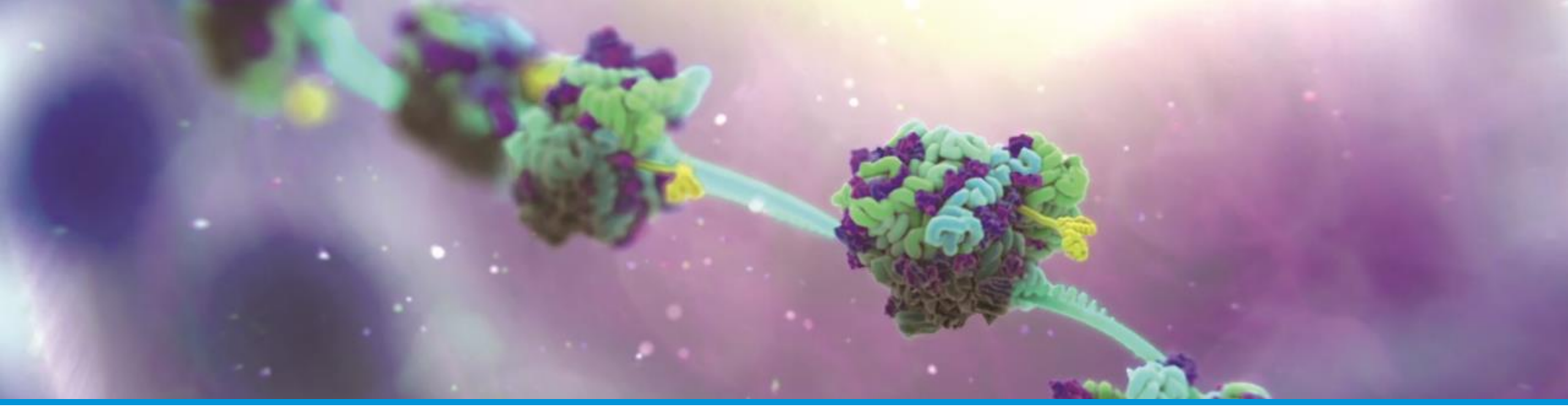

Removing T_0 Constraint Reveals Differences in Specificity of Engineered Gene Editing Proteins

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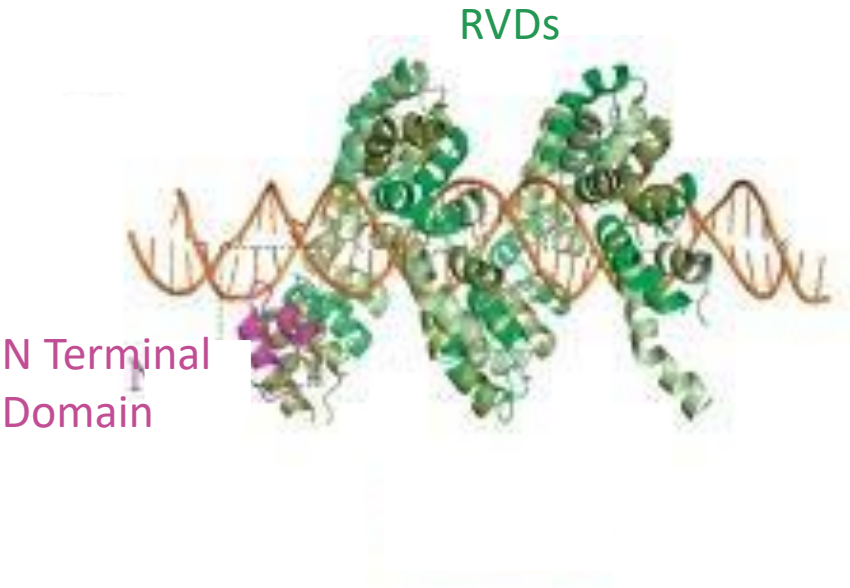
ASGCT 2021

What is the T_0 Constraint?

