Comprehensive Understanding of the latest advancement in CRISPR based gene editing delivery methods

By, Claudio Mussolino, Spela Malensek, Peter Pecan, Tadej Satler Jure Bohinc, Emily Haughton; Karim Benabdellah, Dhanu Gupta, Dusko Lainscek

EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

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Comprehensive understanding of the latest advancement in CRISPR based gene editing delivery methods

Duration: Three days; 26–28 of June 2024

National Institute of Chemistry Slovenia

Ljubljana, Slovenia

PROGRAM AGENDA

Wednesday, 26.6. (7.30-18)

Thursday, 27.6 (9-18)

Friday, 28.6. (10-15)

Organized by the European COST action "Genome Editing to Treat Human Diseases" (GenE–Humdi; action CA21113), an EU–funded network that connects researchers and innovators across Europe and beyond

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Summary:

Led by an esteemed panel of international experts, the workshop delves into the forefront of CRISPR/Cas9 gene editing tool delivery. By exploring delivery modalities and practical applications, the workshop endeavors to facilitate the effective translation of gene editing technologies into clinical use.

Key Points:

- Introduction to the transformative capabilities of the CRISPR/Cas system in genome modification for clinical application
- Exploration of diverse in vitro delivery strategies
- Examination of viral, non-viral, delivery modalities for CRISPR/Cas systems.
- Hands-on training led by experts in lipid nanoparticle (LNP) preparation, mRNA production, and machine learning for targeted delivery.
- Laboratory sessions on LNP-mediated CRISPR/Cas gene editing, offering participants invaluable insights and practical skills.
- Discussions on the clinical applications of nanoparticles, approved gene therapies, and case reports analysis, led by international thought leaders

Trainers:

1. **Dr. Duško Lainscek (National Institute of Chemistry, Slovenia)**
2. **Dr. Dhanu Gupta (Oxford University, United Kingdom)**
3. **Dr. Claudio Mussolino (Freiburg University, Germany)**
4. **Dr. Karim Benabdellah (Fundación progreso y salud, Granada Spain)**

Local Support:

1. Špela Malenšek (National Institute of Chemistry, Slovenia)
2. Peter Pečan (National Institute of Chemistry, Slovenia)
3. Dr. Tadej Satler (National Institute of Chemistry, Slovenia)
4. Jure Bohinc (National Institute of Chemistry, Slovenia)

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Wednesday, 26.6. (7.30–18.00)

Location: Great Lecture Hall, NIC

7.30-8.00 Registration

8.00-8.15. Welcome (Karim Benabdel Lah El Khlanji, PhD; Duško Lainšček,

PhD) 8.15.-8.30 Objectives of the workshop (Duško Lainšček, PhD)

Session 1: CRISPR/Cas system–a powerful tool for genome modification

8.30-9.15 CRISPR/Cas system gene editing tool-introduction (Claudio Mussolino, PhD)

9.15-10.00 CRISPR/Cas delivery in vitro and in vivo-introduction (Dhanu Gupta, PhD)

10.00-10.30 Coffee break

Session 2: Delivery modes for the CRISPR/Cas system

10.30-11.15 Exploring viruses for the CRISPR/Cas system delivery (Duško Lainšček, PhD)

11.15-12.00 Exploring IDLV and Inducible LV-Variants for Versatile Delivery Application (Karim Benabdel Lah El Khlanji, PhD)

12.00-12.45 Extracellular vesicles: a non-viral method for CRISPR delivery (Dhanu Gupta, PhD)

12.45-13.30 Lipid nanoparticles-a new method for genome editing tool delivery (Duško Lainšček, PhD)

13.30-14.30 Lunch

break

Session 3: Practical approaches of LNP usage as a CRISPR tool transfer

PRACTICAL TRAINING with THEORETICAL BACKGROUND

14.30-14.45 Introduction to practical training (Duško Lainšček, PhD)

14.45-15.30 Theory in LNP preparation and subsequent characterization (Špela Malenšek, Peter Pečan)

15.30-16.15 mRNA production for genome editing tools (Claudio Mussolino, PhD)

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16.15-17.00 Using Machine learning and AI for de novo binder design for cell targeted delivery (Tadej Satler, PhD)

17.00-17.30 Cas9 protein isolation-tricks and tips for protein isolation (Jure Bohinc)

17.30-18.00 Wrap up of the first day, presenting case studies for the discussions in groups for CRISPR delivery (Duško Lainšček, PhD)

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Thursday, 27.6. (9–18)

Location: Great Lecture Hall, Department of Synthetic Biology and Immunology, NIC

PRACTICAL TRAINING with THEORETICAL BACKGROUND

9.00-9.15 Presentation of the agenda for the day (Duško)

9.15-10.00 Protocol for LNP preparation (Špela, Peter)

10.00-13.00 CRISPR/Cas gene edit mediated by LNP delivery - working in laboratory
(Špela Malenšek, Peter Pečan, Duško Lainšček,

PhD) 13-14.30 Lunch break

14.30-17.30 CRISPR/Cas gene edit mediated by LNP delivery - working in laboratory
(Špela Malenšek, Peter Pečan, Duško Lainšček,

PhD) 17.30-18.00 Wrap up

Friday, 28.6. (10–15)

Location: Great Lecture Hall, NIC

10.00-10.30. Discussions about the past day; Presentation of the agenda for the day (Duško Lainšček, PhD)

10.30-11.15 Nanoparticles in clinical use (Duško Lainšček, PhD; Dhanu Gupta;

PhD) 11.15-12.00 Approved gene therapies and their delivery (Claudio

Mussolino; PhD) 12.00-13.00 Lunch break

13.00-15.00 Case reports discussions

15.00-15.15 Wrap up, End of the

Workshop

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Report:

1. Dusko Lainšček

Duško Lainšček, PhD presented general knowledge on lipid nanoparticles (LNPs) and their efficient use in various cargo delivery. Composition (ionizable lipids, helper lipids, cholesterol) was elucidated, also the role of PEG lipids and DOTAP addition were discussed in order to aid in special cell specific targeting and increasing RNP encapsulation efficiency respectively. Based on published literature safety aspects of LNP were also investigated regarding the dosage and administration route. Also, clinical aspects of the use of LNPs was discuses ass CRISPR based clinical trials, using LNPs were presented. LNPs can be used as a powerful delivery tool for CRISPR/Cas system in the form of mRNA or RNPs. Jure Bohinc, a PhD student also presented in house established protocol for recombinant Cas9 protein isolation and purification. LNP production and also delivery, biodistibution and uptake mechanisms were presented. Special emphasis was put on in vivo delivery and how passive and active targeting can be achieved, especially for brain delivery in vivo, bypassing the limitations of LNPs and their blood brain barrier crossover.

2. Dhanu Gupta (Half page)

    a. EVs productions use functionalization

Dhanu Gupta described extracellular vesicles (EVs) as naturally occurring nanoparticles surrounded by a lipid bilayer, secreted by all cells, from bacteria to human cells. These vesicles contain a variety of bioactive molecules, including different types of RNA, proteins, and lipids derived from their cells of origin, allowing them to mediate communication between cells. Due to their natural origins, EVs have unique properties, such as immune tolerance, stability in circulation, and the ability to cross biological barriers, including the blood-brain barrier, making them highly attractive for therapeutic applications like drug delivery. He highlight that the had EVs have gained significant attention as potential next-generation drug delivery vehicles. Researchers have developed engineering tools to enhance the therapeutic capabilities of EVs, including methods for loading therapeutic cargoes such as CRISPR-Cas9 either exogenously or endogenously. Exogenous loading involves manipulating the EV membrane after isolation using techniques like electroporation, while endogenous loading involves engineering the producer cells to incorporate therapeutic agents directly into the vesicles during their formation. EVs have shown promise in delivering CRISPR-Cas9 for various therapeutic applications. For example, EVs have been used in gene editing for diseases like Duchenne muscular dystrophy (DMD), leading to improved muscle function and increased dystrophin expression in both animal models and patient-derived cells. Recent studies have also introduced innovative systems, such as VSV-G plus EV-sorting Domain-Intein-Cargo (VEDIC) and VSV-G-Foldon-Intein-Cargo (VFIC), which

enhance cargo delivery efficiency by using proteins that facilitate endosomal escape and efficient cargo release into recipient cells. These systems have demonstrated high recombination and genome editing efficiency. Dhanu point out that, despite these advances, challenges remain in optimizing the stability, targeting, and cargo loading efficiency of EVs to ensure safe and effective use in clinical settings. Future research will focus on overcoming these challenges to fully harness the potential of EVs in therapeutic applications.

3. Claudio Mussolino mRNA Production in house (just a short text, pros, difficulties…tips..)

Transgene delivery using in vitro transcribed mRNA is a simple but efficient modality to transiently equip the cells with a novel gene function. In this Training School, the trainees have received an overview of the different cellular RNA types and focused on messenger RNA (mRNA) which is used to deliver genome editing components inside the cells to induce genomic changes. The trainees have learnt how to generate *in vitro* an mRNA that encodes the Cas9 protein and have received an overview of the different steps involved. Gene delivery via mRNA offers the advantage that the transgene is expressed for short time, but in the case of genome editing, this is sufficient to induce in the cell a long lasting editing (i.e. hit-and-run approach). This is particularly important to reduce toxicity and potential unwanted events that might result by the long-term exposure of the genome to editing components. However, mRNA are very sensitive and degraded fast, thus requiring particular handling precautions. Moreover, the human cells have evolved to fast recognize and destroy foreign RNA, which might resemble an invading virus genome, and therefore it is important to control the impact of the mRNA on cell viability. To this end, the trainees have learnt of different mRNA modifications that can be introduced to mitigate cellular reaction and toxicity.

