CRISPR Gene Editing via Lipid Nanoparticle-RNA complex

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Introduction

Simplifying genetic manipulation has long been a goal of scientists. Recently, CRISPR-Cas9(Clustered Regularly Interspaced Short Palindromic Repeat-Cas9) delivery via LNP has gained attention due to its significant potential for in vivo gene editing and delivery. The region between these palindromic repeats is transcribed to make RNA that can be linked to the Cas9 protein, which cuts a specific part of the gene.

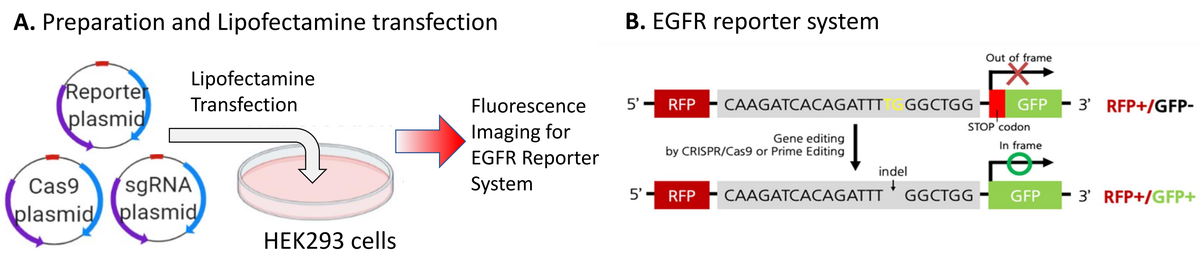
Alternatively, in a reverse approach, if there is a specific part of the gene that we want to modify, by preparing sgRNA that is complementary to specific gene locus, we could utilize this technology for gene editing. Other than CRISPR-Cas9, there have been other gene editing tools-zinc finger, TALEN or siRNA, which required gene-specific protein or mRNA for each modification. However, CRISPR-Cas9 only requires gene-specific sgRNA, which is easier to produce than protein.

In addition, delivery method of this CRISPR components have been discussed for a long time. By using LNP as a delivery method for CRISPR-Cas9 gene editing components, variable gene editing expression can be expected due to the biodegradable nature of LNP. Instead of using a vector that consistently expresses genes, which can sometimes cause off-target effects, LNP can provide specific gene targeting, even to organs, by tailoring the lipid components of LNP.

In this experiment, we attempted to modify the SRD5A2 gene using CRISPR-Cas9 technology and LNP. SRD5A2 encodes the enzyme ' steroid 5 alpha-reductase 2 ' , which converts testosterone into the more potent dihydrotestosterone. This enzyme affects male sexual development, including hair growth and the formation of external genitalia, so editing this gene would contribute to therapeutics for baldness. To achieve these goals, we started by confirming the activity of the Cas9-sgRNA complex with the DNA form of the CRISPR components. We then moved on to SRD5A2 gene editing by setting up different controls-we checked the efficiency of gene editing by making the mRNA form of CRISPR components, with pseudouridine and capping reagent, and without this reagent, which are said to stabilize mRNA expression.

Experimental Methods

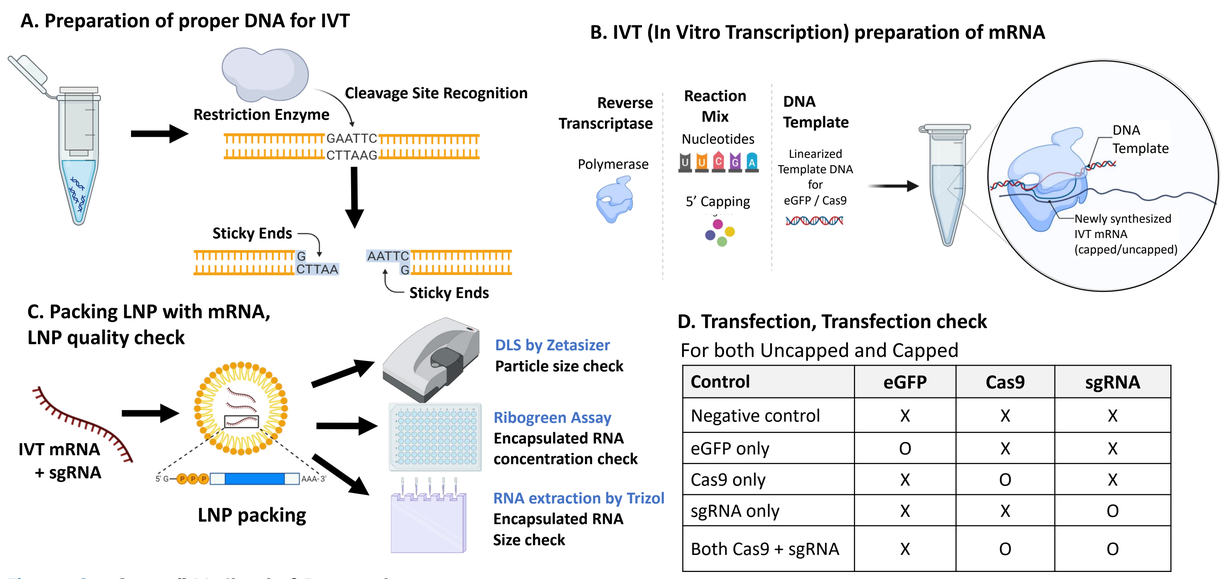
Fig. 1 | Confirming Cas9-sgRNA activity with EGFR reporter plasmid via lipofectamine



