells using different routes of administration (systemic or local injections). These properties make LNPs an attractive option for the delivery of drug products.

LNPs are composed of 4 types of molecules:

- **Active lipid(s)** (cationic and/or ionizable lipids): positively charged lipids interact with negatively charged nucleic acids and mediate disruption of the endosome to allow release of nucleic acid into the cytoplasm,
- **Structural lipid** (phospholipids): ensures rigidity and stability of LNPs,
- **PEG-lipid** (modulatory lipids): provides steric stabilization and prolong blood circulation,
- **Sterol lipid** (modulatory lipids): increases the fluidity and stability of the LNP membrane and promotes membrane fusion with the cells.

The LNP assembly is promoted by the rapid mixing of two miscible phases: one organic lipidic solution and an aqueous phase containing the nucleic acids. Several methods can be used: microfluidic mixing, T-junction mixing, ethanol injection, hand-mixing, etc.

First, cationic or ionizable lipids interact with and encapsulate nucleic acids. The intermediate nanostructures will make hydrophobic lipid tails available to interact with structural and modulatory lipids. The LNP structure will then be finalized by the formation of functional vesicles capable of protecting and delivering nucleic acids (Fig. 1).

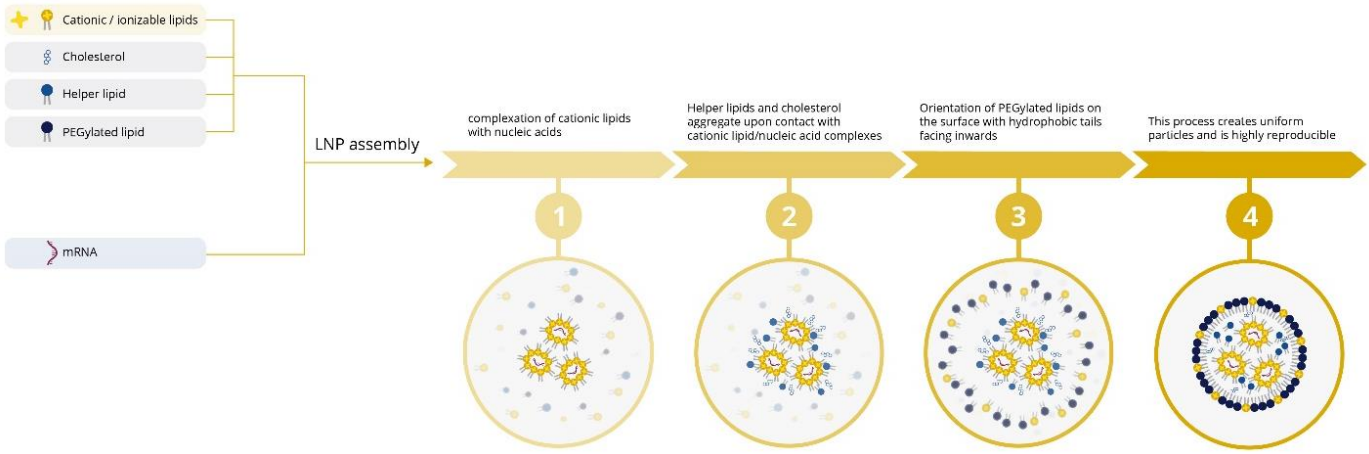

Figure 1: Schematic of mRNA-LNP formulation

2. Material and Equipment

2.1. Material supplied

Table 1. LipidBrick® Library Kit for LNP formulation composed of 8 imidazolium-based lipids

LipidBrick® library	Linear formula	Molecular weight
IM21.7c	$C_{59}H_{117}ClN_2$	890.03 g/mol
IM3c	$C_{41}H_{79}ClN_2$	635.55 g/mol
IM12c	$C_{48}H_{95}ClN_2$	735.75 g/mol
IM13c	$C_{64}H_{127}ClN_2$	960.18 g/mol
IM15c	$C_{53}H_{97}ClN_2$	797.82 g/mol
IM16c	$C_{56}H_{111}ClN_2$	847.97 g/mol
IM22c	$C_{51}H_{101}ClN_2$	777.83 g/mol
IM25c	$C_{57}H_{113}ClN_2O$	877.99 g/mol