Many gene editing proteins have constraints that limit the available target sites

ex. PAM sequence for CRISPR-Cas9

TALENs and NoveSlice require a thymine directly 5' to DNA binding domain (T_0 site)



Can we modify the N terminal domain of the protein to allow for editing at target sites without a T_0?

Image: (Lamb, Mercer & Barbas, 2013)

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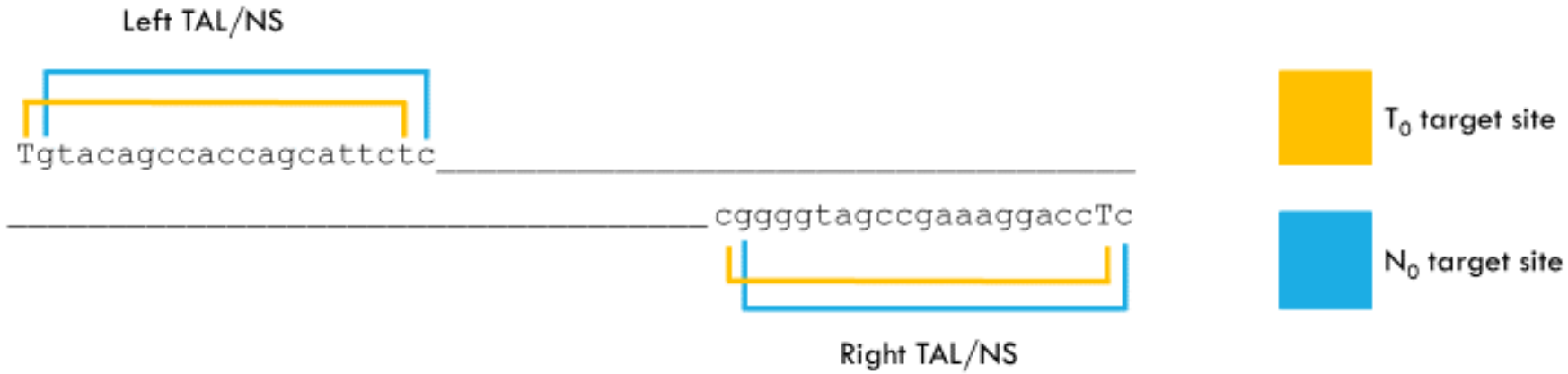
Col7a is a Clinically Relevant Target Site

- Mutations in COL7A1 gene leads to *dystrophic epidermolysis bullosa*
  - Rare pediatric skin disease characterized by fragile skin and blistering
- More than 700 mutations of COL7A1 have been identified
(Wertheim-Tysarowska et. al, The COL7A1 mutation database)



Aims

1. Create TALEN and NoveSlice protein variants comprising novel N terminal domain amino acid sequences

2. Test if the modifications improve editing at N_0 target sites

3. Test efficiency and specificity of TALENs and NoveSlices with these modifications

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Methods – Site Directed Mutagenesis, T7E1 Assay

Creating the modified N terminal region in the protein

- *Site Directed Mutagenesis* of backbone containing N terminal region
- Construct DNA binding domain of gene editing protein with *golden gate cloning*

