Efficiency of SSA Assay in Quantifying CRISPR/Cas9 induced DNA Cutting Activity

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Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated endonucleases (Cas) were first found to be involved in microbial adaptive immunity[1] .
CRISPR and Cas9 are now used for gene editing.

Gene Editing Steps[1]

1. guide RNA (gRNA) is designed to contain a targeting guide sequence.
2. gRNA forms a complex with Cas9 and directs Cas9 to the target site.
3. Cas9 cleaves the target site and make a double strand break.
4. Error prone nonhomologous end joining (NHEJ) happens with insert or deletion.
   Homologous recombination (HR) happens when donor DNA with homologous sequence is present.

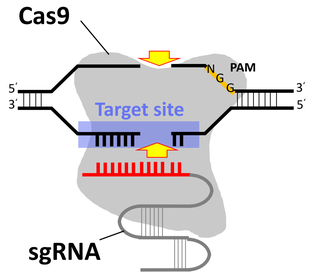


Figure 1. Mechanism of gene targeting using CRISPR/Cas9 (adapted from [2])

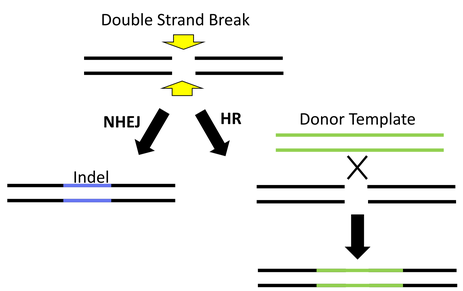


Figure 2. Gene Repair mechanism

Single Strand Annealing (SSA) assay[2]

Quantify the activity of Cas9/sgRNA by luminescence.

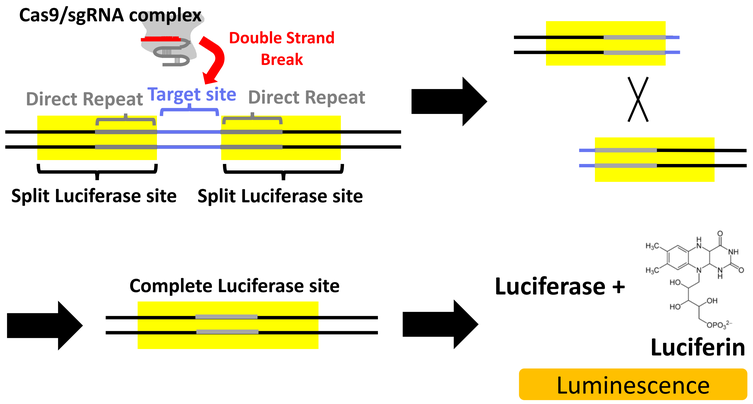


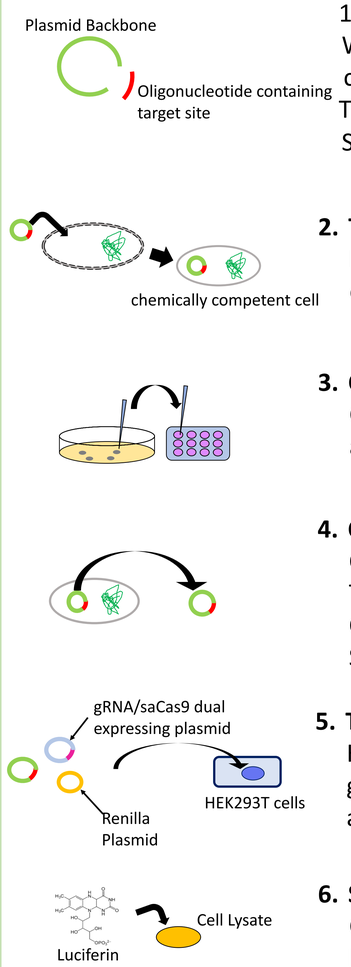
Figure 3. Mechanism of SSA assay

Motivation

- Determine whether SSA assay can be used to assess gRNA /Cas9 activity compared with T7E1 assay.
- Compare the cleavage activity of Cas9/gRNA at different target sites.