2.2. Suggested material and equipment (non-supplied)

Table 2. Third-party material recommended for LNPs formulation using LipidBrick®

Description	Example of possible materials
Structural lipids	DPyPE
	DOPE
	DSPC
Sterol lipid	Cholesterol
PEG lipids	DSG-PEG_{2k}
	DMG-PEG_{2k}
	ALC-0159
Ionizable lipids	DODMA
	DLin-MC3-DMA
	SM-102
	ALC-0315
mRNA	CleanCap® mRNA
mRNA buffer	10 mM acetate buffer, pH 4.0
	50 mM citrate buffer, pH 4.0
mRNA quantification	Quant-it™ RiboGreen RNA Assay Kit

Table 3. Third-party equipment & consumables recommended for LNPs formulation using LipidBrick®

Description	Example of equipment/consumable & supplier
Microfluidic equipment	NanoAssemblr Ignite, Precision NanoSystems
Cartridge	NanoAssemblr Ignite NxGen cartridges, PNI
Centrifugal filter concentrator	Vivaspin® Turbo 4, 10kDa, Sartorius
Syringes	BD Syringes 1 mL & 10 mL, BD Plastipak
Needles	Needles 20Gx1'' & 21Gx1 ½'', Terumo
Filter	Minisart® Syringe Filter, PES, 0.45 µm & 0.22 µm, Sartorius
Bench Top Centrifuge	-
Vortex Mixer	-
Particle size analyzer	Zetasizer Nano-ZS, Malvern Panalytical
Cuvettes for particle size analyzer	UV Cuvettes, VWR

3. Stock solutions preparation with LipidBrick® Library

Before beginning, ensure that all supplies, consumables, and working environments are RNase-free.

- Dissolve LipidBrick® lipids in absolute ethanol in the concentrations indicated in Table 4. We suggest solubilizing the lipids in an ultrasonic bath at 37°C for 30 minutes before use.
- Once fully solubilized, LipidBrick® solutions should be stored at 4°C.

Table 4. Recommended concentrations of the LipidBrick® stock solutions.

LipidBrick® Library	Pack size (mg)	Molecular weight (g/mol)	Concentration		EtOH (µL)
			(mM)	(mg/mL)	
IM21.7c	50	890,03	100	89,00	495
	250				2475
	1000				9902
IM3c	50	635,55	100	63,56	724
IM12c	50	735,75	100	73,58	617
IM13c	50	960,18	100	96,02	458
IM15c	50	797,82	100	79,78	564
IM16c	50	847,97	100	84,80	526
IM22c	50	777,83	100	77,78	580
IM25c	50	877,99	100	87,80	507

4. LNP Formulation protocol using LipidBrick® Library

4.1. LNP Formulation workflow

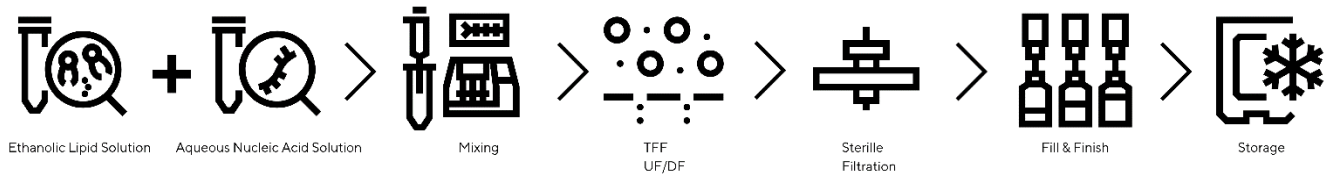

Figure 2: LNP Formulation workflow

4.2. Ethanolic Lipid Solution Preparation

In light of the comprehensive internal research and proof-of-concept studies conducted, we have identified optimal formulations that serve as robust **positive controls** in the development of lipid nanoparticles (LNPs) utilizing LipidBrick® IM21.7c. These formulations, described in Tables 5 and 6 below, have been meticulously selected based on their consistent performance, characterization profile (size, PDI, Zeta potential and encapsulation efficiency), efficacy and safety in preliminary assays. As such, they are recommended as **standard references** to benchmark the performance of novel LNP constructs for your specific application(s).

