Single-Base Editing of Cellular mRNA by CRISPR/Cas9

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Abstract

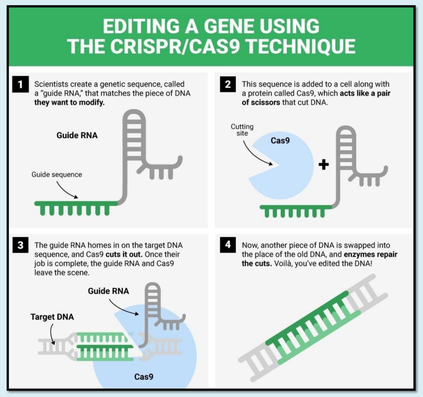
Genetic engineering is currently dominated by CRISPR/Cas9 technology, promising precise multipurpose genome editing. The 2016 WPI iGEM team investigated the potential of adapting this technology to direct single-base editing of mRNA by linking deactivated Cas9 to the C-to-U RNA editor enzyme APOBEC1. We pursued the re-cloning and characterization of the dCas9/APOBEC fusion to identify and eliminate problems related to expression and toxicity. Ultimately, this adapted CRISPR/Cas9 system could provide an advantageous method of editing, particularly for therapeutics.

Theory & Background

A Revolution in Synthetic Biology: The "CRISPR" Way

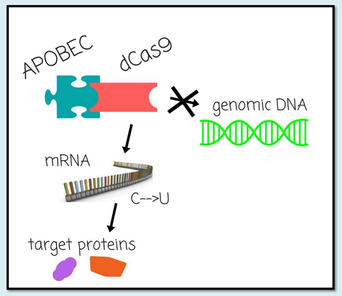
In CRISPR/Cas9 editing, **C**lustered **R**egularly-**I**nterspaced **S**hort **P**alindromic **R**epeats (**CRISPR**s) are recognized by the nuclease Cas9 and cleaved for the purpose of editing genomic DNA.

This system has been specially adapted for the use of targeting virtually any position in the genome by guiding the Cas9 to recognize specific sites of double-stranded DNA (dsDNA), usually via engineered single-guideRNAs (sgRNAs). The figure to the right shows a simplified diagram of how the CRISPR/Cas9 technology works.[1]



Problems & Controversies with Current CRISPR/Cas9 Technology:

- Sequences similar to the target sequence are also cut, resulting in unpredictable and irreversible off-target effects
- Complex post-transcriptional modifications and splicing variation allow for a myriad of uncontrollable outcomes
- Accidental release of genetically modified organisms could lead to ecological disruptions or medical hazards
- Moral dilemma surrounds the idea of editing human genomes [2]



Our Solution: Use dCas9/APOBEC to Target mRNA

We developed an *adapted CRISPR/Cas9 system that targets mRNA*.[3]

In our proposed method of single-base editing, the CRISPR/Cas9 system:

- Foregoes double-stranded DNA cleavage
- Retains editing power through an attached DNA-editing deaminase enzyme called **APOBEC**
  - targets specific bases changes, such as a C→U substitution [4]
- Holds the ability to be reversed

Methodology

Preparation of pcDNA3.1+ [5]

Transformed pcDNA3.1+ vector DNA into competent *e. coli* cells → Incubated on ice, heat shocked, incubated on ice again → Added SOC media and incubated with shaking → Plated on LB w/ Ampicillin plates and incubated overnight → Single colonies picked and grown overnight → Mini-prepped using kit → Products run through agarose gel, cut, and gel-purified by kit

Amplification of dCas9 and APOBEC

First attempt(s) to amplify dCas9 → low DNA yield

Troubleshooting: We then conducted a gradient-temperature PCR reaction to find the optimal annealing temperature for subsequence reactions.

From this, we determined that the optimal annealing temperature for dCas9 PCR reactions is 58.5 degrees.