4. Karim Benabdellah (Half page) IDLV production uses, production, aplication

Karim Benabdellah discussed the use of retroviruses, such as gamma retroviruses and lentiviruses, for gene delivery in gene therapy. Although these viruses can efficiently deliver large amounts of genetic material to cells, their stable integration into the host genome is not ideal for gene editing, which requires transient expression. To address this, Self-inactivating (SIN) and integrase-defective lentiviral vectors (IDLVs) were developed to enable safer and temporary delivery of therapeutic genes. Benabdellah highlighted key studies using IDLVs for gene editing. For example, in 2007, Cathomen and colleagues used IDLVs for gene correction through Homology-Directed Repair (HDR), demonstrating a proof-of-concept approach to rescue a defective EGFP gene. Later, IDLVs were used to correct Artemis deficiency in mouse hematopoietic stem cells and to deliver zinc finger nucleases (ZFNs) for high editing rates in human cells. Further advancements included targeted gene addition in human epithelial stem cells and modification of the human

adenosine deaminase (ADA) gene. Benabdellah also discussed improvements in IDLV design to enhance gene editing efficiency. His team developed enhanced IDLVs with configurations that improved transgene expression and cell specificity. Other studies showed that IDLVs are effective for delivering CRISPR components, such as Cas9 and guide RNA, in vitro and in vivo, including applications for correcting mutations like those causing sickle cell disease. Overall, Benabdellah highlighted the potential of IDLVs as a promising tool for safe and efficient gene editing.

5. Špela Malenšek and Peter Pečan, other team members

On Wednesday (26th of June 2024), Špela Malenšek and Peter Pečan presented theoretical concepts for designing and preparing experiments involving lipid nanoparticle (LNP) production. The presentation covered key parameters affecting LNP-mediated delivery efficiency to target tissues or cells, including lipid composition, formulations, N/P ratio, and low pH buffer requirements. Various LNP production procedures from literature were discussed, with emphasis on microfluidics. The protocol for LNP production and particle quality control was reviewed, followed by an overview of approaches to target LNPs to specific tissues and cell types through lipid composition modification or conjugation with cell type-specific antibodies. Data presented during the workshop were from previously published studies or representative results of our own work.

On Thursday (27th of June 2024), a practical workshop on LNP production was conducted using in-house prepared lipid formulations, mRNA, and the Nanoassembler Ignite+ microfluidic device. LNPs were prepared as previously described in the literature [1,2]. Starting material of mRNA was at least 180ng/µl. For the LNP assembly molar ratio 50:10:39:1 (ionizable lipid MC3:helper lipid DSPC:cholesterol:PEG ioniazble lipid) was used. Flow rate was 12ml/min with flow ratio of 3:1 (aqueous phase: ethanol phase). Post-processing buffer exchange was performed via PBS dilution and subsequent concentration using centrifugal concentration filters (Amicon; 100kDa; 2000g/30min/4°C). LNP size was measured by dynamic light scattering (DLS), and encapsulation efficiency was determined using the QuantiFluor RNA system (Promega). Three types of LNPs were prepared: empty LNPs, LNPs encapsulating eGFP mRNA, and LNPs encapsulating Cas9 mRNA (prepared in house by in vitro transcription kit using HiScribe T7 ARCA mRNA kit and B2M-targeting sgRNA (purchased from Genscript) (4:1 w:w ratio). Encapsulation efficiencies were 78% and 84% for the latter two, respectively. After buffer exchange, average LNP sizes were 69 nm (empty), 79 nm (eGFP), and 85 nm (Cas9-B2M_sgRNA). Polydispersity indices (PdIs) were all below 0.2 (0.172, 0.131, and 0.112, respectively). LNP samples were tested for delivery efficiency on THP-1 cells (acute monocytic leukemia cell line) and on U937 cells (macrophages). The LNP uptake efficiency for eGFP LNP was determined 24 hours post LNP addition and was quantified by flow cytometry, whereas gene editing efficiency was determined by B2M loci amplification with subsequent Sanger sequencing and TIDE analysis.

6. Tadej Satler

Tadej Satler, PhD presented potential targeted delivery of LNPs through the use of minibinder armored LNPs. Strategy for protein binder design relies on cutting-edge AI technologies for de novo protein design. The workflow commences with the generation of binder scaffolds using RFdiffusion[5] to diffuse poly-glycine binder scaffolds towards the target protein. Subsequently, the ProteinMPNN[3], a deep learning-based protein sequence design method, is employed to generate binder sequences based on the diffused protein

backbone. To validate the designed binders, we employ AlphaFold2[4], an advanced deep-learning model for protein structure prediction. This validation stage includes an assessment of AlphaFold2 scores and relevant metrics obtained through Rosetta software. The filtering process, guided by these scores and metrics, enables the identification and prioritization of binders with the highest potential. We have implemented the whole modelling pipeline at the computational cluster at NIC and are using this modelling pipeline routinely and have successfully designed and established the efficiency of de novo designed minibinders in the low nanomolar efficiency.

7. Emily

    a. General notes from the TS (See attached document)

1. Wang, X. *et al.* Preparation of selective organ-targeting (SORT) lipid nanoparticles (LNPs) using multiple technical methods for tissue-specific mRNA delivery. *Nat Protoc* **18**, 265–291 (2023).
2. Weinstein, S. *et al.* Harnessing RNAi-based nanomedicines for therapeutic gene silencing in B-cell malignancies. *Proceedings of the National Academy of Sciences of the United States of America* **113**, E16–E22 (2016).
3. Cao, L. *et al.* Design of protein-binding proteins from the target structure alone. *Nature* **605**, 551–560 (2022).
4. Dauparas, J. *et al.* Robust deep learning–based protein sequence design using ProteinMPNN. *Science (80-.).* **378**, 49–56 (2022)
5. Watson, J. L. *et al.* De novo design of protein structure and function with RFdiffusion. *Nature* **620**, 1089–1100 (2023).

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General notes from Training school:

DAY 1:

Intro to genome editing tools/CRISPR-Cas9 systems (Claudio Mussolino)

-genome editing vs. epigenetic editing

-NHEJ (error prone, 'always on' therefore increased efficacy)

-HDR (increased accuracy, but only active in dividing cells)

Designer nuclease types:

- Zinc finger nulceases
- CRISPR-cas9
- TALENs

Improving CRISPR-cas9 specificity:

- Orthologous Cas9 (longer PAM seq. Means less off target as occurs less in genome)
- Truncated gRNA (typically ~20nt but can reduce to 17nt to decrease mismatches…need at least 16nt for cleavage)
- CRISPR-FokI
- Paired nickases (staggered ds break causes reduced genotoxicity)
- Hifi Cas9

Genome editing process:

1) Retrieve DNA site
2) Find suitable target site (gRNA has to have 5' G/GG for polymerase to bind and Cas has to have PAM site)
3) Delivery of genome editing components
4) Evaluate gene editing (TIDE etc.)

Delivery of CRISPR cas9-overview (Dhanu Gupta)

Issues:

- v. large molecules
- Negatively charged

- Endocytosis does NOT = delivery (must get into cell AND be effective)
- Blood flow decreases massively in liver therefore Kupffer cells etc. Have a long time to sequester 90-99% of dose
- New Crispr systems (e.g. prime editors) are even bigger therefore getting even harder to deliver.

Can deliver as:

1) DNA:

-widely used BUT

-will keep cutting until PAM site is mutated (therefore have long-term expression)

-lot of off-target effects

-often inserts mutations

2) mRNA

-most used in vivo

-transient expression (3-4 days) as is unstable

-Decreased off target

-BUT v. big therefore hard to deliver

3) protein

-rapid expression (therefore decreased off target/ bystander edits)

-hard to deliver

Delivery vectors include:

- Adenovirus
- AAVs
- LVs
- EVs

AAVs:

-size is limiting factor (can package ~4kb/ **MAX** 5Kb) therefore base/prime editors are too big

-can use intein system/ trans splicing (splits cargo into 2) but aren't guaranteed to get both in same cell

-can also compact cargo

-viral toxicity is issue

-different AAVs go to different tissues

-synthetic AAVs/ library screens optimize tropisms

Delivery of modified mRNA:

-can alter stability/ half-life and increase translation through modification

-can lead to immune response

-need to protect from nucleases

Can use LIPID NANOPARTICLES:

-hydrophobic outside (interacts with plasma/ causes protein corona)

-ionizable lipids are only cationic at low PH therefore doesn't interact with platelets etc.