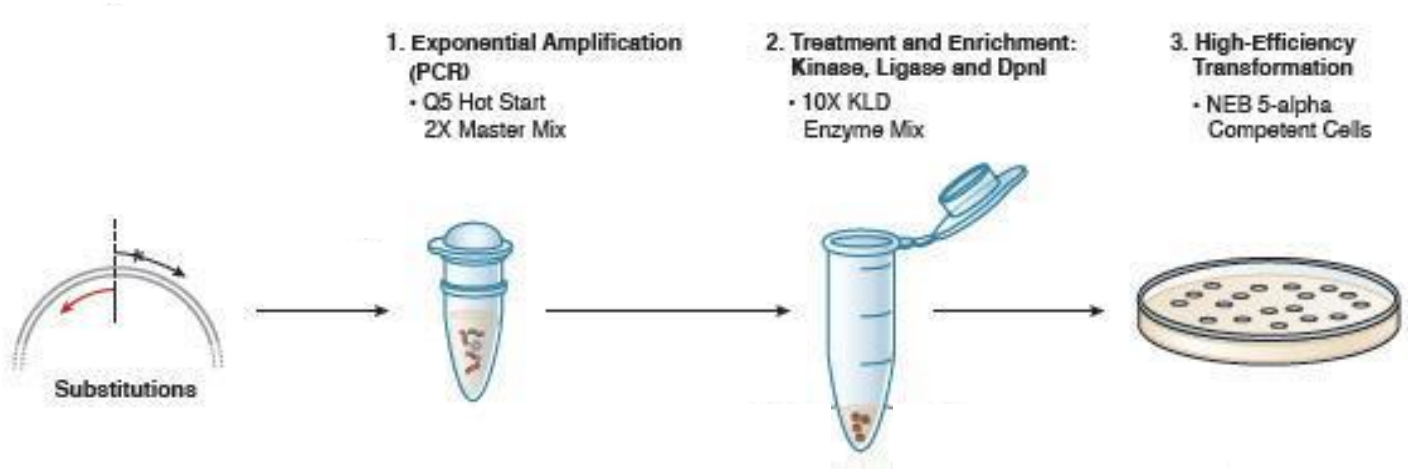

Image: (Figure 2: Q5 Site-Directed Mutagenesis Kit Overview, New England Biolabs)

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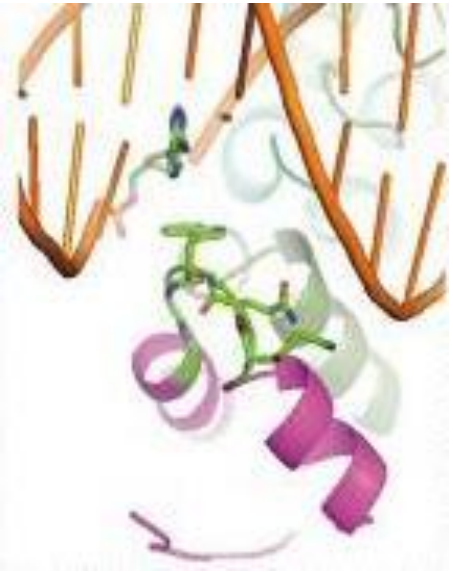
N Terminal Domain Mutations



Name	Amino Acid Sequence
WT	Asp225 - IVGVGKQWSGARAL - Glu240
KR	Asp225 – IVGVGKQKRGARAL – Glu240 (Lamb, Mercer & Barbas, 2013)
GS	Asp225- IVGVGGSKRGAGSGARAL – Glu244

Image: (Lamb, Mercer & Barbas, 2013)

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Methods – Site Directed Mutagenesis, T7E1 Assay

Testing Gene Editing Efficiency – T7E1 Assay

- Electroporated human primary cells with mRNA encoding gene editing pairs
- Genomic DNA isolated
- Target site amplified via PCR
- PCR products hybridized and digested with T7 endonuclease I