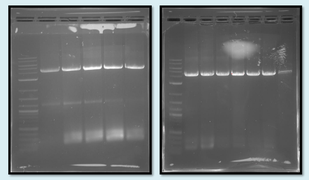


Figure 1. Gradient-Temp PCR Reactions 1-12, spanning from 45 to 62 degrees Celsius

dCas9 PCR Protocol	
1x	95°C for 02:00
30x	95°C for 00:30
	59°C for 00:45
	72°C for 04:30
1x	72°C for 10:00

APOBEC PCR Protocol	
1x	95°C for 02:00
30x	95°C for 00:30
	55°C for 00:45
	72°C for 01:00
1x	72°C for 10:00

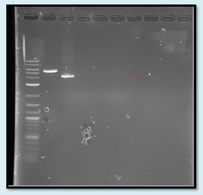
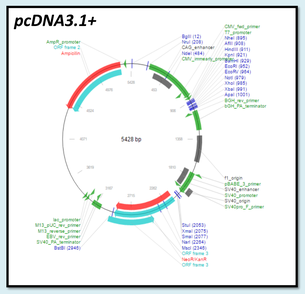


Figure 2. Gel Validation of pcDNA3.1+ Preparation (Lane 1 at 5.4kb) and Amplification of dCas9 (Lane 2 at 4.1kb)

Gibson Cloning of dCas9 into pcDNA3.1+ [6]



- **Mixed DNA into Reagent**
  - pcDNA3.1+ vector
  - dCas9 insert DNA
  - NEBuilder HiFi DNA Assembly Master Mix [7]
- **Incubation & Transform-ation**
  - Incubation in PCR machine for 30 min at 50°C
  - 2 uL product put into competent *e. coli* cells
  - Iced, heat shocked, and iced again
- **Media Addition for Plating**
  - SOC media added to product + cells
  - Incubated at 37°C with shaking for 1 hour
  - Plated on pre-warmed LB w/Amp. plates

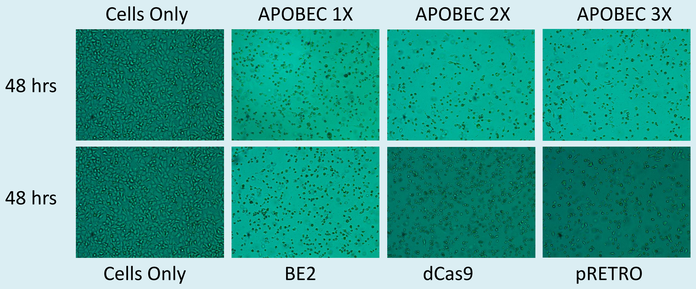
Transfection of dCas9/APOBEC into Cells

Plated 600,000 cells into a 6-well plate and allowed 24 hours for adherence. Added 2 µg DNA + 10 µL TransFectin. Allowed 4 hours before changing media.

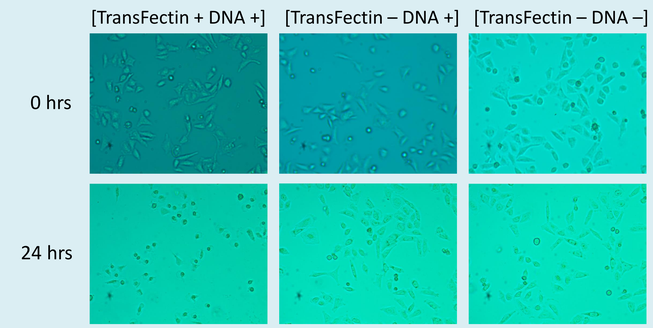
- ***Transfection 1*** — H1299's
- ***Transfection 2*** — MCF7's

Results & Conclusions

Transfection One



Transfection Two



Future Work

Continued Cloning of dCas9/APOBEC into pcDNA3.1+ Vector
Suggestion → future persistence in cloning in the dCas9 and APOBEC inserts with various size linkers into the pcDNA3.1+ and other standard vectors

Determining Alternate Transfection Protocols to Reduce Toxicity
Suggestion → find alternate methods of transfecting the DNA into the cells, in order to accurately determine the effect of the dCas9 construct(s)

References & Acknowledgements

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