EGFR gene editing via CRISPR-Cas9 system and the Lipid nanoparticle packaging

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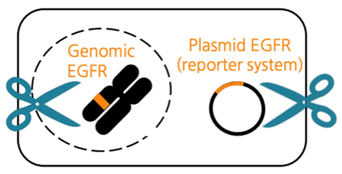
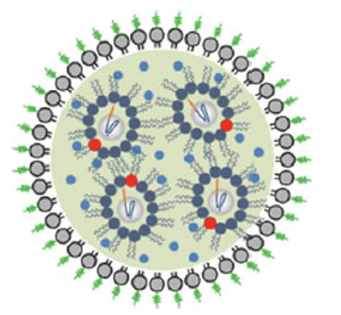
Abstract





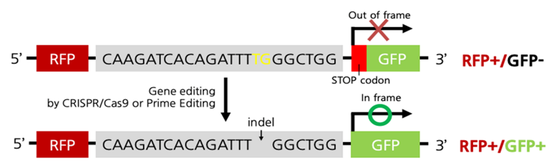
CRISPR genome editing involves the assembly of ribonucleoprotein complexes together with guide RNA to bind and cleave DNA with complete sequence complementarity. CRISPR genome editing holds high promise for precision gene therapy for various diseases, including cancer and genetic disorders, which are caused by DNA mutations within the genome. By locating the mismatches within the protospacer adjacent motif (PAM) sequence, a novel CRISPR genome editing technique was assessed in our study since the PAM sequence has been shown to have high specificity. To this end, we conducted the reporter gene assay to assess the ability of PAM sequence based CRISPR Cas9 system on the endothelial growth factor receptor (EGFR) gene which has a major role in cell growth. So, we transfected the group of cells with different conditions of plasmids that contains EGFR reporter gene (surrounded by RFP and GFP), sgRNA, Cas9 each. After 3 days, we watch the difference of fluorescence in each group and saw the impact of CRISPR complex.

However, despite the obvious benefits, delivery of RNPs is the most challenging method, due to considerable size of Cas9, negative charge of sgRNA, and difficult protection against degradation or denaturation during the whole formulation and delivery process. Based on a fundamental understanding, we designed ionizable lipid nanoparticle that contain cationic lipid (DOTAP) to preserve the integrity of RNP. We calculated the appropriate amount of each lipid component since the concentration of each component of LNP was the major role to successfully encapsulate the RNP. After checking the LNP size with DLS, we transfected the cells to check whether these LNP successfully deliver RNP. We can check the result by performing T7E1 assay, and luciferase assay.

Scheme

- Reporter Gene Experiment

(A)



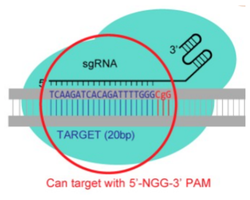


Fig1. (A) Reporter system and Crispr-Cas9 system

(B)

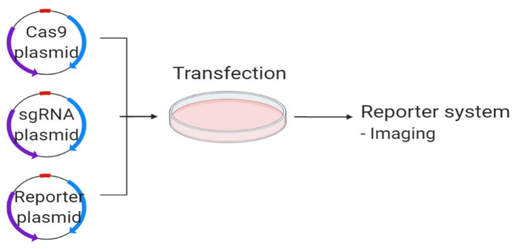


Fig2. (B) Experimental plan and setting

(C)

Lane	Formulation buffer	Treatments
1	PBS Buffer (pH 7.4)	DNA Ladder
2		PBS
3		MC3-DOT-10 Cas9/sgLuc (1/1)
4		MC3-DOT-10 Cas9/sgLuc (1/3)
5		MC3-DOT-10 Cas9/sgLuc (1/5)

Fig3. Experimental conditions of each RNP/LNP complex

Results

- Reporter system Fluorescence (EVOS after 48h)