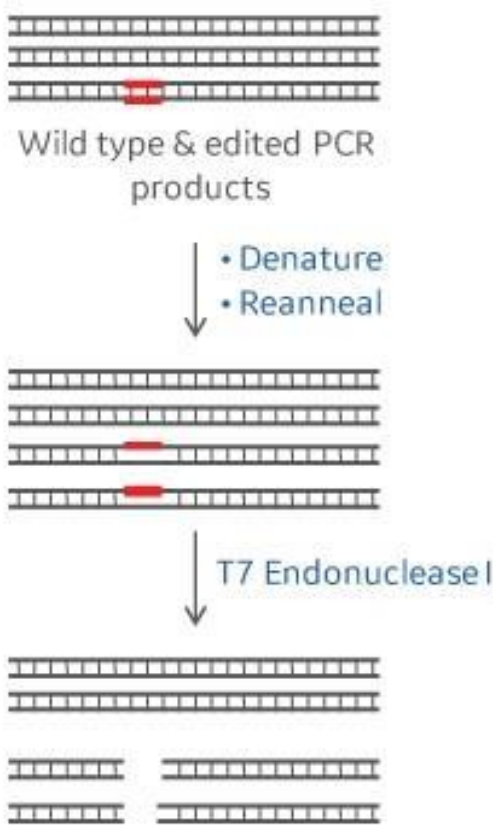

Image: (Kerschgens, Horizon Discovery Biosciences Limited)

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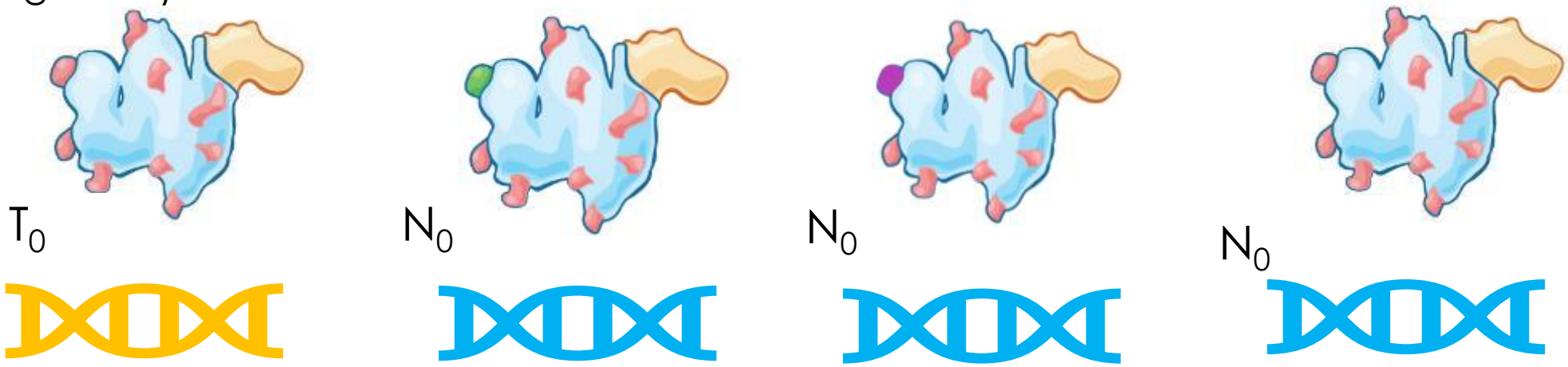
Constructs

TAL/NS-mutation->(target site)



	TAL-WT->(T_0) NS-WT->(T_0)	TAL-KR->(N_0) NS-KR->(N_0)	TAL-GS->(N_0) NS-GS->(N_0)	TAL-WT->(N_0) NS-WT->(N_0)
Target site	T_0	N_0	N_0	N_0
N terminal region modified	✗	✓	✓	✗
Editing Expected	✓	?	?	✗