-v. Fast expression in liver (~½ a day) and is unstable therefore short lasting

-only goes to liver but has good editing

-no real size limit but hard to package protein as result of charge

-can alter backbone to increase stability and decrease off target

VLPs:

-LVs without genome

-fuse Cas9 to cause simultaneous genome and cas9 expression

-hard to put in modified RNA

-seem to have similar editing to AAVs

-different AAVs go to different tissues

-synthetic AAVs/ library screens optimize tropisms

Viruses for CRISPR-Cas delivery (Duško Lainšček)

Safety (genome integration/ immunogenicity):

AAV>AdV>LVs

Note: LVs can cause oncogenesis

Expression duration:

- LVs-several years
- AdV< 2 months
- AAVs- several years (in nondividing cells)

-Can modify capsid to increase targeting

-need to consider immunogenicity

AAVs:

- Small genome
- 100 serotypes in nature-have differing tropisms
- Don't cause inherent disease in humans
- Can't replicate without helper virus
- 30% of US residents have neutralizing antibodies
- Can use sense / antisense cargo (~4.7kb)
- Can cause genome integration
- Most used =AAV9
- Enter cells via clatherin mediated endocytosis
- Can split Cas OR have nuclease in one and RT in another

AdVs:

- Can cause (mild) human disease
- ~57 serotypes
- Ad2/Ad5 (sp.C) most commonly used
- BUT Most people have neutralizing antibodies
- Non-envelope dsDNA virus (26-45kb)
- 90-100nm
- Can infect both dividing and non-dividing cells (use multiple receptors)
- Cell dies after release of virus (can be harnessed for cancer therapies)
- Has early and late-stage lifecycle with different genes involved (can knock out different genes to target different functions/ life stages)
- Production involves 3 plasmids…pAdeasy/ shuttle vector+GOI/ pAd+GOI

LVs

- +ssRNA virus
- 80-120m,
- V. efficient (can infect dividing and non dividing cells)
- Can engineer to recognize any receptor (e.g. Use VSVG for LDLR)
- Structural genes: gag (virion assembly), pol. (virus replication), env. (binding/fusion)
- Accessory genes help formation/ regulation etc.
- Are 4 generations (3&4 are replication deficient)

Inducible LV variants/ IDLV (Karim Benabdel Lah El Khlanji)

Viral vectors :

-v. Efficient for large cargo

-low immunogenicity and high tropism

-reduced need for specialized equipment

Retroviruses:

- Envelope
- SsRNA
- Integrate into host genome
- Can be simple (e.g gamma retrovirus) or complex (e.g.LVs)

Inducible LVs:

- Use trans-activators (e.g. tet-on/ doxycycline)
- Should be reversible
- Inducibility is v. dependent on cell type (e.g. KS62)
- IN CART cells toxicity is issue, therefore inducibility would be v. useful (increased IL6 expression would stop CART expression)

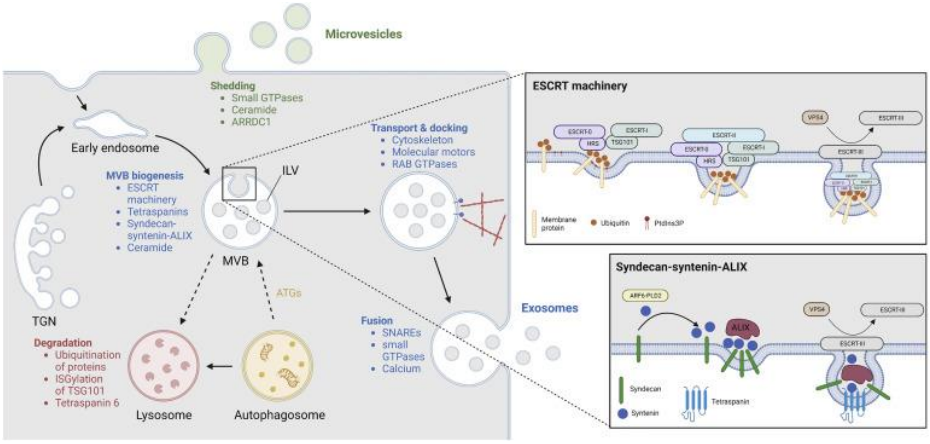
IDLVs

- ‘integration deficient LV)
- Can be used as unbiased method for detecting off-target AND as delivery vector
- Use of IS2/WPRE increases transgene expression of IDLVs

Extracellular vesicles as delivery vectors

-made in every cell therefore v. low safety profile (can repeatedly dose etc.

-Biogenesis pathways of extracellular vesicles:



- Note: There are also apoptotic bodies

Can endogenously engineer:

-fuse cargo to EV sorting domain to load

(hard to load after creation /electroporate etc.)

Luminal engineering

- Membrane interacting proteins (e.g BasP1/ARRDC1/ myristolation tag etc.)

- Soluble proteins (syntenin/ ALIX etc.)
- Tetraspanins (CD63/ CD81 etc.)
- Single transmembrane proteins (PTGFRN/PDGFR etc.)
- Oligomeric transmembrane proteins (TNFR-foldon-syntenin)

surface engineering

- Tetraspanins (CD63/ CD81 etc.)
- Single transmembrane proteins (PTGFRN/PDGFR etc.)
- Oligomeric transmembrane proteins (TNFR-foldon-syntenin)
- Outer membrane interacting proteins (GPI anchors etc.)

Lipid nanoparticles as delivery tool

5 types:

- Liposomes
- Lipid nanoparticles
- Lipid emulsion
- Solid lipid nanoparticles
- Nanostructured lipid carrier

are NON-viral

-low immunogenicity

-application flexibility (10-500nm)

-easier to assemble (spontaneous self-assembly)

-but CAN'T cross bbb

Nanoparticles:

- Nanospheres: homogenous makeup
- Nanocapsules: shell structure containing payload

LNP composition:

- Ionizable cationic lipid …endosomal escape
- Cholesterol

- Helper lipid (DOPE/DSPC) …loading and endosomal escape
- PEG lipid… can be anchored/ conjugated

Ionizable lipids:

- head group (often amine group)
- Stable biodegradable linker
- tail with differing carbon length
- have +ve charge under acidic PH (loading) but neutral charge at physiological PH (laminar vs hexagonal phase)
- Properties influence biodegradability, immunogenicity, potency, control of nucleic acid encapsulation and endosomal escape

Note: loading RNPs need DOTAP or guanidium etc.

Helper lipids:

-Phospholipids/ cholesterol/PEG

-cause:

- Increased stability
- Destable endosome
- Increased integrity
- Increased cell uptake
- Increased biodist.
- Decreased endosome fusion
- Decreased aggregation (via zeta potential)

Uptake does NOT= protein expression

Lots of barriers:

- enzyme degredation
- plasma protein sequestration
- RES entrapment
- high renal clearance

ONLY 2-3% ESCAPE ENDOSOMES

Targeted delivery:

-couple to antibodies

-need modification to cross bbb (as bbb doesn't have LDLR)…can use TfR/insulin receptor etc.

-'trojan horse'

-can use GalNac (uptakes via ASGPR) to try avoid liver

LNP production theory:

4 main components:

1) ionizable lipid

2) neutral lipid

3) cholesterol

4) PEG lipids

Molar ratios have to be adjusted based on target tissue/cell

- N/P ratio is usually 1-6:

(Amine groups of ionisabel lipid: total number of –ve phosphate groups in nucleic acid payload)

There are 4 main manufacturing methods:

- Ethanol dilution
- Manual mixing

(both cheap and easy to do BUT low reproducibility and decreased encapsulation efficiency)

- T mixing
- Microfluidics

T mixing:

-control flow rate (increasing reproducibility)

-need large sample size

-PDI is high

Microfluidics:

-have features that cause mixing

-Can control LNP size (20-200nm)

-low PDI

Evaluation:

1) DLS (dynamic light scattering)
   - Shows size and size distribution
   - PDI (indicates size range)... <0.2 is good
   - Zeta potential (+ve surafce charge =binding to cell membrane/ unspecific binding to normal tissues)

2) Encapsulation efficiency

3) Delivery assessment
- Use mRNA for reporter proteins (Fluc/eGFP/iRFP)

Targeting LNPs:

Passive targeting:

- Deliver to tissues without modifying surface (intratissue administration)
- SORT (changes surface charge/ high organ specificity but low level cell specificity)

Active targeting:

- Can facilitate cellular uptake of LNPs
- Can use antibodies (or derivative/ ScFV/ minibinders)

2 methods:

1) Anchor lipid (e.g. PEG/ malemide)-> BUT can get incorrect orientation
2) Post insertion of hydrophobic antibody derivative (usually lipid-ligand conjugate)

mRNA production (Claudio Mussolino)

RNA types:

- LncRNA
- mRNA
- siRNA
- premRNA
- tRNA
- SncRNA
- rRNA
- SnoRNA

…So many types that its difficult to mimic in vitro

In vitro transcription:

1) Polymerase binds to promotor to initiate transcription (TATA)
2) Protection
   -5’ capping
   -pol II c-terminal domain is essential for capping enzyme activation

3) Splicing
   -removes introns
   - can be simultaneous to transcription
   -½ life of an intron=7 min

4) Polyadenylation
   -protects
   - stops polymerase (5’-3’ exonuclease)

Splicing :

- U1 interacts with U2 (snRNA base pairs with corresponding GU site)
- U6 brings ends close enough to base pair (recruitment of U4/5/6 & release of U1)
- Nucleophilic attack to 5’ G (creates lariat)
- Nucleophilic attack of 5’-3’ brings ends together
  can be error prone

-there are many alternative splicing options (cause ~20% of total mature transcripts) …e.g exon skipping/ intron retention etc.

Different cap analogues:

Typical->cap 0

…Turns into cap 1 (triggers decreased immunogenicity)

…If v. long lived, turns into cap 2

Mimic using cleancap (TM)-cap1 (A&G)

ARCA-cap0 (G)

different caps may give different efficiency

Have to choose promotor based on cleancap etc.

Technical procedure:

1) Template prep:
   -PCR (FWD primer with T7 AG promotor and rev. Primer with polyA tail)
   -plasmid linearization (contains T7 AG promotor/ use RE)

2) IVT
   -add nucleotides
   -incubate at 37 degrees

3) DNA degradation
   -DNAse1 at 37 degrees

4) PolyA tailing
   -polyA buffer/ polymerase/ ATP (37 degrees)

Machine learning/ AI for de novo binder design

RFdiffusion

- Can generate protein scaffold out of noise
- Hotspot and 2ndary structure binding (provide protein binding domain)

Protein MPNN

- Inverse folding –give structure/seq

Alphafold (AF)

- Predicts protein structure from seq.

