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A Novel CRISPR/RNA-Aptamer-Mediated Base Editing System with Potential Therapeutic Value

By

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A dissertation submitted to the School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements for the degree of Doctor of Philosophy

Graduate Program in Cellular and Molecular Pharmacology

Graduate Programs in Molecular Biosciences

Written under the direction of

Shengkan Jin, PhD

And approved by:

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New Brunswick, New Jersey

January 2019

ABSTRACT OF THE DISSERTATION

A Novel CRISPR/RNA-Aptamer-Mediated Base Editing System with Potential Therapeutic Value

by JUAN CARLOS COLLANTES VELA

Dissertation Director: Shengkan Jin

Nuclease-dependent precise genome editing such as correction of point mutations requires introduction of targeted DNA double strand breaks (DSB) and activation of homology dependent repair (HDR), limiting its application to proliferating cells. To expand GE capabilities for therapeutic use in non-dividing somatic cells it is necessary to precisely modify nucleotides avoiding DSBs. Recently, Cas9-cytidine deaminase fusions, also known as based editors (BE), were shown to precisely modify target bases at certain genomic loci. To expand the base editing toolbox, we sought to engineer a novel base editing system based on RNA-aptamer mediated recruitment. To this end, we engineered a nuclease-deficient CRISPR/Cas9 system as a recruitment platform for non-nuclease DNA/RNA editing enzymes that catalyze C·G→T·A conversions by cytidine deamination. Targeted nucleotide modification was achieved with high precision in prokaryotic and eukaryotic cells. In bacteria, we tested our system targeting the rifampicin resistance determining region of the *rpoB* gene. Survival in rifampicin reached over 1000-fold higher than untreated cells. To examine whether the system can correct loss of function mutations in human genome, we treated a stably integrated non-fluorescent *EGFP* gene containing an A·T→G·C mutation on the chromophore sequence.

Fluorescence was efficiently restored in treated cells, detecting around 10% of GFP positive cells after treatment. Next generation sequencing confirmed a G·C→A·T conversion in 60% of reads at the target position, restoring the wild type sequence, with low by-stander effect. Exome-wide sequence analysis revealed no detectable off-target effects. Targeting of endogenous loci also resulted in highly efficient nucleotide conversion at the desired C positions. We also show that our system can destroy the 3' splice acceptor site of intron 50 in human *DMD* gene, potentially inducing exon 51 skipping, providing evidence of a therapeutic application to treat Duchenne muscular dystrophy. Taken together, the data show that our GE system represents a safe and promising technology for editing specific nucleotides, correcting genetic mutations or other clinically relevant applications, independent of DSB and HDR, with potential therapeutic value in non-dividing cells.

ACKNOWLEDGEMENTS

I would like to thank Dr. Shengkan Jin for the wonderful opportunity he has given me to work in this exciting project. With his advice and guidance, this has been the most rewarding professional experience of my career.

I want to also thank the members of my committee, Dr. Debabrata Banerjee, Dr. Joseph Fondell and Dr. Richard Padgett. Their input, advice and positive feedback have been fundamental to guide my work.

This project would not have been possible without the contributions of many friends and colleagues. I want to thank my lab mates that collaborated with me, directly or indirectly, to advance this project. Thanks to Huiting Xu and Victor Tan for their instrumental contribution to lab work. To Dr. Roman Wernyj for his contribution with early experiments that paved the way for the success of this project. To Dr. Hanlin Tao, Dr. Amer Alasadi and Dr. Jingjing Guo for their advice and friendship. To Dr. Katarzyna Tyc and Dr. Jinchuan Xing for their contribution for some of the bioinformatics work. To Husam Al-hraishawi for developing the stable nfEGFP cell line.

I would also wish to extend my appreciation to faculty and staff members of the Pharmacology Department, SGS-Biomedical Sciences and Molecular Biosciences for the continuing the support I have received during my time at Rutgers.