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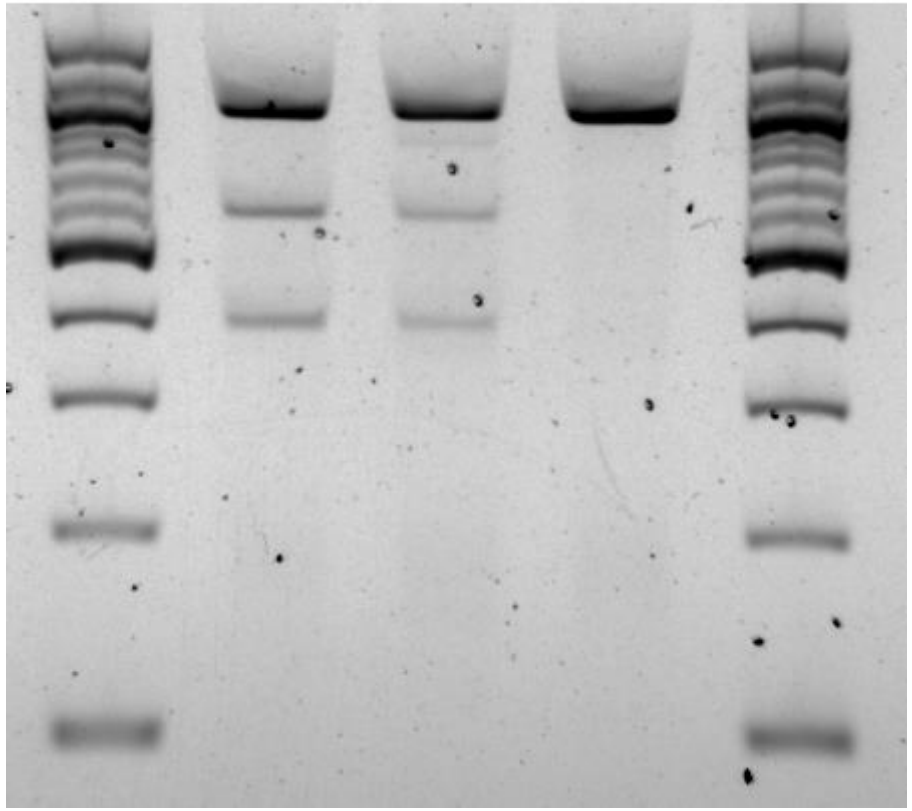
Editing is Possible with Modified N Terminal Region

Cell-Free Amplicon Cutting Assay

- In Vitro Translation of RNA coding for gene editing pairs
- Add target editing sequence amplified from WT gDNA
- Run resulting DNA on a gel

Left	TAL>(T_0)	**TAL-KR>(N_0)**	TAL>(T_0)
Right	TAL>(T_0)	TAL>(T_0)	



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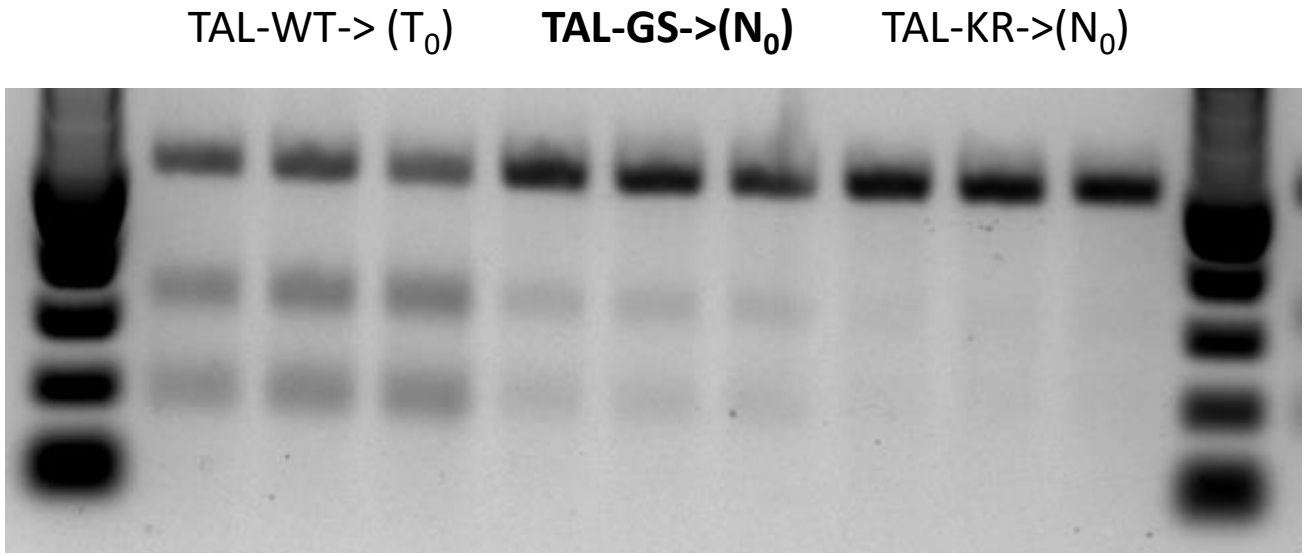
Testing Gene Editing Efficiency of N Terminal Region Mutations