Manually decide target/pocket--> RF diff--> MPNN/AF2

Most binder /target predictions from AF will be off target (~2% will be desired protein/ +ve hits)

-filter results using AF and Rosetta

-do partial diffusion of backbone (add noise then refine again)

Cas9 protein isolation tips:

Can use RNPs rather than plasmids/ mRNA

- controls dose
- decreased nucleic acid in cells
- faster delivery (no transcription etc.)
- works better in some cells

In vitro experiments;

- Can quickly validate gRNA seq.
- validate novel genome edits in vitro

Can use Cas9 as restriction enzyme/ in cloning

Not all Cas9 proteins are commercially available

- Can modify Cas9 proteins/ use Cas9 from uncommon spp.

1) Fermentation:
   -if bacteria not growing spike with glucose
   -ferment at low temp. (18C) overnight
2) Lysis:
   -keep everything on ice

-check for colour changes

3) NINTA column purification:
- Also keep cold
- Use v. high salt buffer

4) SEC with FPLC:
   - Analyse fractions with PAGE

5) Dialysis and concentration :
   -try high concentration storage buffer (2X)
   -dilute in 50%glycerol to help with storage

6) Validate

Day 2

CryoEM

Samples can be:

- proteins
- vesicles
- artificial lipids etc.
- Complexes

Freezing in h2O means samples can imaged over the course of days

Use amorphous solid water

- Samples MUST be vitrified
- Must have NO crystals otherwise unable to see anything
- Use ‘vitrobot’ …use grids

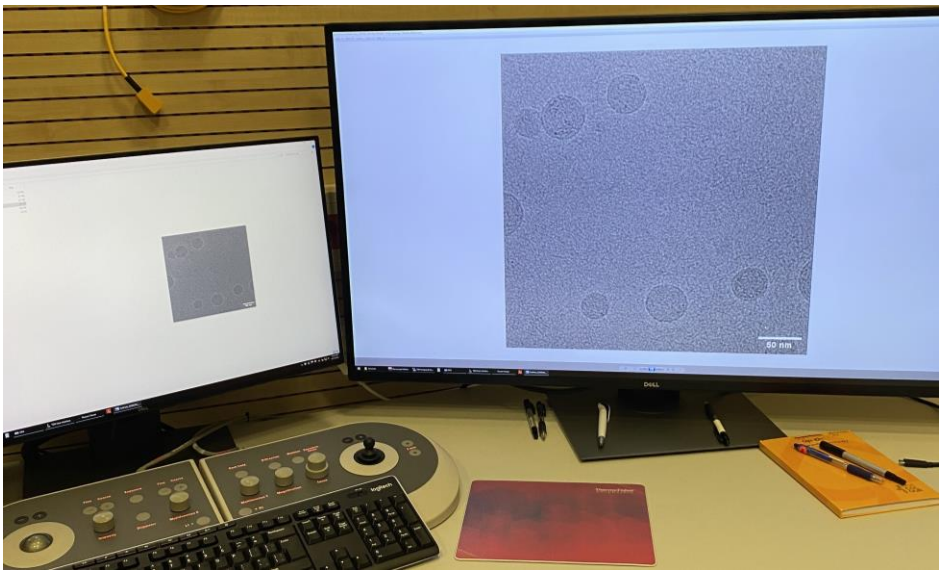
Image sample in ‘holes’ of grids (50-10nm of ice)

Take 2D averages of particles in many orientations

- Combine together to create density map/ 3D structure
- Can get atomic resolution

Can produce 1.5 terabytes of data in 24 hours.

(CryoElectron microscope)



(image of LNPs under cryoEM)

LNP production

*****TEST YOUR CARGO- very important!!!!! LNPs won't work if cargo is bad******

1) Reagents preparation

-sterile filtration of buffers (PBS/ citrate buffer)

Lipid mix (prepared in advance):

- Molar ratio 50:10:39:1 (ionizable lipid:helper lipid:cholesterol:PEG)
- Total concentration: 12.5mM

Formulation	
MC3	51
DSPC	10
Chol	38
DMG-PEG2000	1

Volumes for 2mL total	
MC3 (50mg/mL)	142
DSPC (4mg/mL)	550
Chol (4mg/mL)	774
DMG-PEG2000 (50mg/mL)	15
EtOH to 2mL	*519*

- lipid mix

- Nucleic acid solution

-note: can premix and freeze, but better to use fresh

Nucleic acid solution:

- N/P ratio: total N of ionizable lipid amine groups (N) to total number of negatively charged phosphate groups (P)

(1) $N = ionz.\ lipid\ c\ in\ etoh\ mix * lipid\ mix\ inj.vol * N_A * number\ of\ N\ per\ molecule$

(2) $\frac{N}{P} =$

(3) $n_{mRNA} = \frac{P}{N_A * number\ of\ P\ per\ bp * number\ of\ bp}$

(4) $\gamma_{mRNA} = \frac{n_{mRNA} * Mr_{mRNA}}{mRNA\ inj.volume}$

5

-cas9mRNA:sgRNA = 4:1

NOTE: often lipid formulations that work in vitro DON'T translate into in vivo –use literature instead

2) LNP production

-syringe filling

-take sample for DLS (QC control) before AND after centrifugation

Note: per70nm lipid particle, payload= 1-2mRNAs

-set start waste (mixing will be sub optimal at start therefore get rid of ~100ul depending on syringe)

-We created 3 types of LNPs

- Cas9mRNA+sgRNA (4:1 ratio)
- eGFP mRNA
- Empty LNPS

Note: LNPs should not look milky as this indicates aggregation.

3) post processing

-buffer exchange/ concentration (use 30kda amicon centrifugal filters ->2000G->30+min @4 degrees)

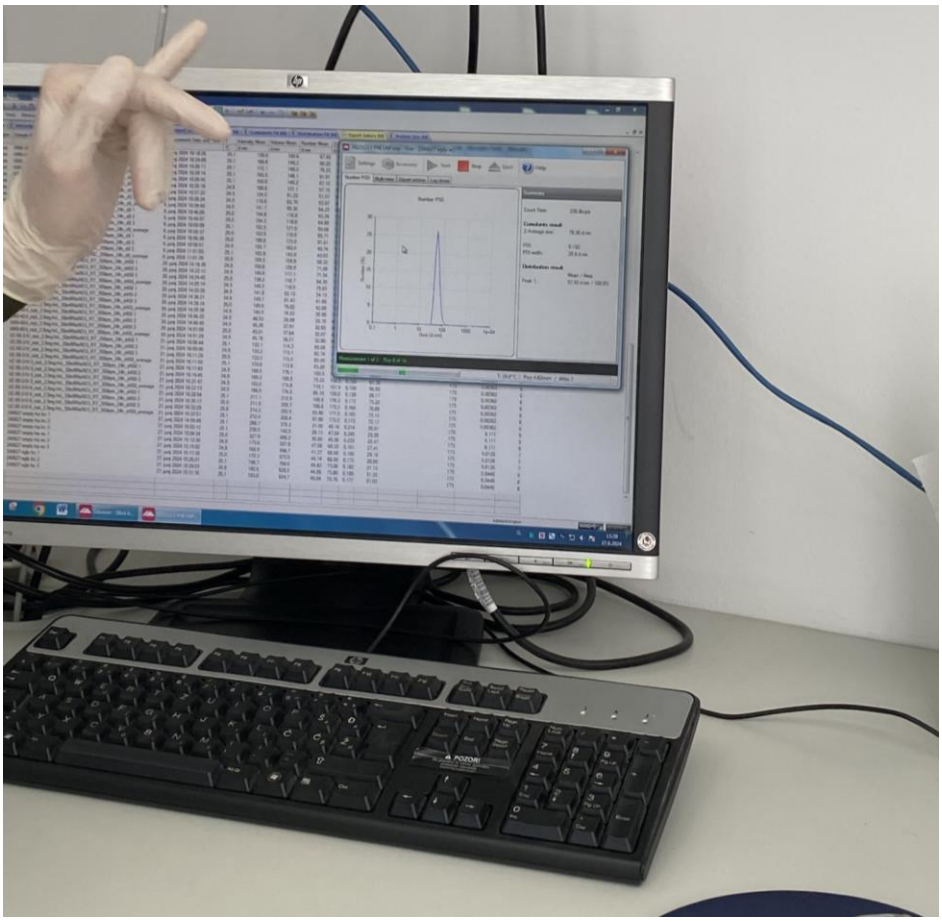
-conjugation

-freezing in 20% sucrose

4) Final QC

-DLS

- Average size, polydispersity and size distributions
- PDI=normalised value that indicates size range and indicates sample quality (<0.2 =acceptable)



-encapsulation efficiency

- QuantiFluor RNA system
- Measures 0.1-500ng/ul RNA
- Wider dynamic range
- Use 2% tritonX-100 to destroy LNPs (must take triton blank)
- LNP only shows non-encapsulated mRNA

DAY 3

Approved Gene therapies and their delivery (Claudio Mussolino)

Transplant of healthy donor cells:

-v. Successful in primary immunodeficiency (related donors 90% success, unrelated 60% success)