I wish to thank my family, my beautiful wife Meli, my son Gabriel and my daughter Elena (on her way to this world) for their endless love, support and motivation to keep me going through this hard but beautiful journey. I am also grateful to my parents, Carlos

and Ana María, my siblings, Daniela and Sebastian, my cousin Andrés, and my grandma and one of my best friends, Chivita, for always supporting me and encouraging me to pursue this dream.

Finally, this work was supported by Fulbright – SENESCYT grant No. 15130816, US NIH/NCI R21 CA216604, and Rutgers University – School of Graduate Studies "Biomedical Completion and Acceleration Fellowship".

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CHAPTER I

Introduction and Rationale

Humans have had the ability to manipulate and modify genomic sequences for almost half a century, since the advent of recombinant DNA technology.[1] In the early 1990s, Pavletich and Pabo published the first crystal structure of a zinc finger protein interacting with its target DNA.[2] The elucidation of zinc finger DNA recognition mechanism paved the way to design chimeric DNA binding proteins with novel functions.[2,3] By fusing engineered zinc finger domains to nuclease domains from restriction enzymes such as FokI, also known as zinc finger nucleases (ZFN), researchers were able to introduce programmable and highly specific DNA double strand breaks,[4] thereby launching the gene editing era. In the years that followed, many research applications of these customizable designer nucleases were reported.

Right from the start, the potential use of GE technologies in biomedicine was appreciated.[3] Recently several studies, including a phase 1 clinical trial,[5] have successfully shown that GE technologies may be suitable to treat human diseases, including viral infections as well as inherited disorders.[6] However, this possibility may be fully achieved when existing technologies overcome the challenges and limitations they currently face regarding safety, efficacy and delivery.[7,8]

Currently, there are four GE platforms: ZFN, transcription activator-like effector nucleases (TALENs), meganucleases and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 (CRISPR/Cas9).[6,8,9] A common feature among these methods is the introduction of sequence specific DSBs, which in turn activate DNA repair pathways, mainly HDR and non-homologous end joining (NHEJ).[6,8,10] HDR is an error free pathway, and its activation allows for precise sequence modification when a donor template with the desired modification, flanked by homologous sequences to the target region, is delivered together with the designer nuclease.[10,11] Nonetheless, this method can be applied exclusively to actively dividing cells, specifically throughout S/G2 phases , because HDR is absent in post mitotic cells.[6,12] NHEJ is active in mitotic and post mitotic cells throughout the cell cycle. However, it is an error prone pathway that often introduces insertions and deletions of nucleotides (indels) in the target sequence. If the break occur on coding regions of the target gene, the effective outcome is disruption of the ORF, thus deleting the gene.[6] Therefore, when it comes to treating terminally differentiated cells, current designer nuclease platforms are restricted to gene knockout, which limits their application in the clinical setting. By harnessing the DNA targeting features of GE platforms, independently from their nuclease activity, engineering them as delivery systems of non-nuclease DNA interacting enzymes, we hypothesize that some of such limitations could be potentially overcome.

Monogenic disorders could potentially benefit from clinical applications of GE technologies, since the disease phenotype can be reversed by the correction of a single gene in specific tissues.[13] The World Health Organization (WHO) estimates that over 10,000 human diseases are monogenic (WHO, 2018; retrieved from http://www.who.int/genomics/public/geneticdiseases/en/index2.html), and these disorders affect both dividing and differentiated cells. Some of these disorders arise from changes in single nucleotides, known as single nucleotide polymorphisms (SNPs).[14-16] Developing GE platforms capable of introducing precise modifications in terminally differentiated cells, at the single nucleotide level, will allow researchers treat the disease causing mutation on the DNA sequence, potentially moving forward GE therapeutics.