Electroporated human keratinocytes with gene editing pairs

WT: IVGVGKQWSGARAL

GS: IVGVGGSKRGAGSGARAL

KR : IVGVGKQKRGARAL



Col7a T7E1 results from a 48hr incubation with modified gene editing proteins at 33C in human keratinocytes T22, in triplicate

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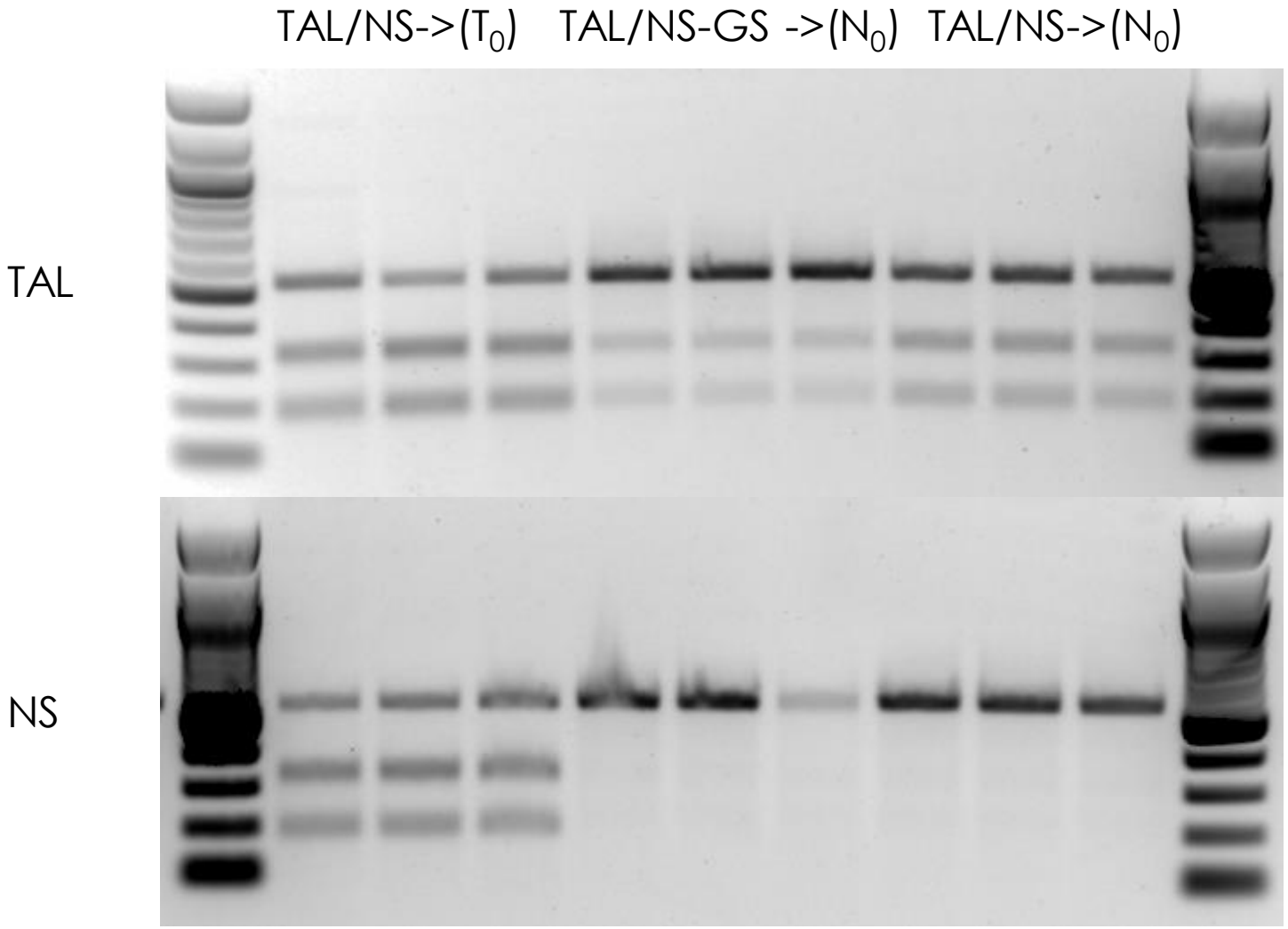
TALEN vs. NoveSlice Gene Editing Efficiency with N_0 Target Sites