Donor--->stem cell isolation---> ex vivo gene correction--->stem cell reinfusion

SCID-X1

- Complete loss of T, B and NK cells
- IL2RG gene complete loss

- Cured via LV gene therapy BUT occasionally resulted in cancer development

X-CGD

- Mutation in gp91 phox gene causes loss of function of macrophages (via NAPDH)
- Epigenetic silencing selected for GFP NOT therapeutic gene … effect decreased after a while
- Also gave rise to cancer due to proto-oncogene proximity

use self-inactivating vectors-> decreases adverse effects

Hemoglobinopathies

- Among most common inherited diseases worldwide
- Through embryonic development/life, method of O2 consumption differs and therefore so does globin type

B-thalassemia

-point mutation/ deletion in HBB

-severity depends on mutation (normal->reduced globin synthesis->no globin synthesis)

SCA

-level is normal but globin are sickle (causes blood to clot in capillaries)

-other mutations can 'compensate' for SCA

- HPFH reactivates gamma globin (increases globin in blood)
- Can harness this…introduce compensatory mutation mimicking HPFH
- Target BCLIIA intron enhancer (only active in disease cells) using CRISPR-gamma globin not repressed
- Patients are transfusion independent

Nanoparticles in clinical use (Duško Lainšček; Dhanu Gupta)

-~100 CRISPR based clinical trials (most ex vivo for cancer trials eg. CART)

-1st FDA approved CRISPR therapy=Casgevy

LNPs in clinical trials:

1) VERVE-101 (in vivo phase1)

-ABE inactivate PCSK9 in liver
-for HeFH, ASCVD and patients with uncontrolled LDL-C levels
-decreases heart attack and stroke risk

2) VERVE-102
-similar but different LNP formulation (GalNac)
-LDL-R independent pathway
-ANGPTL3 gene

3) CTX310 (phase 1)
-ASCVD
-knocks out ANGPTL3 (regulates lipid metabolism)
-Cas9 mRNA and gRNA in lipids
-LDL-R independent
-mice and NHP studies are promising
(CTX320 is similar)

4) NTLA-2001 (phase III)
-ATTR (protein folding disease) caused by TTR mutation (damages peripheral nervous system)
-LNPs containing cas9 mRNA and gRNA used
5) NTLA-2002 (phase I/II)
-for HAE
-disables KLKB1 gene using Cas9/gRNA LNPs
-3 patients have shown dose dep. Decrease of KLKB1 (25/50/75mg mRNA/kg dose)

NOTE: ALL trials are in LIVER (/lungs)!!!! No targeted delivery yet

Ways to deliver gene therapies:

1) In vivo-> IV route/ direct brain injection
2) Ex vivo
3) In situ (tumor injection etc.)

-sequencing of human genome led to increase of gene therapy

-only 30% of vectors in clinical use are non-viral

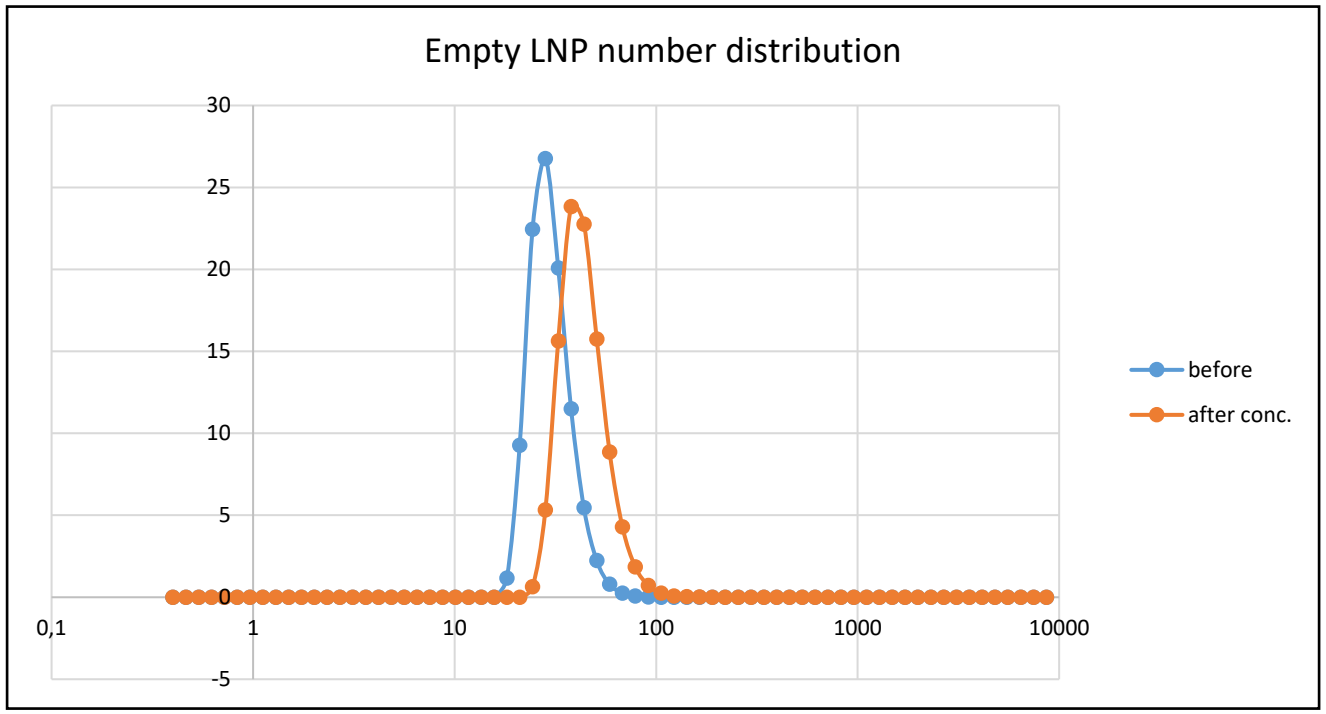
Non-viral vector examples:

- Microneedle patch
- Antibody-drug conjugation
- LNPs
- Inhalable device
- Nanoparticle
- PH response capsule .....

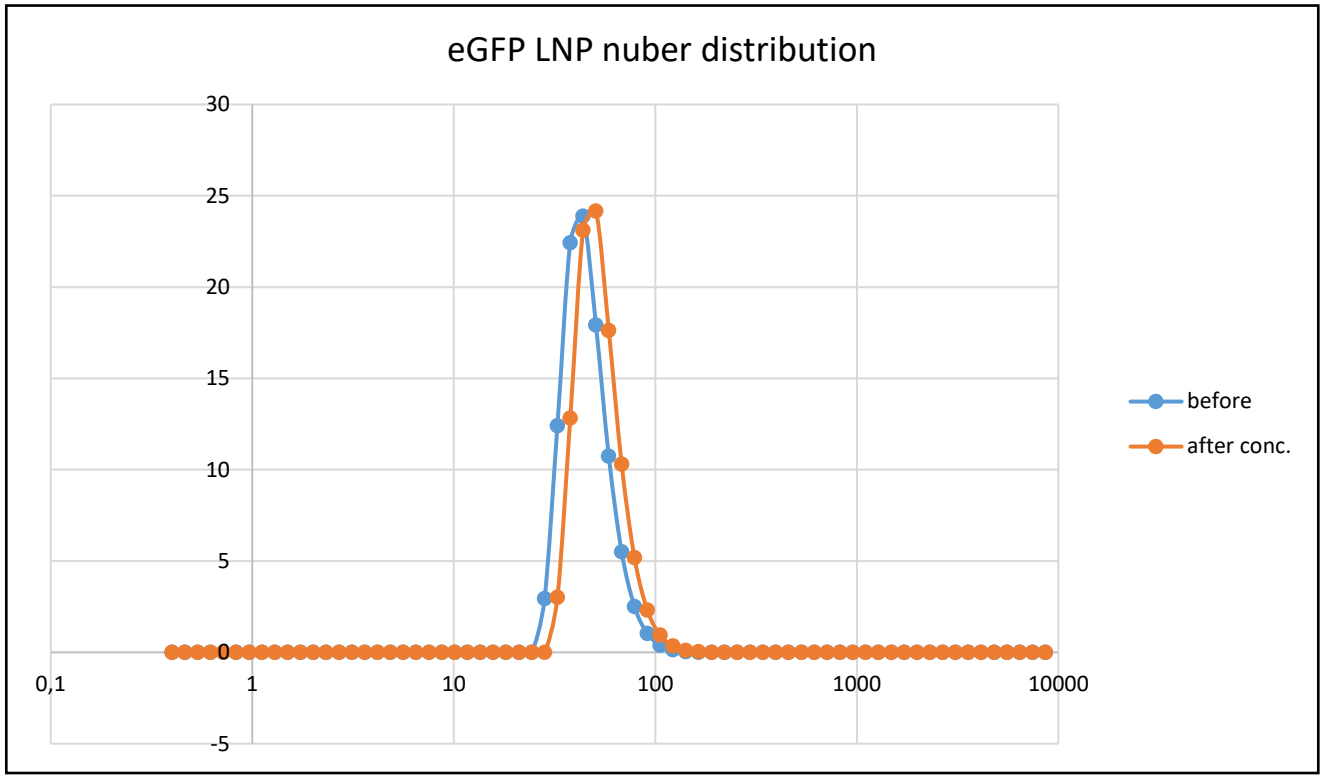
Oligonucleotide drugs

- 15 approved by FDA -> most gapmers (use RNAse H therefore act in nucleus)
- Modifications lead to inreased affinity and stability
- WAVE biosciences have new successes with ASOs
- Can increase delivery using
  -LOCs (e.g. galNac)-increase specificity
  - Antibody-oligo conjugates (TfR moist used as this increases delivery to the brain 20/30 fold)