The recent discovery of the DNA targeting capabilities and ease of use of the bacterial and archaeal adaptive immune system, called CRISPR/Cas9,[17,18] has revolutionized the research field and its potential therapeutic applications are being explored.[6,19,20] In contrast to ZFN, TALENs and meganucleases, CRISPR/Cas9 does not require labor-intensive and costly protein engineering processes to program every new target since it is an RNA-guided engineered endonuclease system (RGEN), greatly facilitating GE experimentation.[17,21] CRISPR/Cas9 can be easily programmed to target specific genomic regions provided that strand complementary to the target sequence (protospacer, normally 20 nucleotides in length) is flanked by a 3' protospacer adjacent motif (PAM), which sequences differ depending on the bacterial species.[22] Most laboratories are

engineering *S. pyogenes*' CRISPR system because of its relatively simple PAM sequence requirement, i.e. NGG.[17,22] However, one major drawback of this and other GE platforms is the introduction of off-target, potentially cytotoxic DSBs.[23] Several strategies to improve the specificity and safety of CRISPR have been reported.[24-33] Despite the robustness and versatility of CRISPR systems, a better safety profile is required in order to improve its specificity and its potential clinical applicability.[10,32] For example, specificity is improved when engineered Cas9 nickases (nCas9) are delivered with paired, offset gRNAs targeting opposite DNA strands.[24,31,34] Off-target effects produced from nickase targeting are likely well tolerated, since single strand breaks (SSB) are resolved efficiently by base excision repair pathway (BER)[35-37] or by an alternative HDR pathway,[38] however the latter might be irrelevant for terminally differentiated cells. Similarly, fusion of catalytically dead Cas9 (dCas9) to the monomeric nuclease domain of FokI targeted with paired gRNAs has been reported as an alternative mechanism to improve CRISPR specificity.[27,28,33] Both of these strategies enhance specificity by relying on CRISPR's targeting capabilities without generating DSBs.

Numerous CRISPR/Cas9 nuclease-independent applications have been described thus far, including regulation of gene expression by transcriptional activation,[39-41] repression[42,43] and epigenome modulation,[44,45] visualization of defined genomic loci,[46,47] immunoprecipitation and isolation of specific genomic sequences,[7] among others. These efforts have opened the path to increase the repertoire of CRISPR technology applications beyond generating DSBs. This prompted us and others[48] to engineer *S.*

pyogenes' CRISPR/Cas9 as a targeted recruitment system for several non-nuclease DNA modifying proteins capable of introducing highly specific sequence modifications at the single nucleotide level, giving rise to a new generation of genome engineering application: base editing. Phage RNA scaffolds that are synthetically fused to the 3' end of the gRNA molecule mediate the recruitment mechanism.[40,41,47] Our platform, called CRISPR-RNA-Aptamer-Mediated Correction/Modification (CRC) system, is able to target and modify specific nucleotides in prokaryotic genome, as well as extrachromosomal DNA and endogenous loci in human cells in a programmable fashion, circumventing the generation of DSB. To test CRC in a clinically relevant target, we also aimed the system to abrogate the 3' splice acceptor site of *DMD*'s intron 50 to induce exon 51 skipping in *DMD* gene. This strategy has been shown to restore dystrophin expression in patients diagnosed with Duchenne muscular dystrophy using a morpholino-based antisense oligonucleotides targeting the mRNA,[49-51] inducing a transient effect. Our results show that it is possible to use CRC to permanently delete exon 51, potentially extending the beneficial effect of therapeutic genome editing. Together, the data suggest that CRC system could be a promising therapeutic tool.

CHAPTER II

Construction of CRC System and Proof of Concept in Prokaryotic Genome

CRISPR/Cas9 are modular DNA targeting systems that have been engineered for targeted gene editing[17], as well as many other nuclease-independent applications such as gene expression regulation[41,52], dynamic genomic labeling[46,53], epigenome editing[54,55], and even as diagnostic tools,[56] etc. In this Chapter, a nuclease-deficient CRISPR/Cas9 system derived from *Streptococcus pyogenes* was used as a recruitment platform for activation-induced cytidine deaminase (AID),[57] a DNA editing enzyme that catalyzes C→U (T) conversions in genomic DNA.[58,59] The recruitment mechanism is mediated by engineered gRNAs synthetically fused to RNA-aptamers[41] which bind to specific RNA-interacting proteins fused to AID. We call our system CRISPR-RNA-Aptamer-Mediated Correction/Modification system (CRC; **Figure 1**). We achieved efficient nucleotide conversion in *E. coli*'s genome using a negative selection system (**Figure 2, 3**). These findings represent a proof of concept for a novel gene editing approach and pave the way for the development of mammalian applications.