Control	EGFR reporter	Cas9	sgRNA
Negative control	O	X	X
Cas9 only	O	O	X
sgRNA only	O	X	O
Both Cas9 + sgRNA	O	O	O

Figure 1 | EGFR Reporter System Method.
A) Required plasmid DNA were cloned into DH5a E.Coli for selection and amplification. Plasmid DNA was purified by Promega Miniprep Kit. Transfection in HEK293 cell culture was performed with Lipofectamine3000. **B)** Diagram showing the EGFR reporter system. After gene editing by CRISPR-Cas9, we could observe GFP by frameshift of EGFR reporter gene.

Fig. 2 | Transfecting Cas9-sgRNA complex to SRD5A2 gene via LNP



Control	eGFP	Cas9	sgRNA
Negative control	X	X	X
eGFP only	O	X	X
Cas9 only	X	O	X
sgRNA only	X	X	O
Both Cas9 + sgRNA	X	O	O

Figure 2 | Overall Method of Research.
A) Preparation of proper DNA for IVT. To guarantee sufficient amount of DNA, midiprep is used for DNA purification. Then, DNA linearization is proceeded for linearized template of plasmid DNA, which is used in IVT process.
B) IVT(In Vitro Transcription) to make mRNA, mRNA quality check. With Linearized DNA template, nucleotides, and capping agent, we could produce massive amount of mRNA. In this experiment, we made mRNA with and without pseudouridine and 5' capping agent, which would stabilize mRNA.
C) Packing LNP with mRNA, LNP quality check. We packed LNP with Cas9, eGFP mRNA from IVT, and also with sgRNA for SRD5A2 gene. After making LNP, ribogreen assay, DLS(Dynamic Light scattering), and RNA extraction with trizol was held, to confirm that LNP made is appropriate for transfection.
D) Transfection. After transfection, we checked fluorescence for eGFP LNP, and T7E1 assay for Cas9+sgRNA LNP.

Results

Fluorescent Imaging of EGFR Reporter Cas9 activity in HEK293 cells at 48 hours

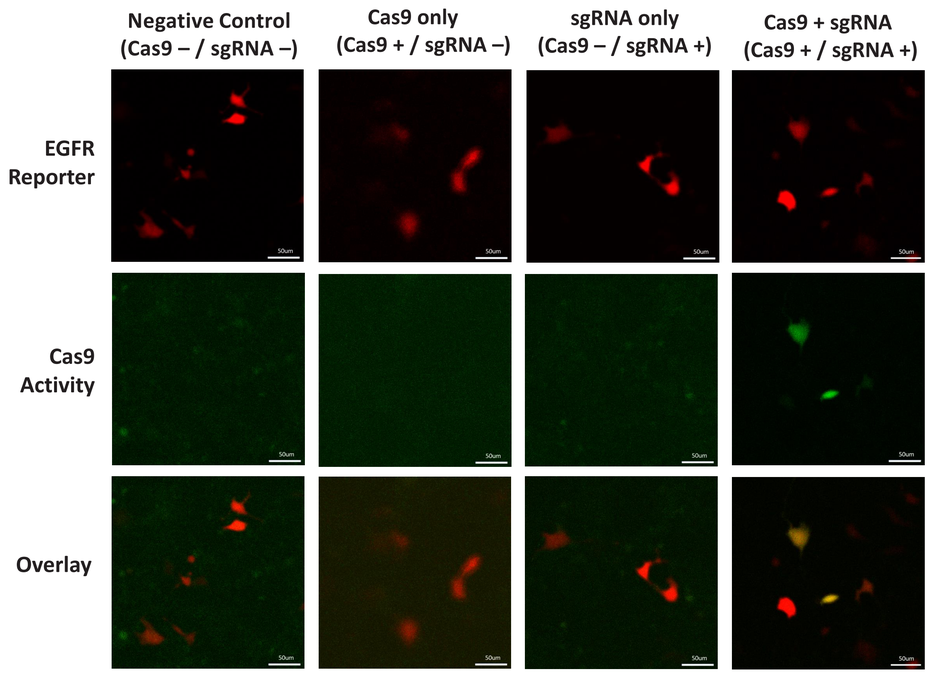


Figure 3 | Zeiss Image of HEK cell 48 hr after transfection. Fluorescence from EGFR reporter expression (red) was observed in all groups. Successful cleavage by Cas9 (green) was only observed when EGFR, Cas9, and sgRNA were transfected together