Tables 5 and 6 exemplify the preparation process of the **Ethanolic Lipid Solution** making it possible to prepare **1.0 mL of final LNP solution** (based on 1.5 mL preparation with 0.5 mL of waste volumes) for these **LNP formulations recommended as standard references**.

- To this aim, dilute the required volume of each constituent into absolute Ethanol using a single-use Eppendorf tube. Ensure homogeneity by pipetting the mixture up and down several times.

Table 5. Organic phase preparation of the 4-lipid LNP formulation recommended as standard reference

	IM21.7c	DOPE	Cholesterol	DMG-PEG$_{2k}$	Absolute EtOH
Stock solution concentrations (mM)	100	30	50	10	N/A
Organic phase composition (µL)	75	50	115.5	22.5	112
Organic phase concentration (mM)	20	4	15,4	0,6	N/A
Theoretical lipid concentration in final formulation (mM)	**5**	**1**	**3,85**	**0,15**	**N/A**

Table 6. Organic phase preparation of the 5-lipid LNP formulation recommended as standard reference

	IM21.7c	DODMA	DPyPE	Cholesterol	DSG-PEG$_{2k}$	Absolute EtOH
Stock solution concentrations (mM)	100	50	30	50	10	N/A
Organic phase composition (µL)	60	90	50	55.5	22.5	97
Organic phase concentration (mM)	16	12	4	7,4	0,6	N/A
Theoretical lipid concentration in final formulation (mM)	**4**	**3**	**1**	**1,85**	**0,15**	**N/A**

To assist you in preparing your LNPs formulations, we encourage you to use our **LipidBrick® In Lab LNP Formulation Spreadsheet**, which allows for **automatic volume calculations tailored to your specific purposes**. Please feel free to **contact our technical support team** (polyplus-support@sartorius.com) or your **dedicated Field Application Specialist for access**. They will be happy to provide you with the tool and offer you their support in your LNP project!

4.3. Aqueous Nucleic Acid Solution Preparation

We have internally tested and validated the compatibility of LNP formulations using our LipidBrick® lipids with two different aqueous buffers (10 mM Acetate buffer, pH 4.0 & 50 mM Citrate buffer, pH 4.0). Therefore, we recommend the use of one of these 2 buffers, following the preparation protocol provided below.

4.3.1. Preparation of the Aqueous Buffer

Given the volumetric ratio of **1:3 (organic to aqueous)** recommended for the mixing process, we recommend preparing 4X concentrated aqueous nucleic acid solutions.

- 1L of Acetate buffer (40 mM, pH 4.0):
  1. Weigh anhydrous sodium acetate (MW=82.03 $g.mol^{-1}$, 744.4 mg) and dissolve it into ultrapure water (800 mL).
  2. Adjust the pH to 4.0 by addition of pure acetic acid (MW=60.05 $g.mol^{-1}$, 1857 mg).
  3. Measure the pH and possibly adjust it slightly by addition of 1M NaOH or 1M HCl solution to reach pH=4.0.
  4. Complete to 1L with ultrapure water.
  5. The buffer can then be stored at 4°C for long-term use.
- 1L of Citrate buffer (200 mM, pH 4.0):
  1. Weigh sodium citrate dihydrate (MW=294.10 g mol^{-1}; 19.86 g) and citric acid (MW=192.12 $g.mol^{-1}$; 25.45 g).
  2. Dissolve both compounds into 800 mL of ultrapure water.
  3. Measure the pH and possibly adjust it slightly by addition of 1M NaOH or 1M HCl solution to reach pH=4.0.
  4. Complete to 1L with ultrapure water.
  5. The buffer can then be stored at 4°C for long-term use.

4.3.2. Nucleic Acid dilution in Aqueous Buffer

- To prepare the Aqueous Nucleic Acid Solution, dilute the desired amount of RNA in one of the previously described buffers and gently pipette up and down several times to achieve a homogeneous mixture.
- To prepare 1.0 mL of final LNP solution, **prepare at least 1125 µL** of the Aqueous Nucleic Acid Solution at the chosen concentration.
- The final RNA concentration should be determined based on the desired N/P ratio for your specific application (Refer to Table 7).
- For optimal conditions using mRNA, we recommend the use of chemically modified mRNA, with a preference for commercially available mRNA. If

synthesizing mRNA in-house, we recommend using phenol-chloroform precipitation method followed by isopropanol purification and avoid salt-based precipitation techniques. Ensure that the mRNA is diluted and aliquoted in RNase-free water.