During the course of this work, several studies were published by David Liu and colleagues describing a similar gene editing approach, named base-editing.[48,60-62] Their systems, dubbed base editors (BE), use cytidine deaminase APOBEC1 as effector module to catalyze C→T conversions. In base editors, the effector proteins are directly tethered to Cas9. Since BE and CRC have the same goal but key differences in terms of

recruitment mechanism and effector modules, we decided to compare them side-by-side in our mammalian cell experiments (Chapters III and IV).

Results

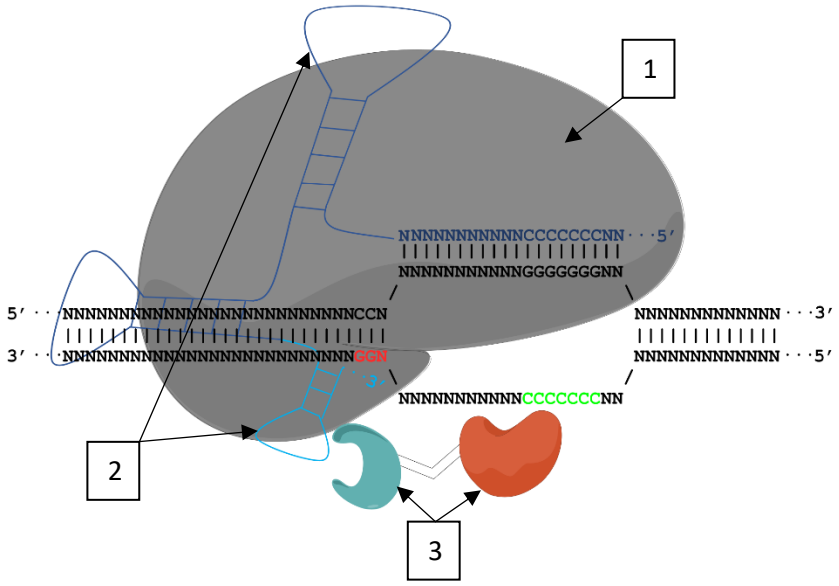
CRC System: A Modular Base Editing Platform

The CRC base editing system contains three functional modules illustrated in **Figure 1A**: (1) a nuclease deficient Cas9 protein; (2) a programmable chimeric RNA scaffold containing gRNA (for sequence recognition and Cas9 binding) and a recruiting RNA motif (aptamer) for effector module recruitment; and (3) the effector module containing a cytidine deaminase (effector) fused to a small RNA binding protein that specifically interacts with the recruiting RNA-aptamer described in (2).

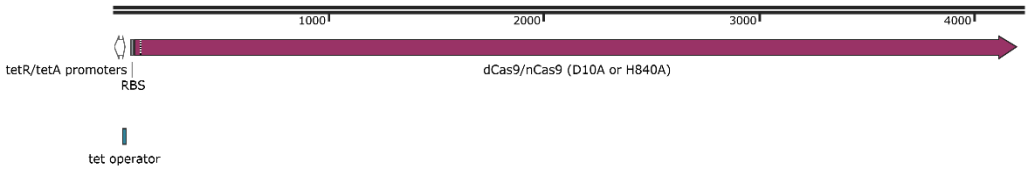
In our prototype system, we used a catalytically dead Cas9 (dCas9) protein derived from *Streptococcus pyogenes* ($nCas9_{D10A}$) as the CRISPR targeting platform. Activation induced cytidine deaminase (AID), an enzyme that catalyzes C→U (T) conversion in DNA context, was used as the effector protein. MS2-MCP (RNA-aptamer – RNA binding protein pair) was used for the RNA mediated effector recruitment.[41] To construct the system, we synthetically fuse MS2 RNA motif to the 3' end of the CRISPR RNA scaffold for recruiting the effector module, AID_MCP fusion protein. As a result, the effector AID

