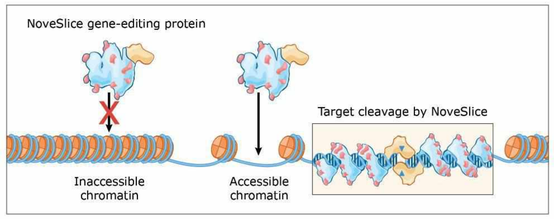
NoveSlice: A Novel Chromatin Context-Sensitive Gene-Editing Endonuclease

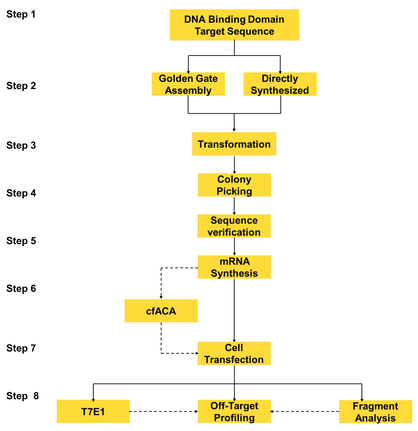
Mitchell R. Kopacz[1], Franklin Kostas[1], Craig Mealmaker[1], Jasmine K. Harris[1], Christopher B. Rohde[2], Matthew Angel[2]
[1]Novellus, Inc. Cambridge, MA [2]Factor Bioscience Inc. Cambridge, MA

Introduction

Genome-editing endonucleases are currently undergoing early clinical evaluation for the treatment of a wide range of diseases. However, *in vivo* use of gene-editing endonucleases is limited by the risk of potentially harmful off-target effects. It has been previously shown that gene-editing endonucleases are blocked by heterochromatin and show reduced efficiency in nucleosome-associated targets.



Methods



Kits Used by step	
Step	**Kit**
1	-
2	Golden Gate Assembly - NEB Enzymes Second Party Gene Synthesis
3	NEB Stable Stable Competent E. coli (High Efficiency) (Cat. No. C3040H)
4	-
5	GeneWiz Plasmid Sequencing
6	NEB T7 RNA Synthesis (Cat. No. E2040)
Optional 6.5	Cell-Free Amplicon Cutting Assay utilizing Promega Rabbit Reticulocyte Lysate (Cat. No. L4960)
7	Synthetic mRNA is transfected using the ThermoFisher Neon Transfection System, ThermoFisher Lipofectamine 3000, or ToRNAdo™. Cells are extracted using a Promega Maxwell® RSC Cultured Cells DNA Kit (Cat. No. AS1620), Qiagen QIAamp DNA Micro or Mini Kit (Cat. No. 56304 or 51306), or Zymo Quick-DNA Miniprep Kit (Cat. No. D3024).
8	T7E1 Mismatch assay - KapaBiosystems HiFi HotStart PCR Kit (cat. No KK2502) followed by NEB T7 Endonuclease I (Cat. No. M0302L) Novel Off-Target Assay Fragment Analysis by GeneWiz

Figure 1 & Table 1: Flow Chart of NoveSlice creation and testing process. The processing and testing procedure for NoveSlice is shown above. The workflow has been streamlined to efficiently process and test gene-editing mRNA. The workflow includes target sequence selection to assess target specificity, off-target effects, insertion rates, and targeting efficiency.

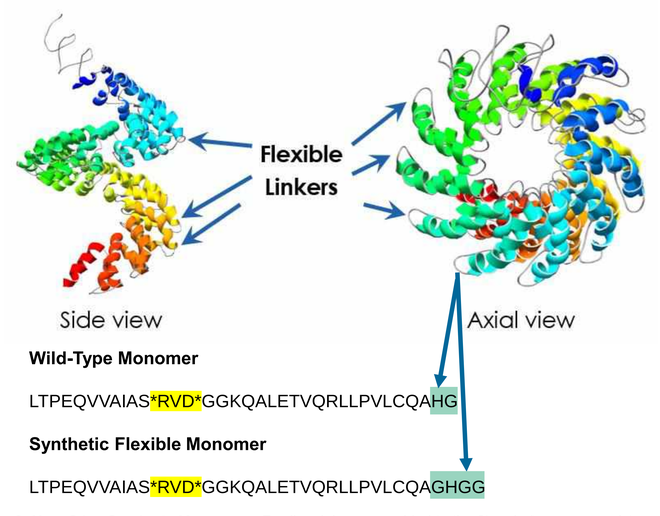


Figure 2: NoveSlice Synthetic Monomers. Flexible linkers are added to the 3' end of monomers. Alternating or non-tandem arrays of synthetic and wild-type monomers were assessed for editing efficiency, toxicity, and chromatin-context sensitivity. The linkers are shown by arrows.