Results

IVT mRNA purification

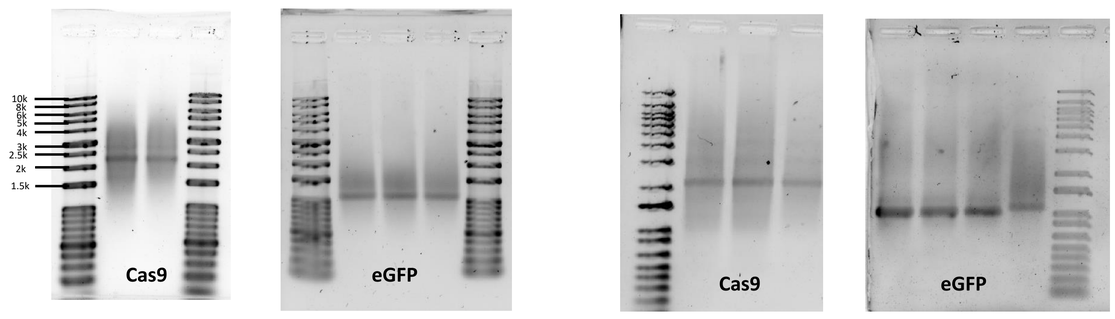
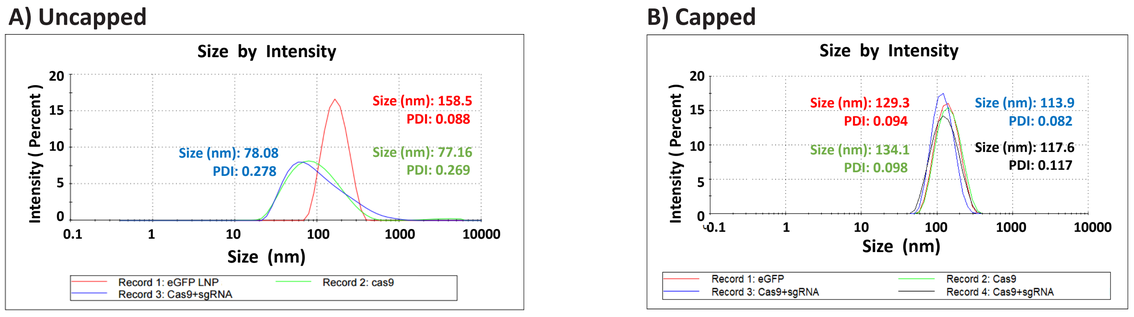


Figure 4 | Result of IVT mRNA purification.

Physicochemical Properties of LNPs

1. Ribogreen Assay and DLS analysis Data



	Uncapped eGFP	Uncapped Cas9	Uncapped Cas9+sgRNA	Capped eGFP	Capped Cas9	Capped Cas9+sgRNA(1)	Capped Cas9+sgRNA(2)
Encapsulation efficiency (%)	94.11%	75.53%	83.75%	57.52%	99.51%	95.55%	96.07%
Encapsulated RNA concentration (ng/µl)	99.55 ng/ul	83.34 ng/ul	121.83 ng/ul	93.47 ng/ul	133.72 ng/ul	207.13 ng/ul	192.00 ng/ul

Figure 5 | Result of Ribogreen Assay and DLS analysis.

2. RNA extraction by Trizol

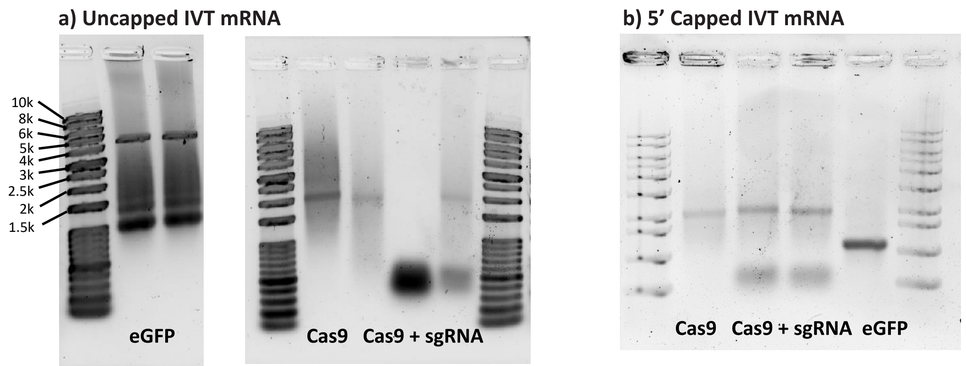


Figure 6 | Result of RNA extraction by Trizol.