protein could be recruited to any target sequence (specified by the guide RNA) where it could convert a C to U (T). In **Figure 1**, the effector is shown as a monomer; however, in cells, AID forms a dimer at the action site. A potential advantage of the RNA based effector recruitment design compared to a direct fusion of the effector to Cas9[48] is that proteins such as AID and other members of the APOBEC family of cytidine deaminases usually require dimerization or other types of oligomerization for enzymatic activity.[63-65] The AID_MCP fusion monomer protein can form an oligomer relatively efficiently (MCP is a small peptide, 13 kDa) while multimerization for the Cas9-cytidine deaminase fusion is expected to be inefficient (Cas9 is bulky, 160 kDa, and one Cas9-cytidine deaminase monomer is pivoted to DNA).

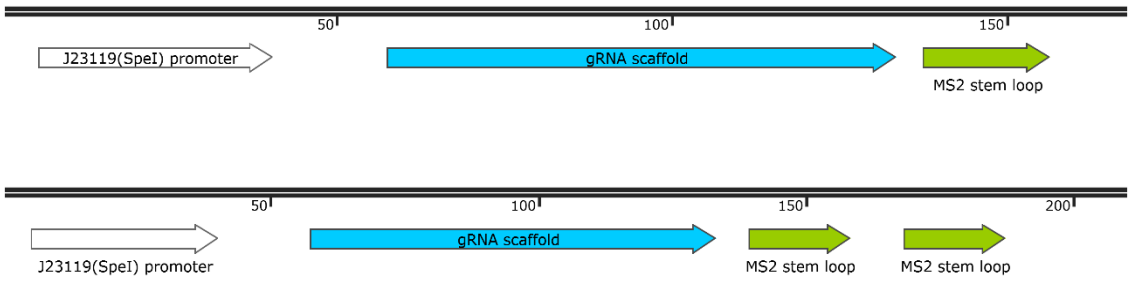
A



B.



C.



D.