Table 7. RNA concentration in Aqueous Nucleic Acid Solution depending on desired N/P ratio

RNA concentration in Aqueous Nucleic Acid Solution	N/P ratio = 6	N/P ratio = 9	N/P ratio = X
Based on the **4-lipid LNP formulation standard reference at 10mM** (see Table 5)	**0.363 µg.µL^{-1}**	**0.242 µg.µL^{-1}**	*Please refer to LipidBrick® In Lab LNP Formulation Spreadsheet or contact our Technical Support team*
Based on the **5-lipid LNP formulation standard reference at 10mM** (see Table 6)	**0.509 µg.µL^{-1}**	**0.339 µg.µL^{-1}**	
Other LNP formulation	*Please refer to LipidBrick® In Lab LNP Formulation Spreadsheet or contact our Technical Support team*		

Please note that the mRNA concentrations mentioned here are higher than those in the final LNP formulation. This accounts for the dilution that occurs during the LNP assembly step.

Of note, the (cationic lipid) **nitrogen/**(nucleic acid) **phosphate (N/P) molar ratio** describes the stoichiometry between the positively charged amine of the cationic lipid(s) and the anionic phosphate groups in the nucleic acid. It is important to conduct an **N/P ratio optimization** for each specific application.

4.4. Mixing

LNPs are prepared by mixing the Ethanolic Lipid Solution with the Aqueous Nucleic Acid Solution. There are several suitable methods for producing LNPs at a laboratory scale, including microfluidic mixing, T-junction mixing, ethanol injection, hand-mixing, among others.
For consistency with our internally generated data, we recommend the use of a **microfluidic mixing device**, such as the NanoAssemblr® Ignite™.

- Set up your microfluidic mixing device following the manufacturer's instructions to proceed with the LNP preparation.
- We recommend initiating the process with a **flow rate of 10 mL/min**; however, please note that this parameter may require optimization.
- Additionally, a volumetric ratio of **1:3 (organic to aqueous)** is recommended for the mixing process.

4.5. Concentration & Filtration

Upon completion of the mixing step and the obtention of formulated LNPs, a few additional steps are recommended to exchange the buffer (for use and storage purposes), concentrate and stabilize the LNPs solution and ensure that LNPs are free of any biological contaminants or residual chemical.

- Transfer the content into a Vivaspin® centrifugation unit that has been pre-rinsed with ultrapure water.
- Fill the tube with the 3 mL of the selected final buffer (*e.g.* 1X PBS, sucrose-based cryoprotectant, etc.).

- Conduct a centrifugation cycle for 30 minutes at 4000 g and 20°C. After centrifugation, discard the filtrate.
- Replenish the tube with 4 mL of the chosen final buffer and conduct a second centrifugation cycle under the same conditions (30 minutes at 4000 g and 20°C).
- If the sample is adequately concentrated, with an approximate remaining volume ≤1 mL, it is ready for the next step (considering that the targeted final volume is 1 mL). If the concentration is not yet appropriate (volume >1mL), repeat the centrifugation cycles as necessary.
- Post-centrifugation, collect the concentrated sample from the cassette into a pre-weighed Eppendorf tube. Adjust the weight to reach 1 gram using the final buffer (considering that the targeted final volume is 1 mL).
- Transfer the concentrated solution into a 10 mL syringe fitted with a PES filter (select 0.45 µm for *in vitro* applications or 0.22 µm for *in vivo* applications) and filter the solution into a sterile tube.

4.6. Storage

LNPs can either be used immediately or stored for later use. Based on our in-house data with LipidBrick® lipids, LNPs can generally be stored at 4°C for up to 3-4 weeks. For longer-term storage, we recommend storing LNPs at -20°C, using a cryoprotectant in the buffer (≥3 months stability with our 4-lipid standard reference LNP formulation described in Table 6).