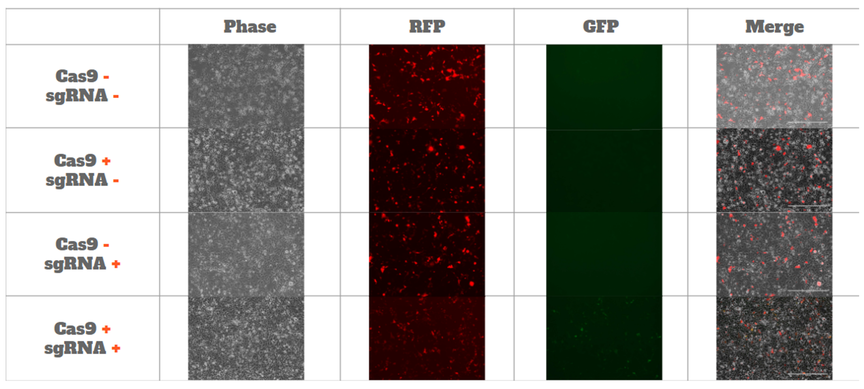


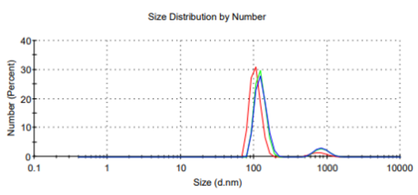
Fig4. Transfected HEK293 imaged by EVOS after 48h. Cells with both Cas9 and sgRNA plasmid have GFP.

- LNP Formation / DLS size measurement

Paper: 500ul of DMEM + 100ul of Nanoformation (Final concentration of sgRNA is 24nM.)
About 250nm is appropriate.

➢ 1000ul of DMEM + 200ul of Nanoformation (Final concentration of sgRNA is 40nM.)

(A)



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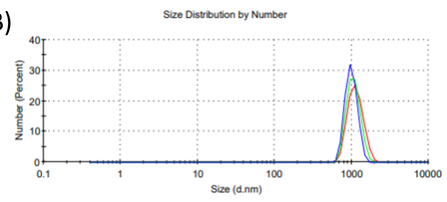


(C)

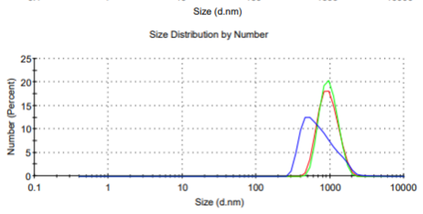
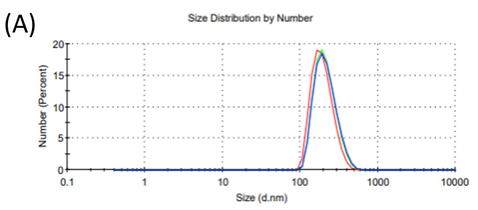


Fig5. Size distribution of 5A2-MC3-10 encapsulating Cas9/sgLuc with molar ratio of (A)1/1, (B)1/3, and (C)1/5. (A) ~1000nm (B) ~700nm (C) ~600nm

➢ 1000ul of DMEM + 200ul of Nanoformation (Final concentration of sgRNA is 4nM.)

(A)



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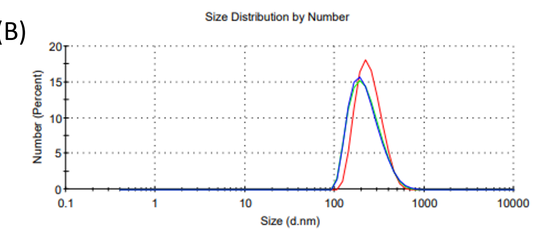


(C)

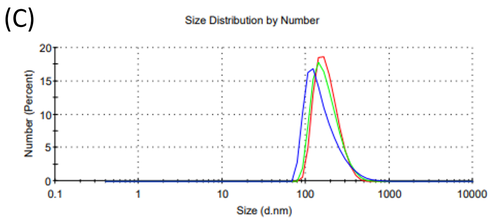
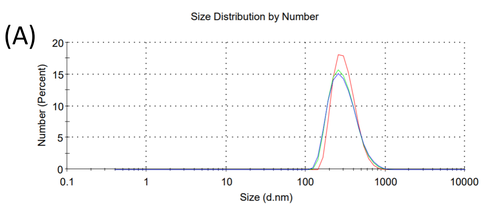


Fig6. Size distribution of 5A2-MC3-10 encapsulating Cas9/sgLuc with molar ratio of (A)1/1, (B)1/3, and (C)1/5. (A)~249nm (B) ~250nm (C)~250nm

➔ We thought maybe the concentration of nanoformation is important.

➢ 600ul of DMEM + 600ul of Nanoformation (Final concentration of sgRNA is 24nM.)