Col7a T7E1 results from a 48hr incubation at 33C, in human keratinocytes triplicates

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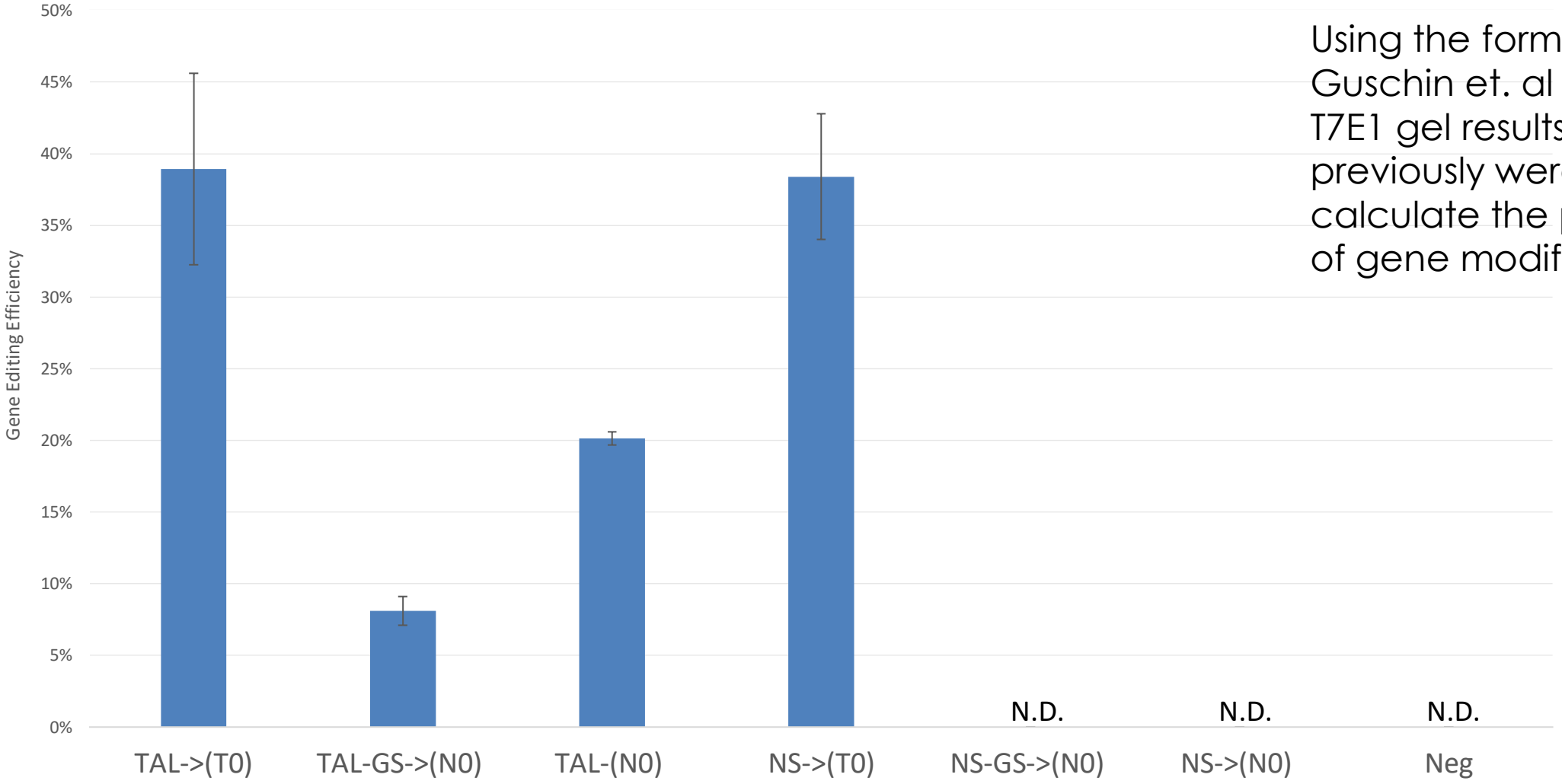
Gene Editing Efficiencies at N_0 Target Sites