T7E1 assay/Transfection of eGFP LNP to NIH3T3 cell

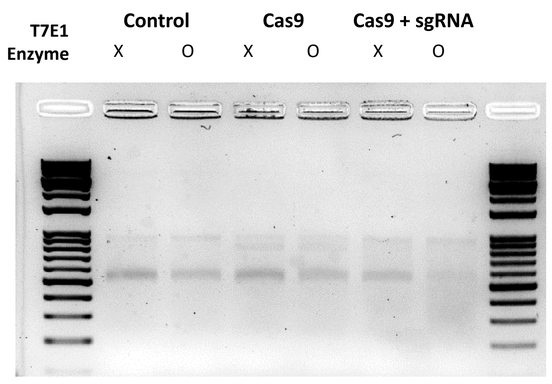


Figure 7 | Result of T7E1 assay.

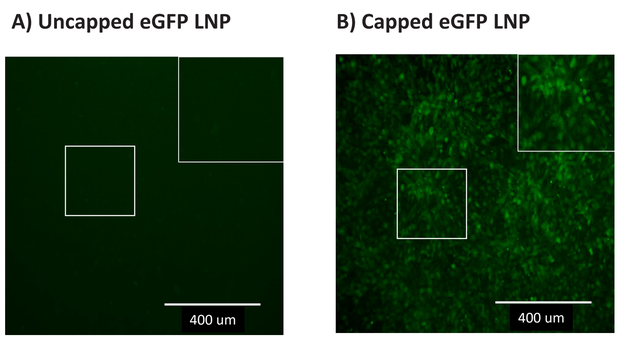


Figure 8 | EVOS image of NIH cell after 48H of transfection.

Conclusion & Further Study

In this study, we initially confirmed DNA form CRISPR components' (Cas9, sgRNA, and reporter gene) activity via lipofectamine, a well-established method for stable cell transfection. From Zeiss image, we could obviously observe GFP expression only for cells with both Cas9 and sgRNA, which would lead to the conclusion that Cas9-sgRNA complex is normally working.

After checking Cas9-sgRNA complex activity, we attempted to formulate LNP containing mRNA form of CRISPR components(Cas9, eGFP), and sgRNA. For mRNA, two types of mRNA were synthesized by IVT(in vitro transcription)-mRNA with pseudouridine and capping, and other without these modifications. This differentiation aimed to ascertain the necessity of pseudouridine and capping reagent for expression stabilization. Following IVT, we purified mRNA and ran bleach gel to confirm that the synthesized mRNA is correct in size.

Subsequently, we encapsulated the synthesized mRNA into LNP to transfect cells. DLS(Dynamic Light Scattering), ribogreen assay, and RNA extraction by Trizol were done right after LNP formulation to each check LNP's particle size, concentration of encapsulated RNA, and band size of encapsulated RNA.

For DLS, particle size of 50~150 d.nm is considered to be optimal for transfection. From the data, we could conclude majority of the LNPs fell within the range. Additionally, for ribogreen assay, we could see relatively high encapsulation efficiency and encapsulated RNA concentration. Furthermore, by looking at the band for RNA extraction with Trizol, we could conclude that mRNA has correctly encapsulated without serious deformation because the band size for trizol assay matches with the band size for RNA purification that we did previously.

Finally, the validated LNPs were employed to transfect NIH cells, and the outcomes were analyzed using EVOS imagingand T7E1 assay. For eGFP LNP, the significance of pseudouridine and capping reagent was evident through distinct GFP expression levels in EVOS images. For T7E1 assay, we wanted to check whether capped Cas9+sgRNA worked well. Although expected result was additional two more small bands, the faint band led us to cautiously infer successful gene modification. However, more experiments should be conducted. For the absence of smaller two bands that should have existed, we are assuming that the annealing temperature for pcr was inaccurate. More studies should conducted to check proper condition for pcr and T7E1 assay procedure.

Reference

- Adli, M. The CRISPR tool kit for genome editing and beyond. Nat Commun 9, 1911 (2018).
- Wei, T., Cheng, Q., Min, YL. et al. Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing. Nat Commun 11, 3232 (2020).
- Bae et al. Molecules. 'Specificity Assessment of CRISPR Genome Editing of Oncogenic EGFR Point Mutation with Single-Base Differences' (2020).
- Duan L, Ouyang K, Xu X, Xu L, Wen C, Zhou X, Qin Z, Xu Z, Sun W and Liang Y (2021) Nanoparticle Delivery of CRISPR/Cas9 for Genome Editing. Front. Genet. 12:673286. doi: 10.3389/fgene.2021.673286