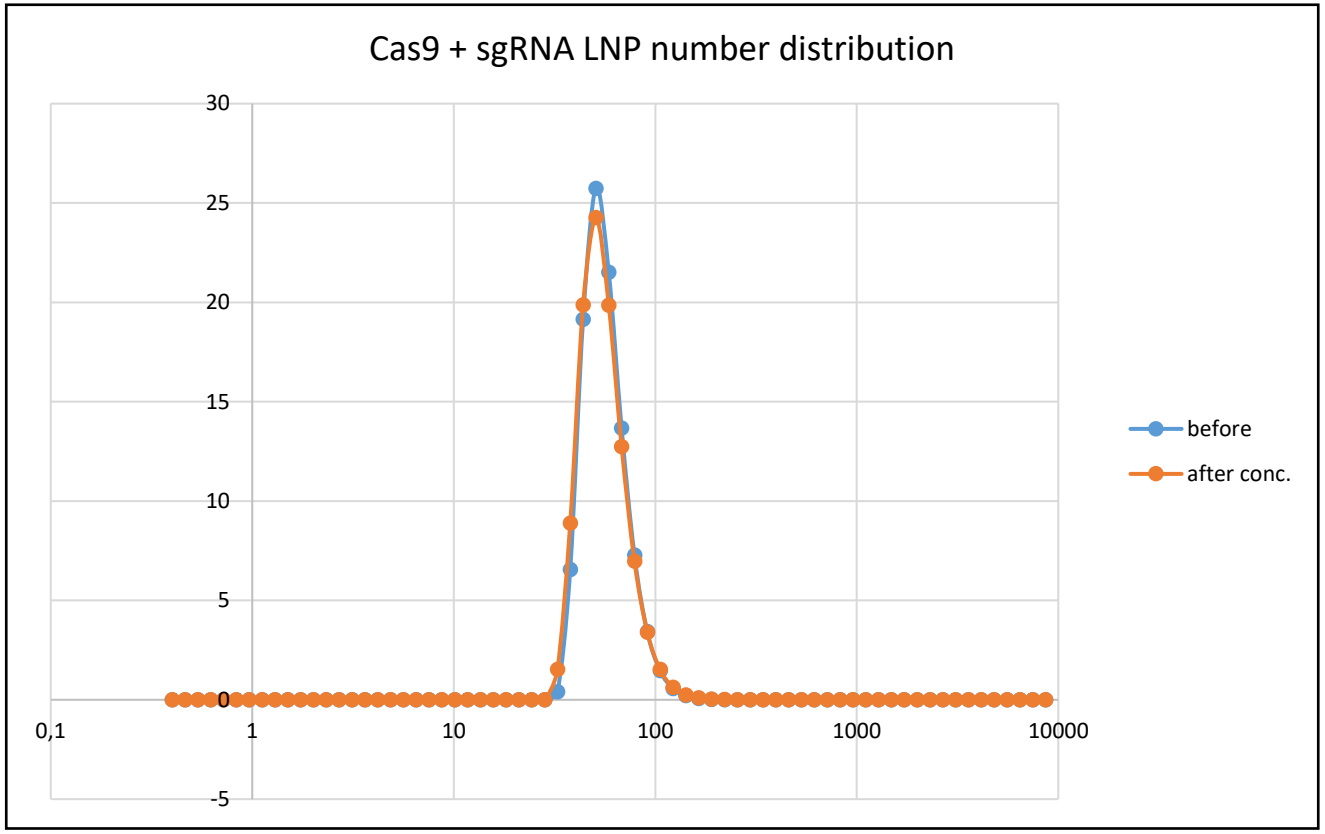
* there are also many other nanoparticles (polymersome, dedrimer, synthetic particles etc.)



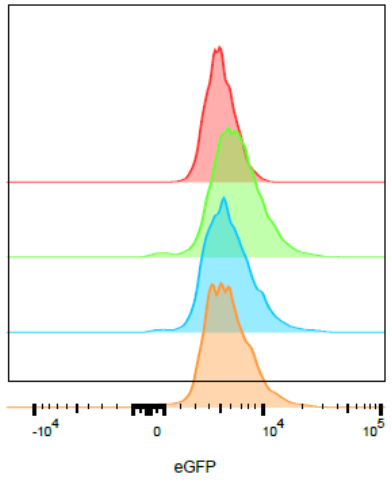
	Z-Ave	PdI
empty before conc.	46,25	0,231
empty after conc.	68,63	0,172



	Z-Ave	PdI
eGFP before conc.	73,54	0,18
eGFP after conc.	79,1	0,131



	Z-Ave	PdI
cas9 before conc.	87,18	0,188
cas9 after conc.	85,84	0,112



	Sample Name	Subset Name	Count
	Unstained (Cells).fcs	Ungated	7783
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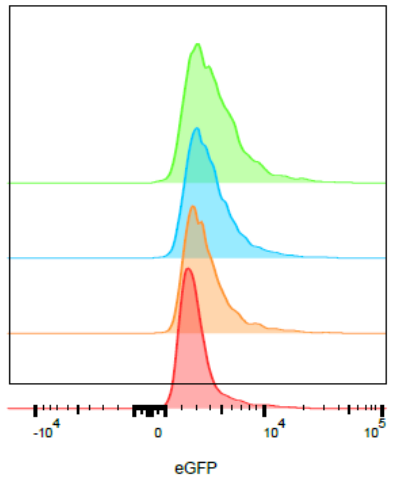
	1ug.fcs	Ungated	5000
	2ug.a.fcs	Ungated	5000



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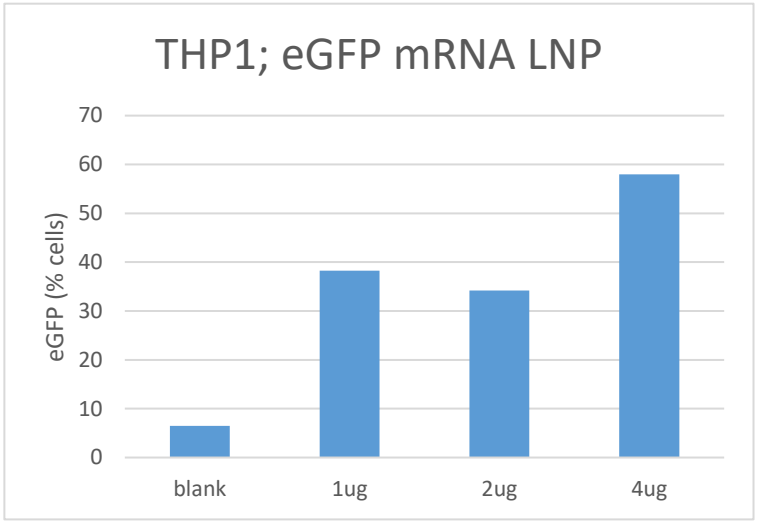