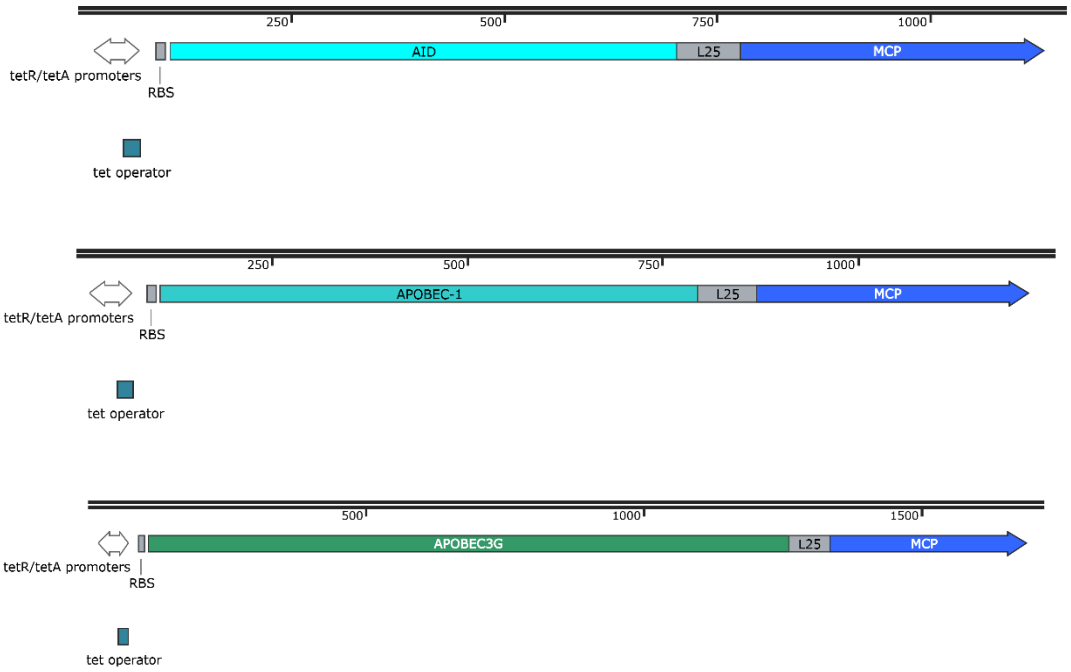


Figure 1. CRC system description

A. Components of the platform: (**1**) DNA targeting module dCas9 or nCas9$_{D10A}$, (**2**) Chimeric RNA scaffold containing a guide RNA motif (for programmable sequence targeting, blue sequence), CRISPR motif (for Cas9 binding, blue scaffold), and recruiting RNA motif (for recruiting effector-RNA binding protein fusion, light blue scaffold), and (**3**) effector-RNA binding fusion protein. In red is shown the PAM sequence. **B – D.** Schematic representation of constructs used in this Chapter, including DNA targeting module encoding for Cas9 variants dCas9, nCas9$_{D10A}$ or nCas9$_{H840A}$ (**B**; component (**1**) in **Figure 1A**); gRNA/recruiting module containing one or two RNA aptamer motifs (**C**, top and bottom, respectively; component (**2**) in **Figure 1A**); and effector module, encoding for fusion proteins AID_MCP, APOBEC1_MCP or APOBEC3G_MCP (**D**, top, middle,

bottom respectively; component (**3**) in **Figure 1A**). For bacterial experiments shown in this chapter, each module is encoded in a single vector.

CRC Proof of Concept in Prokaryotic Cells: Editing an Endogenous Bacterial Gene

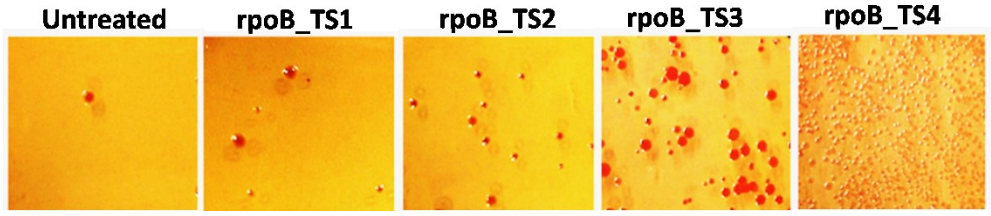
In bacteria, we tested our system using a negative selection approach using the antibiotic rifampicin for selection. This drug binds near the catalytic pocket of the subunit β of bacterial RNA polymerase, encoded by the *rpoB* gene, inhibiting transcription by physically blocking RNA elongation.[66] Well defined mutations along a specific segment of the *rpoB* gene have been associated with rifampicin resistance. This region is known as rifampicin resistance determining region[67] (RRDR; **Figure 2A**). Furthermore, it has been shown that AID is capable of introducing mutations in RRDR, and surviving colonies can be individually assessed for *rpoB* gene mutations,[58] making it an ideal target to test CRC.

Four gRNAs were designed for these experiments targeting the template strand (TS1 – TS4; **Figure 2A, Table 1**), using catalytically dead Cas9 (dCas9) as DNA targeting module and 1xMS2 as recruiting module. The system expressing AID_MCP and dCas9 as effector and targeting modules, respectively, is noted as $^{A}CRC_{d}$. Survival fraction in rifampicin reached 40-fold over the untreated background after CRC treatment with gRNA TS4, while the remaining gRNAs resulted in fewer surviving colonies. The higher efficiency observed in TS4 treated cells might be due to the position of the targeted C within the