Using the formula by Guschin et. al (2010), the T7E1 gel results shown previously were used to calculate the percentage of gene modification

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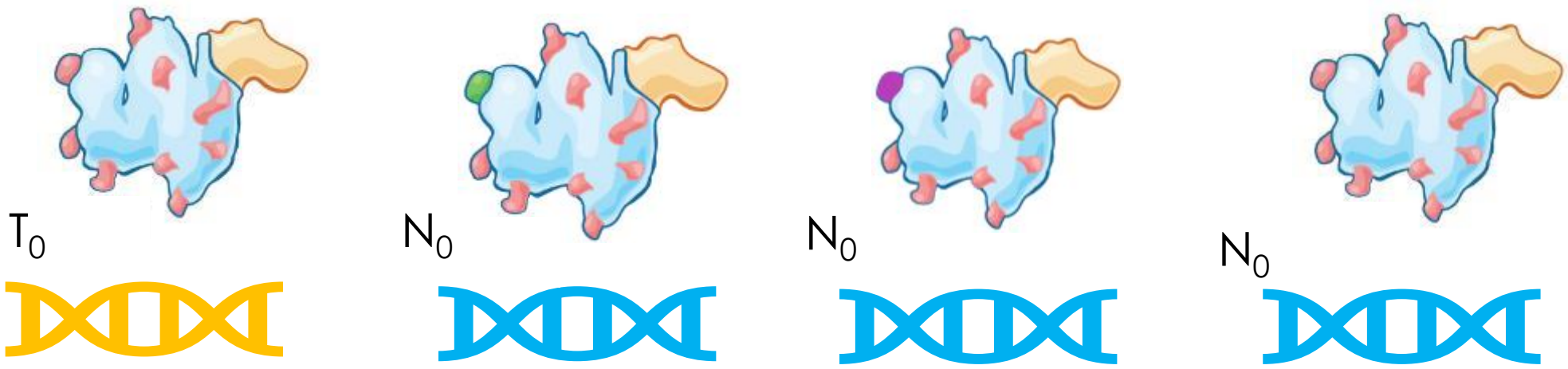
Conclusions



	TAL-WT->(T_0) NS-WT->(T_0)	TAL-KR->(N_0) NS-KR->(N_0)	TAL-GS->(N_0) NS-GS->(N_0)	TAL-WT->(N_0) NS-WT->(N_0)
Editing Expected	✓	?	?	✗
Results	✓	Partially relieves T_0 constraint in TALENs	Lead to more editing than KR mutation in TALENs	We observed editing in TALENs and no editing in NoveSlice

NoveSlice has lower "off-target" editing in a clinically relevant target site and thus could offer an advantage in development of ex-vivo and in-vivo gene therapies

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Acknowledgements

Thank you to the team at Factor Bioscience Inc.

Disclosures: MA and CR are inventors on multiple patents covering NoveSlice.

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