Methodology

Figure 4. Overview of experiments



1. **Cloning**
   15 disease causing targets were selected. When mutated these genes result in cystic fibrosis, tyrosinemia and SCID These target sites were cloned into the SSA vector.
2. **Transformation**
   Ligated plasmids were inserted into chemically competent *E.coli*.
3. **Cell culture**
   Cells were grown O/N, colonies picked and grown O/N in liquid media.
4. **Colony PCR, Miniprep, Sequencing**
   Clones screened by colony PCR.
   Target vector was extracted from cells.
   Correct clones were confirmed by Sanger Sequencing.
5. **Triple Transfection**
   HEK293T cells were co-transfected with gRNA/saCas9 dual expressing plasmid and Renilla plasmid.
6. **SSA assay**
   Cleavage of the target resulted in luminescence.

Results

SSA assay

2 target sequences were correctly inserted inside the SSA target vector. The targets were for genes that result in hemophilia A (*F8*) and cystic fibrosis (*CFTR*) when mutated.

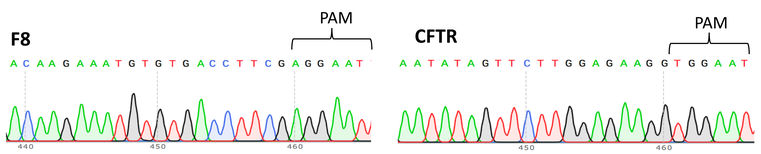


Figure5. Result of sequencing(Left: F8, Right: CFTR)
TGGAAT and AGGAAT are the PAM sequence

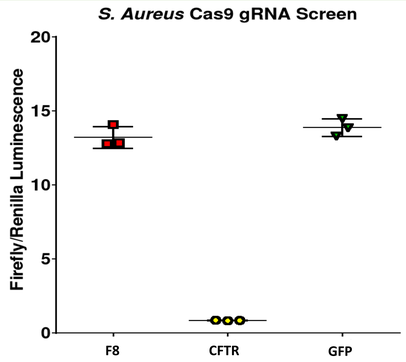


Figure 6. Luminescence measured by luminometer. For normalization, Renilla luciferase expressing plasmid was also transfected into cells. GFP targeting Cas9/gRNA was also transfected as positive control.

F8 Luminescence ratio : 13.2
CFTR Luminescence ratio : 0.9
GFP Luminescence ratio : 13.8

T7E1 assay

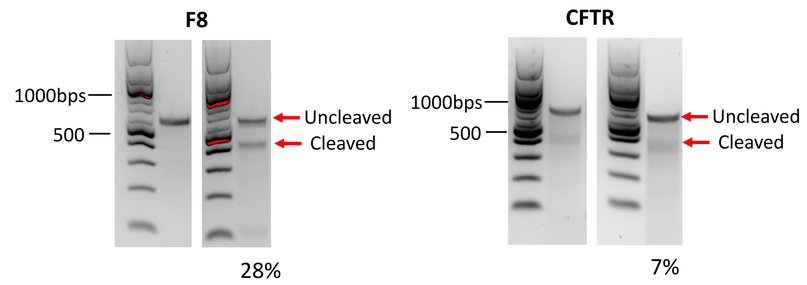


Figure 7. Gel images of DNA after T7E1 assay

Conclusions

- Successfully cloned target sequences.
- Used the SSA assay to demonstrate gRNA/Cas9 activity.
- Showed a correlation between SSA data and T7E1 assay data.
- Showed that the SSA can be used as an alternative to T7E1 assay.

Future Work

- Expand the number of guides that are screened by the SSA assay in order to accurately determine the robustness of the assay.
- Use assay to screen large numbers of gRNA/Cas9 complexes instead of traditional T7E1 assay and Surveyor assay.

Acknowledgments

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References

[1] Patrick D. Hsu, *Cell* (2014)
[2] Yi Yang. et.al., *Appl Biochem Biotechnol.*(2016)