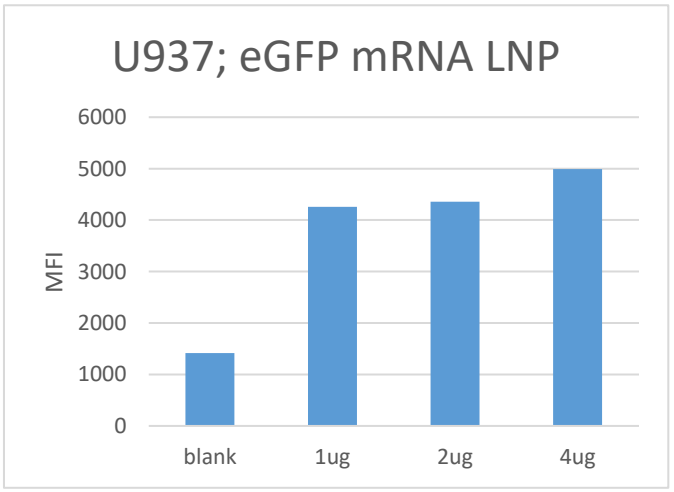

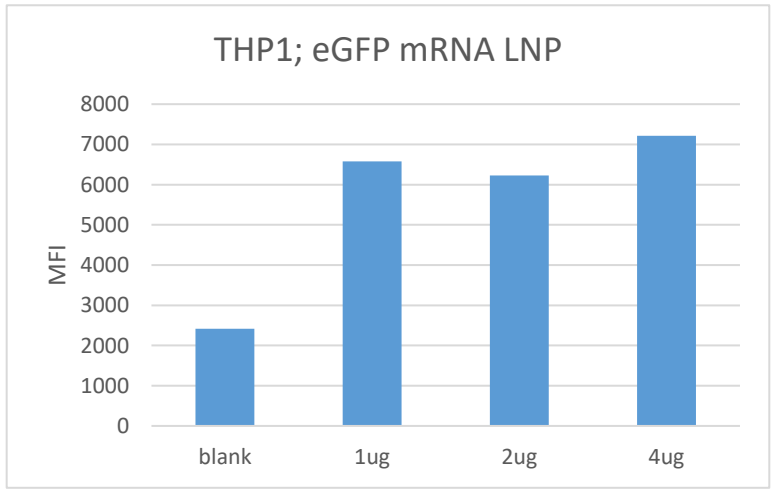

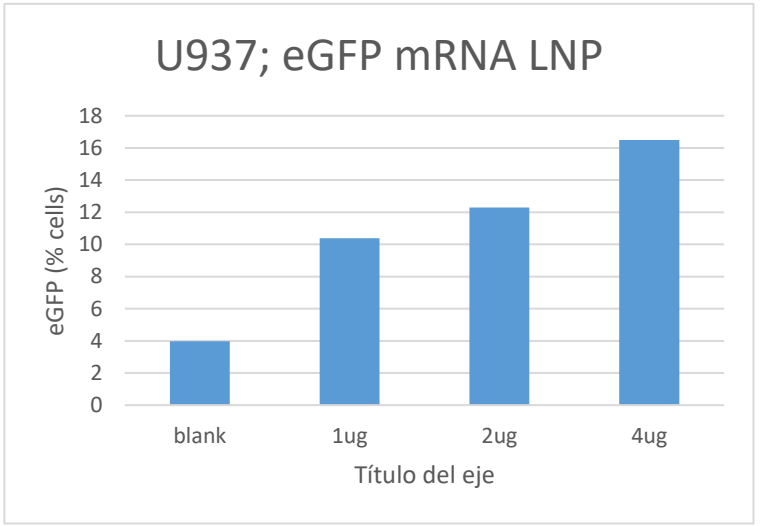

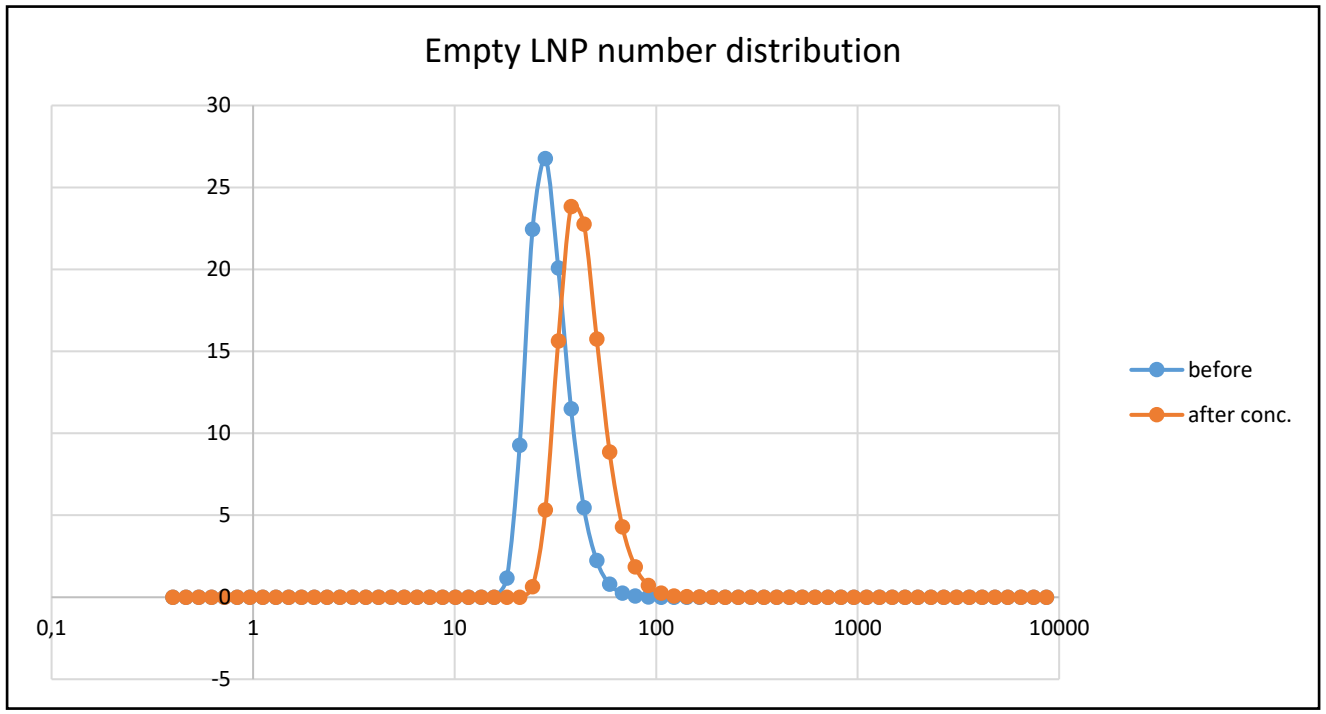

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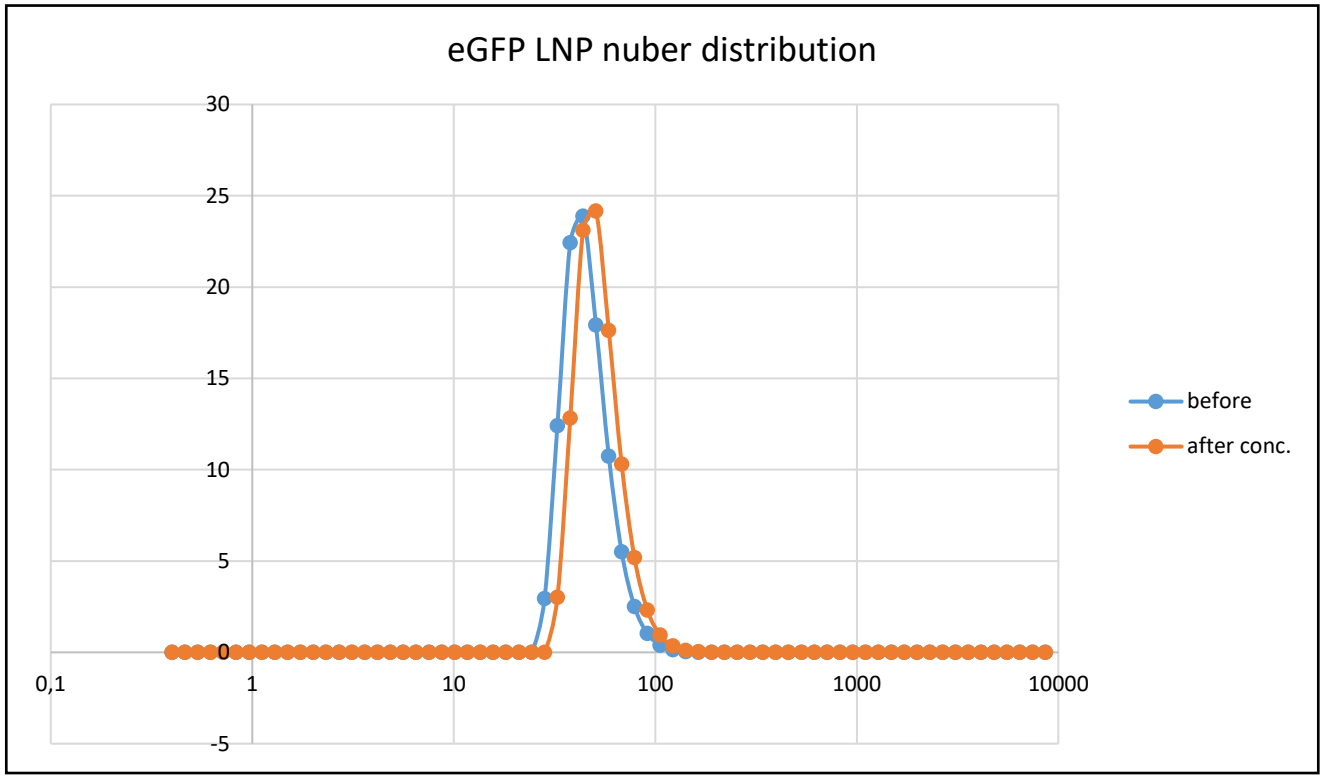

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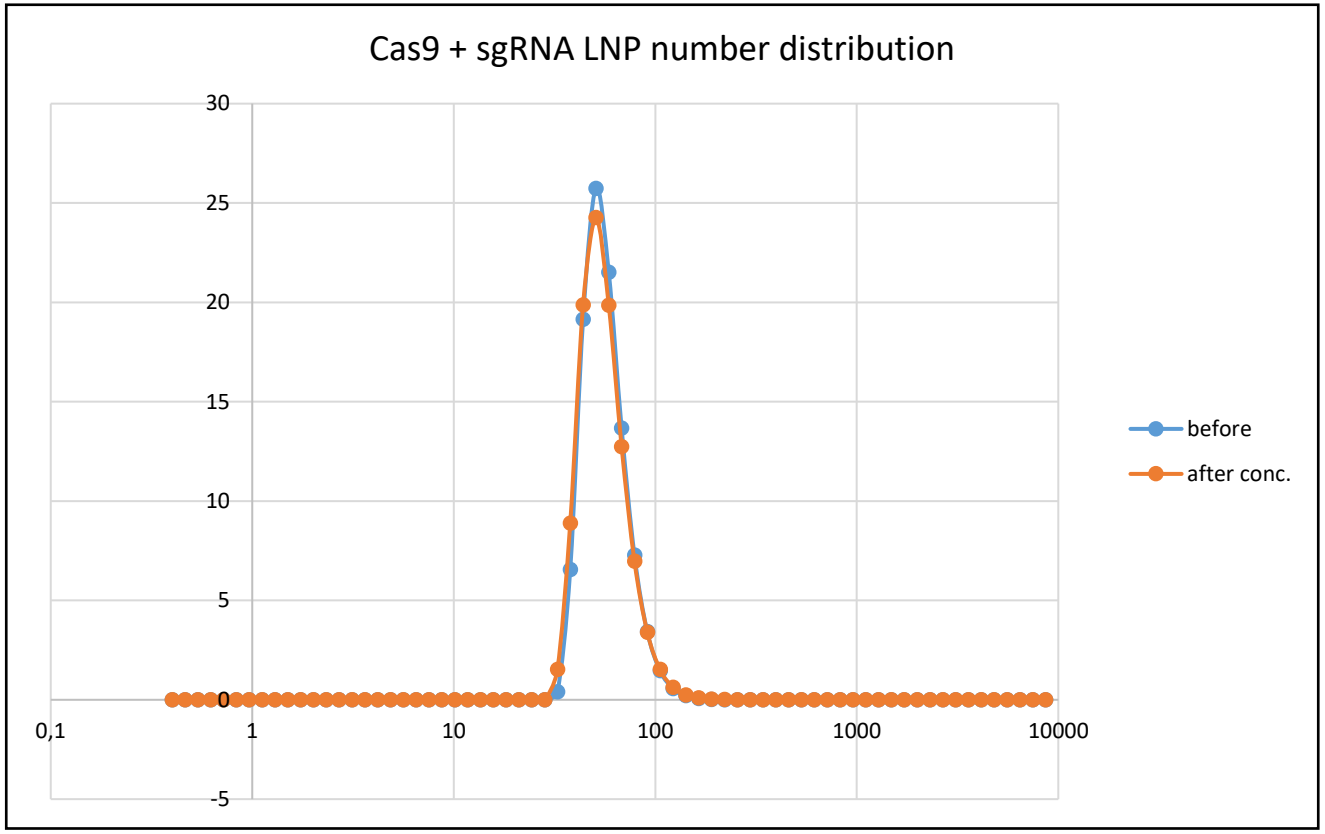