Of note, LNP formulation, buffer, cryoprotectant and storage temperature may need to be optimized. Avoid freeze-thaw cycles.

5. Evaluation of LNPs

5.1. Characterization of LNPs

We recommend starting the evaluation of formulated LNPs by characterizing their properties before their use *in vitro* or *in vivo* to ensure their correct formulation and reproducibility.

Table 8. Recommended LNPs characterization methods to be performed prior to *in vitro* or *in vivo* use

Attribute	Assay(s)	Recommended equipment/material (not supplied)	Expected range of results
Particle size & Polydispersity Index (PDI)	Dynamic Light Scattering (DLS)	Zetasizer Nano-ZS, Malvern	**Size: 25-100 nm PDI: <0.2**
Zeta potential	Electrophoretic Light Scattering (ELS)	Zetasizer Nano-ZS, Malvern	**8-15 mV**
Encapsulation Efficiency	Fluorescent dyes for mRNA quantification	Quant-it™ RiboGreen RNA Assay Kit, ThermoFisher	**>95%**

Once the LNPs are characterized with properties in the targeted ranges, they can be used for *in vitro* or *in vivo* delivery applications.

5.2. Use of LNPs for *in vitro* transfection

For optimal mRNA transfection conditions of cell lines, we recommend using cells which are 60 to 80% confluent at the time of transfection. Typically, for experiments in 24-well plate, between 40 000 to 100 000 adherent cells are seeded per well in 0.5 mL of cell growth medium 24 h prior to transfection.

1. On the day of transfection, add 250 ng to 500 ng of mRNA (Refer to Table 9) to each well (the volume of mRNA-LNP to be added should be determined based on the RiboGreen assay results). Introduce the mRNA-LNP solution dropwise onto the cells in their growth medium and distribute evenly.
2. Gently rock the plate back and forth. Return the plate to the incubator (37°C with 5% CO_2).
3. Analyze gene expression 24 - 48 h after the transfection.

Table 9. mRNA-LNPs transfection guidelines conditions for various cell lines

Cell type	Cells	Number of cells to seed per well (24-well plate)	Amount of mRNA (ng)
Epithelial	Caco-2	40,000	500
	A549	60,000	500
	HeLa	50,000	250
	HEK-293	50,000	250
	PC-3	50,000	500
Hepatocyte	HepG2	100,000	500

Of note, if your objective is to transfect **primary human T cells**, we have developed an **optimized protocol using LipidBrick®** that is accessible on our website. For additional information, please feel free to reach out to our Technical Team (polyplus-support@sartorius.com).

5.3. Use of LNPs for *in vivo* applications

For *in vivo* applications, an N/P ratio between 6 and 9 is recommended as a starting condition; however, for optimal results, we encourage to perform an N/P value optimization for every application targeted.

The quantity of mRNA to be administered must be tailored to the specific animal model, the route of administration, and the organ targeted. For guidance on mRNA delivery in mice, please refer to Table 10. Depending on the application, multiple injections may be required.

For other *in vivo* applications (different administration routes, other animal models, etc.), please contact our Technical Support at polyplus-support@sartorius.com for advice.

Table 10. Recommended conditions for most common injection routes in mice

Animal	Site of injection	mRNA Amount (µg)
Mouse	Intravenous (IV) injection Tail vein/Retro-orbital	10
	Intramuscular (IM) injection	5
	Intraperitoneal (IP) injection	20
	Intranasal (IN) injection	5