Results

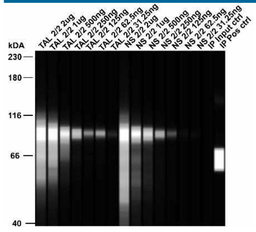
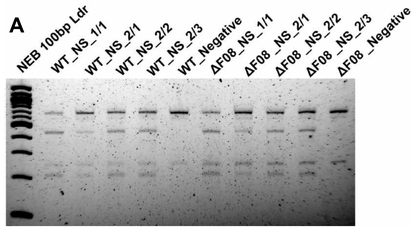
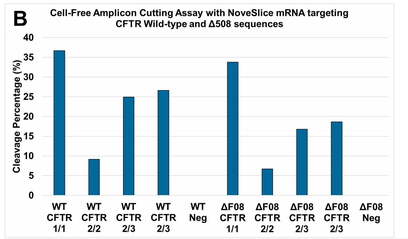


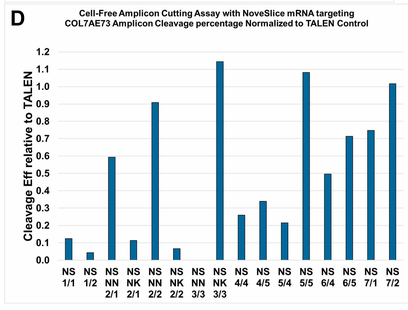
Figure 3: Protein Detection. NoveSlice proteins include a FLAG-tag. Shown here is the result of FLAG-based detection of both NoveSlice and TALENS in a rabbit reticulocyte lysate following immunoprecipitation. After immunoprecipitation, samples were run using FLAG-Tag antibodies on the Biotechne Protein Simple Wes device. Both NoveSlice and TALENs were robustly detected.





C Col7A1E73 Spacing Chart

	NS R1 20mer	NSR2 20mer	NSR3 18mer	NSR4 18mer	NSR5 16mer
NSL1 20mer	18	16	31	4	6
NSL2 20mer	20	18	33	6	8
NSL3 18mer	5	3	18	-8	-5
NSL4 19mer	32	30	45	18	20
NSL5 18mer	0	24	39	12	14
NSL6 18mer	28	26	41	14	16
NSL7 18mer	20	18	33	6	8



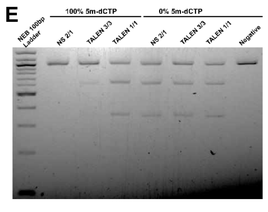


Figure 4: Cell-Free Amplicon-Cutting Assay (cfACA). We developed a rapid method for assessing gene editing proteins using a rabbit reticulocyte lysate system. **A.** gBlocks targeting the WT and ΔF08 mutation in the CFTR gene were synthesized by IDT. **B.** Using ImageJ, cleavage percentage was measured and used to assess different gene-editing pairs. **C.** This table shows spacings of gene-editing protein pairs that were designed to target the splice acceptor for COL7A1 exon 73. **D.** Cleavage efficiency was normalized to the positive control. In this experiment, the effects of converting all NN RVDs to NK were also tested. **E.** dCTP was replaced by 5m-dCTP to create an amplicon that would mimic hypermethylated DNA in the target region. In this case TALEN cut the hypermethylated amplicon, while NoveSlice did not.

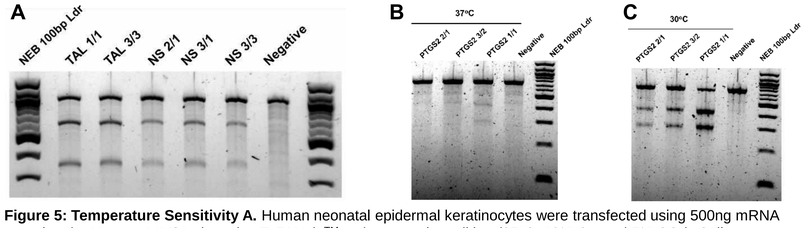


Figure 5: Temperature Sensitivity A. Human neonatal epidermal keratinocytes were transfected using 500ng mRNA targeting the Human AAVS1 site using ToRNAdo™ under normal condition (37ºC, 19% O_2, and 5% CO_2). Cells were harvested and DNA was extracted 48 hours after transfection. Surveyor PCR and T7E1 was performed. **B-C.** iPSCs were transfected using 500ng mRNA under two different temperature conditions 37ºC and 30ºC, both at 5% O_2, and 5% CO_2. These experiments demonstrate the temperature sensitivity of NoveSlice, and that transfection at low temperature can restore efficient cutting of targets that are not cut at 37ºC.

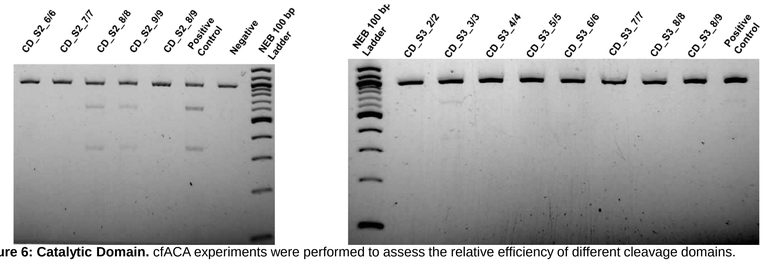


Figure 6: Catalytic Domain. cfACA experiments were performed to assess the relative efficiency of different cleavage domains.