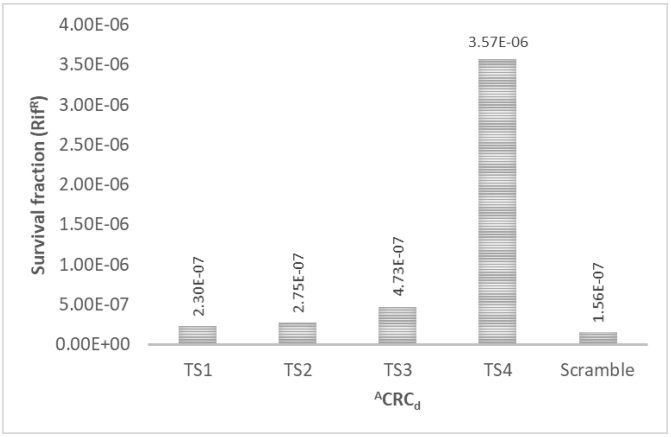
protospacer (the unpaired DNA strand left by CRISPR R-loop), which in this case sits on position 8 from the 5' end. These observations are consistent with base editing activity window, which suggests that for efficient C→T conversion, the relative position of the target C should be between nucleotides 4 and 8 of the unpaired strand.[48] Sequence analysis of isolated colonies treated with $^{A}CRC_{d}$/rpoB_TS4 revealed that the system introduced targeted C→T mutations in codon 531, changing a serine for phenylalanine, a mutation known to induce rifampicin resistance[67,68] (**Figure 2 A – C**). From these results we conclude that CRC's targeting mechanism requires the target C to be on the unpaired DNA strand left by the CRISPR R-loop, closer to the 5' end, as shown in **Figure 1**. Taken together, the data show that targeted nucleotide modification is a feasible approach for gene editing independent of DNA double strand breaks.

A.

B.



C.



D.

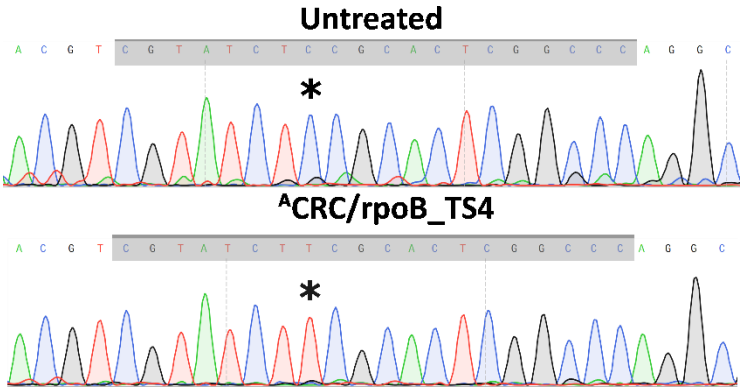


Figure 2. Site-specific nucleotide conversion in bacterial cells. *E. coli* MG1655 cells were treated with four gRNAs and selected in plates containing rifampicin. **A.** Schematic of representation of gRNAs targeting the RRDR Cluster I region of *E. coli*´s *rpoB* gene is shown, where PAM sequences in target DNA are shown in green and mutable positions in red. Black bars represent gRNA targeting sites. **B.** Panels show representative surviving colonies after treatment with CRC targeted with the indicated gRNAs. **C.** Survival fraction was calculated from similar experiments shown in **A. D.** Representative sequencing results from untreated cells (top) and ^{A}CRC treatment with *rpoB*_TS4 gRNA (bottom). Target position in CRC treated and untreated DNA are indicated with black arrows. This C1592>T mutation results in S531F change in protein sequence, a mutation known to induce rifampicin resistance.[68]

Table 1. rpoB targeting gRNA sequences

Name	Target strand	Target gene	Sequence	PAM
TS1	Template	rpoB	GCAGCAGTGAAAGAGTTCTT	CGG
TS2	Template	rpoB	CAGCCAGCTGTCTCAGTTTA	TGG
TS3	Template	rpoB	AAACGTCGTATCTCCGCACT	CGG
TS4	Template	rpoB	CGTATCTCCGCACTCGGCCC	AGG