6. Troubleshooting

Table 11. Troubleshooting for LNP formulation and *in vitro* transfection

Observations	Actions
Formulation concerns	• Utilize our recommended LNP formulations (Tables 5 & 6) as standard references for benchmarking the properties of novel LNP constructs. • If your LNPs exhibit properties (Size/PDI/Zeta potential, EE%) outside the expected ranges (refer to Table 8), review the parameters employed (*e.g.* mRNA buffer, final buffer, centrifugation, mixing technique and instructions (*e.g.* flow rate, wastes, etc.)). • Ensure that the quality of your mRNA and lipids is optimal. • If it does not solve your issues, please contact our Technical Team: polyplus-support@sartorius.com
Unsatisfactory Transfection results	• Utilize our recommended LNP formulations (Tables 5 & 6) as standard references for benchmarking the performance of novel LNP constructs. • Employ *in vivo*-jetRNA®+ as a positive control. This ready-to-use solution consists of pre-formulated lipid-based nanoparticles containing LipidBrick® IM21.7c and provides a convenient and reliable standard for immediate use. • Fine-tune the quantity of mRNA utilized in the delivery assay to achieve optimal transfection efficiency. • Adjust the N/P ratio to optimize the encapsulation and delivery of mRNA. • Screen and fine-tune the LNP formulation by experimenting with various lipids (use the LipidBrick® Library kit; different helper lipids or PEGylated lipids) and/or lipid ratios to determine the most effective combination for your specific application. • Incorporate a common reporter gene-encoding mRNA, such as Luciferase or GFP, as a positive control to validate transfection efficacy. • Ensure the mRNA used is of the highest quality. It is preferable to source mRNA from an oligo supplier rather than using in-house transcribed mRNA. The OD260/280 ratio should exceed 2 to indicate purity. • Consider using chemically modified mRNA, such as 5' capped and incorporating modifications like pseudouridine, 5' methylcytosine, and 5' methoxyuridine, to enhance gene expression levels.
Cellular toxicity	• Replace medium 4 h after transfection. • Analyze transfection at an earlier time point (*e.g.* at 24 h instead of 48 h). • Decrease the amount of mRNA-LNP added per well. • Adjust the N/P ratio.
Toxicity *in vivo*	• Fine-tune the dose of mRNA-LNP to achieve optimal safety results while maintaining high transfection efficiency. • Reduce and optimize the N/P ratio. • Screen and fine-tune the LNP formulation by experimenting with various lipids (use the LipidBrick® Library kit; different helper lipids or PEGylated lipids) and/or lipid ratios. • Ensure that the mRNA preparation is endotoxin-free.

7. Product information

7.1. Ordering information

Table 12. LipidBrick® Offering

Product	Part N°	Quantity
LipidBrick® IM21.7c	**101000232**	50 mg
LipidBrick® IM21.7c	**101000172**	250 mg
LipidBrick® IM21.7c	**101000173**	1 g
LipidBrick® Library Kit	**101000241**	8 LipidBrick® lipids x 50 mg

7.2. Reagent use and limitations

For research use only. Not for use in humans.

7.3. Quality control

Each batch of LipidBrick® product is tested for compliance with the relevant quality controls and specifications. The certificates of analysis are available online in your customer area: https://myaccount.polyplus-sartorius.com/my-account-home/certificates-of-analysis

7.4. Formulation and storage

LipidBrick® should be stored at -20°C upon arrival to ensure long-term stability.

7.5. Trademarks

Polyplus® and LipidBrick® are registered trademarks of Polyplus-transfection S.A.

How to cite us: “LipidBrick® IMXX (Polyplus, Illkirch, France)”.

7.6. Contact information

Do you have any technical question regarding your product?

- Website: https://www.polyplus-sartorius.com/
- Email: polyplus-support@sartorius.com
- Phone: +33 3 90 40 61 87

Contact our friendly Scientific Support team which is composed of highly educated scientists, PhDs and Engineers, with extensive hands-on experience in cell culture and transfection. The Scientific Support team is dedicated to help our customers reach their goals by offering various services such as: protocol optimization, personalized transfection conditions, tailored protocols, etc.

Please note that the Polyplus-transfection® support is available by phone from 9:00 am to 5:00 pm CEST.

For any administrative question, feel free to contact our administration sales team:

- Reception Phone: +33 3 90 40 61 80
- Fax: +33 3 90 40 61 81
- Addresses:

Polyplus® locations	Addresses
Transfection reagent manufacturing site	75, rue Marguerite Perey 67400 Illkirch France
Plasmid Design Site	80 Rue du Dr Yersin 59120 Loos France
US Sales Office	1251 Ave of the Americas 34th fl. New-York - NY 10020 United States
China Sales Office	Room 1506, Tower B, Sunyoung Center No. 28 Xuanhua Road Changning District, Shanghai China