Alias	Sequence
NS_Mod_1	AHDG
NS_Mod_2	GAHD
NS_Mod_3	GTHG
NS_Mod_4	GSGS
NS_Mod_5	NHGG
NS_Mod_6	GSGG
NS_Mod_7	RDHG
NS_Mod_8	IVHG
NS_Mod_9	VHGA
NS_Mod_10	GHGP
NS_Mod_11	RHGD
NS_Mod_12	THGG
NS_Mod_13	GGHD
NS_Mod_14	PHGG
NS_Mod_15	LHGA
NS_Mod_16	GGGG
NS_Mod_17	GRGG
NS_Mod_18	RHGE
NS_Mod_19	AHGA
NS_Mod_20	HRGE
NS_Mod_21	PHDG
NS_Mod_22	GPHD
NS_Mod_23	GKGG
NS_Mod_24	PHGP
NS_Mod_25	IHGM
NS_Mod_26	GPYE

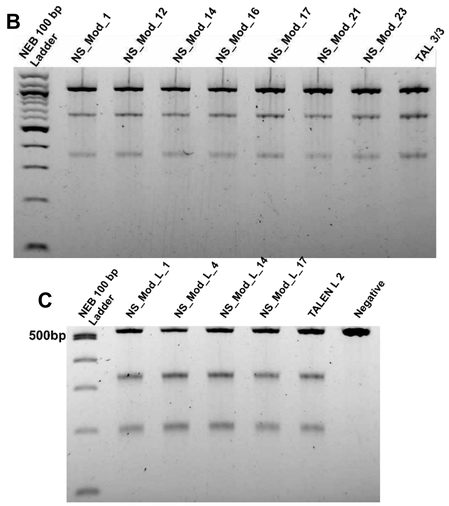


Figure 7: Additional Linkers. A. Various linkers were designed and tested. **B**. Cells were transfected with gene editing pairs in which the left gene editing protein contained a modified linker in the second-to-last position. The pairs were designed to target the Human AAVS1 site. Transfections where performed using ToRNAdo™ and 500ng of mRNA on Neonatal Human Epidermal Keratinocytes. Cells were extracted 48 hours after transfection and a T7E1 was performed. **C**. iPSCs were transfected gene editing pairs in which the left gene editing protein contained multiple alternative linkers. The pairs were designed to target the human Col7A1 exon 73 splice acceptor site. Transfections where performed using Lipofectamine 3000 and 2ug of mRNA per well of a 6-well plate. Cells were extracted 48 hours after transfection and a T7E1 was performed.

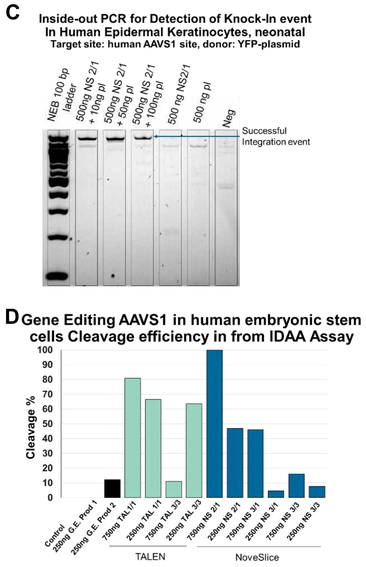


Figure 8: Insertion and Editing Efficiency Quantification. A-B. Human neonatal epidermal keratinocytes were transfected and imaged using an Operetta High-Content Imaging System. **C.** DNA was extracted and an inside-out PCR was performed to assess integration. **D.** The IDAA Assay (Yang Z, Steentoft C, Hauge C, et al. Fast and sensitive detection of indels induced by precise gene targeting. Nucleic Acids Res. 2015;43(9):e59. doi:10.1093/nar/gkv126) was used to quantify the editing efficiency of NoveSlice.

Discussion

While several gene-editing proteins efficiently cut various targets in primary human cells, these data show that the novel NoveSlice gene-editing endonuclease is more sensitive to the chromatin context of the target than an equivalent TALEN pair. The risk of potentially harmful off-target effects has restricted the clinical translation of gene-editing technologies. A gene-editing endonuclease with reduced activity in inaccessible regions of the genome could exhibit reduced off-target effects, and could thus represent a powerful tool for the development of gene-editing therapies. Here we present a novel gene-editing endonuclease that exhibits enhanced sensitivity to the chromatin context of the target. NoveSlice may serve as an important tool for the development of new precision medicines, including *in vivo* gene-editing therapies.

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