(A)



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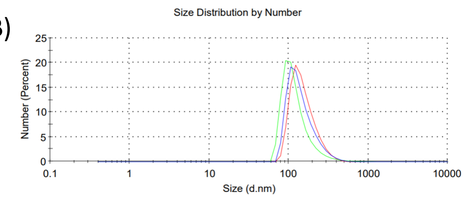
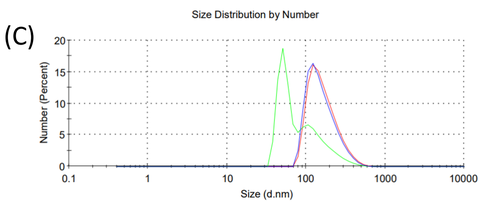


(C)



(D)

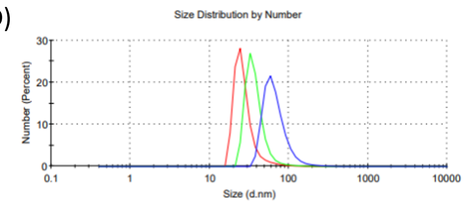


Fig7. Size distribution of MC3-DOT-10 encapsulating Cas9/sgLuc with molar ratio of (A)1/1, (B)1/3, (C)1/5, and (D)empty. (A)~351.1nm (B)~211.8nm (C)~225nm (D)~139nm

- T7E1 assay

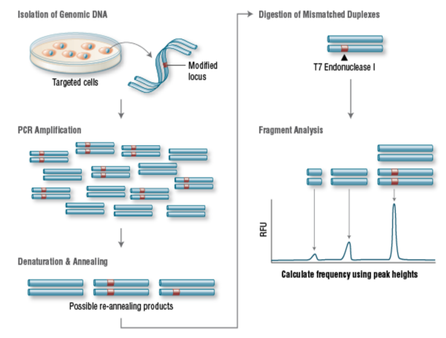


Fig8. T7E1 assay protocol

- Luciferase assay

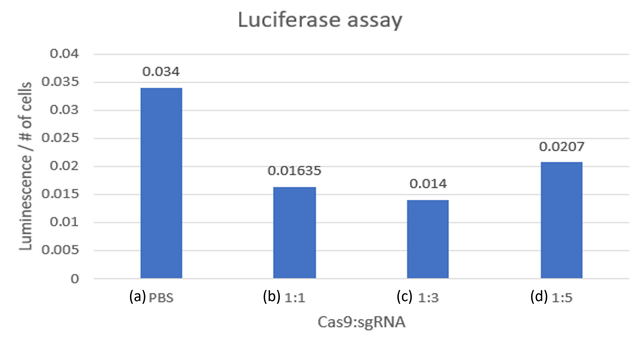
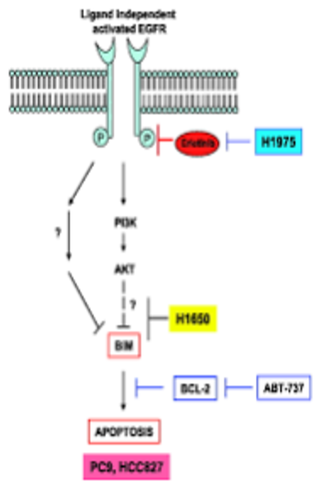


Fig9. Result of Luminescence/number of cells .
(a) PBS: 0.034 (b) 1:1 : 0.01635 (c) 1:3 : 0.014 (d) 1:5 : 0.0207

Conclusion / Discussion



We found that the CRISPR-Cas9 system edits the EGFR gene through the reporter gene experiment. By comparing the degree of GFP expression through Evos, we were able to find GFP expression in all control groups except for the experimental group, and through this, we were able to conclude that the CRISPR-Cas9 system was targeting the EGFR gene properly. Based on this, we tried to compare the transmission of CRISPR complex with lipofectamine and the transmission with LNP encapsulation, but we could not proceed with the experiment to compare it due to the absence of sgRNA targeting EGFR. We also found that BCL2 gene exists among the downstream genes of EGFR gene, which plays an important role in regulating cell death. Therefore, we thought that the efficiency of gene editing could be determined by examining the change in mRNA amount after the use of CRISPR complex as a Further study.

Through the LNP packing experiment conducted after that, we were able to perform LNP formation with cationic lipid and transform the CRISPR Cas9 complex to HEK293 cells with luciferase gene added. When the cell treat concentration was 4nM based on 12 well plates, the survival rate was the highest, and through this, the LNP treatment concentration of T7E1 assay was set. And our group looked for the most efficient complex by varying the ratio of sgRNA and Cas9, and the results were based on how the degree of Luminescence changes. In addition, it was found that the content concentration of lipid nanoparticles and the volume of buffer showed very different capsulation effects, and the higher the concentration of lipids, the higher the size of the nanoparticle, showing poor capability. Through this experimental process, it was concluded that minimizing the toxicity of cells in the LNP packaging process remains a further study.

References

[1]Bae et al. *Molecules. 'Specificity Assessment of CRISPR Genome Editing of Oncogenic EGFR Point Mutation with Single-Base Differences'. 2020*
[2] Wei et al. *Nat. Comm. 'Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing*'. 2020