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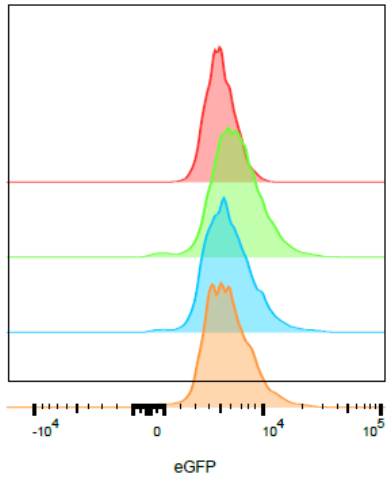

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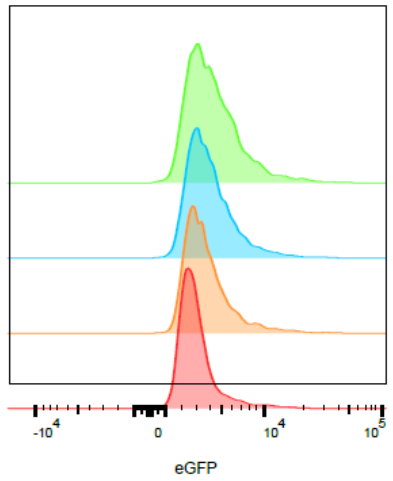

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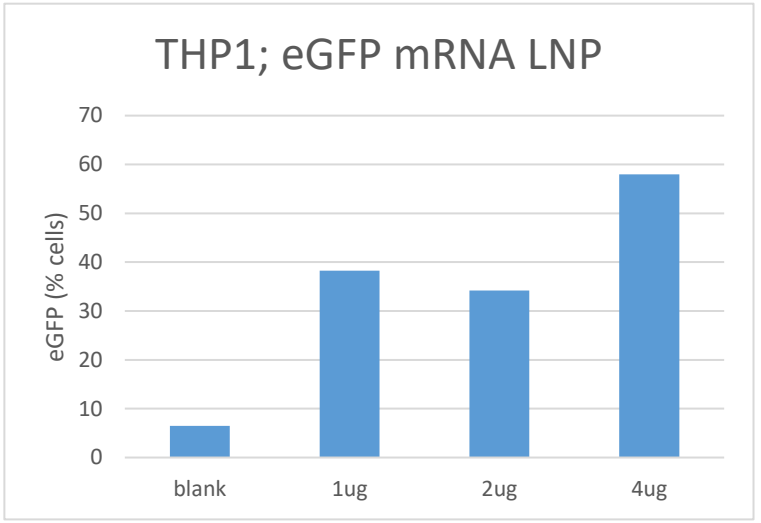
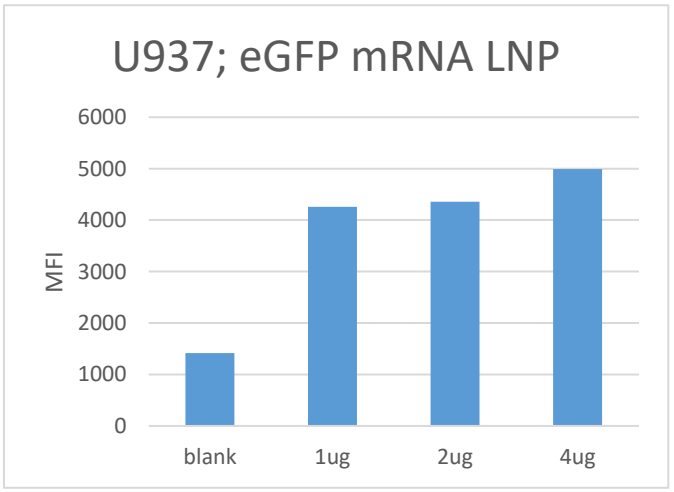
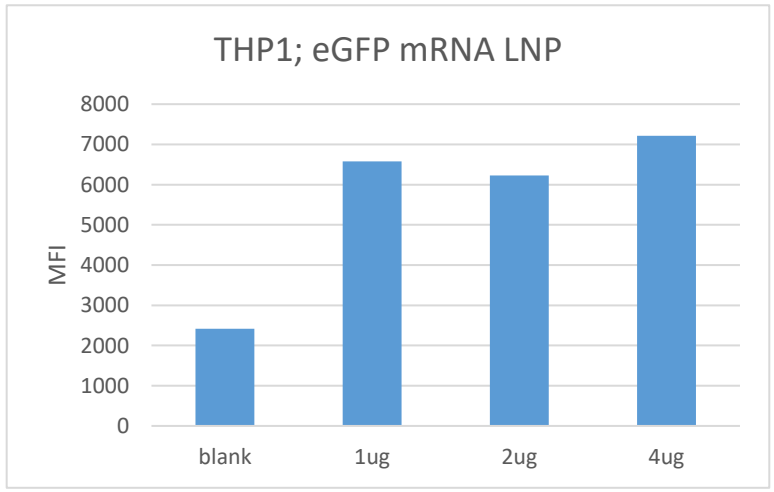
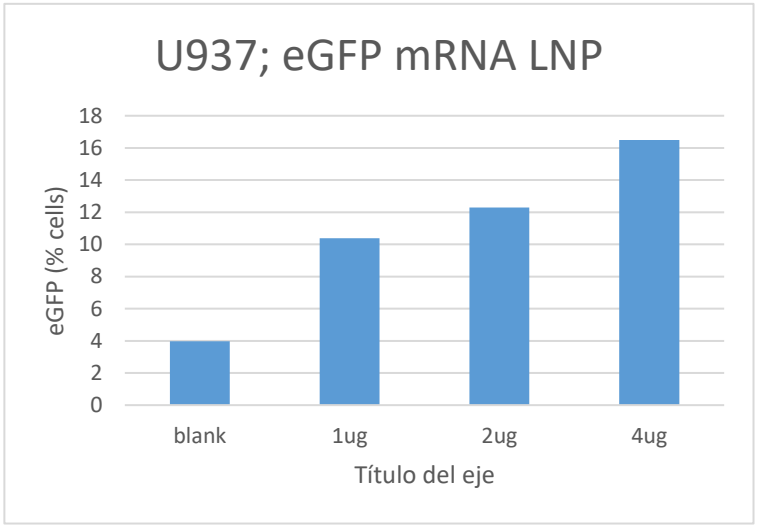








Comprehensive understanding of the latest advancement in CRISPR based gene editing delivery methods

Duration: Three days; 26–28 of June 2024

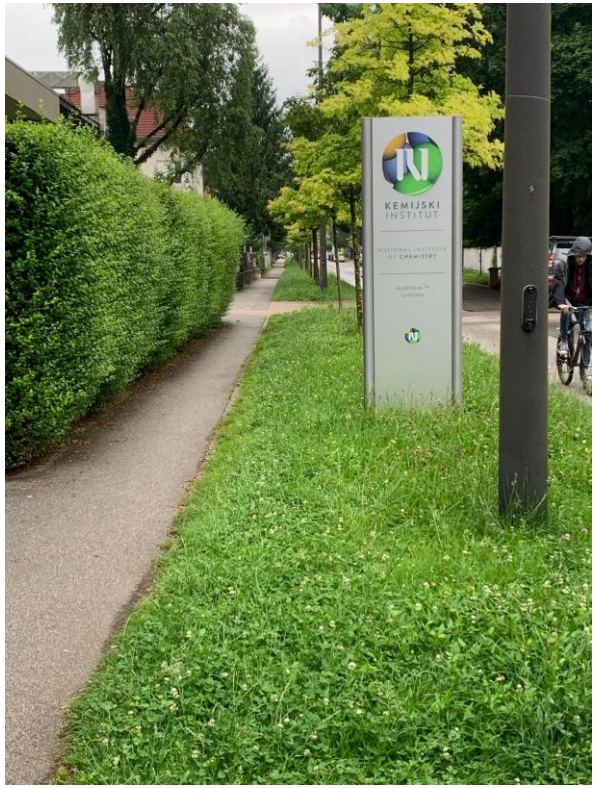
National Institute of Chemistry Slovenia

Ljubljana, Slovenia

GenE-HumDi

NATIONAL INSTITUTE OF CHEMISTRY

Junta de Andalucía
Consejería de Salud y Familias
Fundación Progreso y Salud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