System Optimization: Engineering Individual Modules Enhances Efficiency

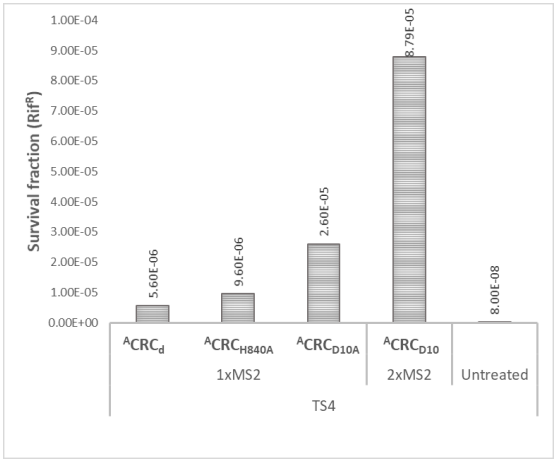
The positive results from our exploratory experiments prompted us to further engineer CRC system to increase its targeting efficiency using gRNA rpoB_TS4. Switching Cas9 module from dCas9 ($^{A}CRC_{d}$) to nickase $Cas9_{D10A}$ ($^{A}CRC_{D10A}$) or $Cas9_{H840A}$ ($^{A}CRC_{H840A}$) dramatically increased the number of surviving colonies (**Figure 3A**). Another strategy to enhance the system's efficiency is to increase the number of recruiting RNA scaffolds. Survival fraction was boosted to 1000-fold higher than untreated cells by doubling the number of MS2 sequences (**Figure 3A**). We decided to continue optimizing the system by testing different spatial configurations of the effector module, in combination with all Cas9 variants developed so far. To this end, various flexible linkers, with different lengths, were used to separate AID from MCP (**Table 2**). The 25 amino acid linker (L25), derived from the hinge region of immunoglobin gamma 3 (IgG3), showed the highest efficiency, although the variations between the different linkers were relatively small, especially for $^{A}CRC_{D10A}$ (**Figure 3B**). These results suggest that the separation between AID and MCP in the effector module does not play a critical role in overall targeting efficiency.

Table 2. Effector module linker sequences

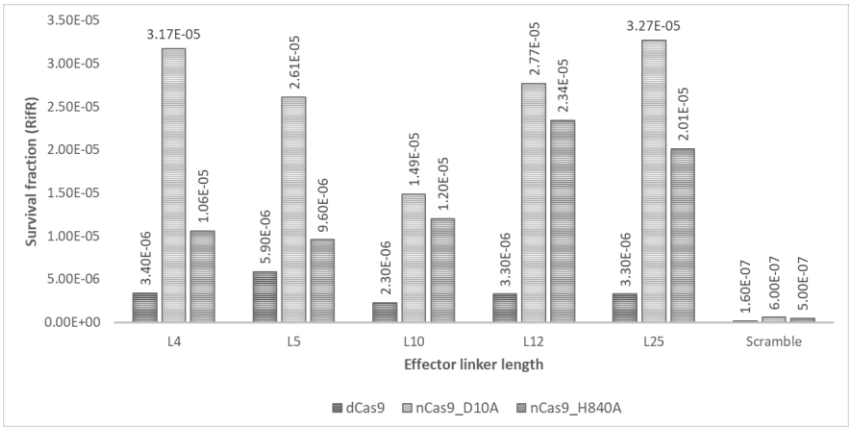
Linker name	Length (aa)	Sequence
L4	4	GSGS
L5	5	GSGRA
L10	10	GSGSGSGSGS
L12	12	GGGGSGGGGSGGGGS
L25	25	ELKTPLGDTTHTSPPCPAPELLGGP

Although $^{A}CRC_{H840A}$ modestly increased the survival fraction compared to $^{A}CRC_{d}$ (**Figure 3A, 3B**), sanger sequencing analysis of individual clones revealed that its targeting accuracy was lower compared to both $^{A}CRC_{d}$ and $^{A}CRC_{D10A}$, which targeted invariably the residue C1592 in codon 531 (**Figure 3C**). On the other hand, $^{A}CRC_{H840A}$ induced mutations not only on the target region, but several nucleotides upstream at high frequency (**Figure 3C**). For this reason, we decided to adopt $nCas_{D10A}$ as targeting module for further research and development in prokaryotic as well as in mammalian cells.

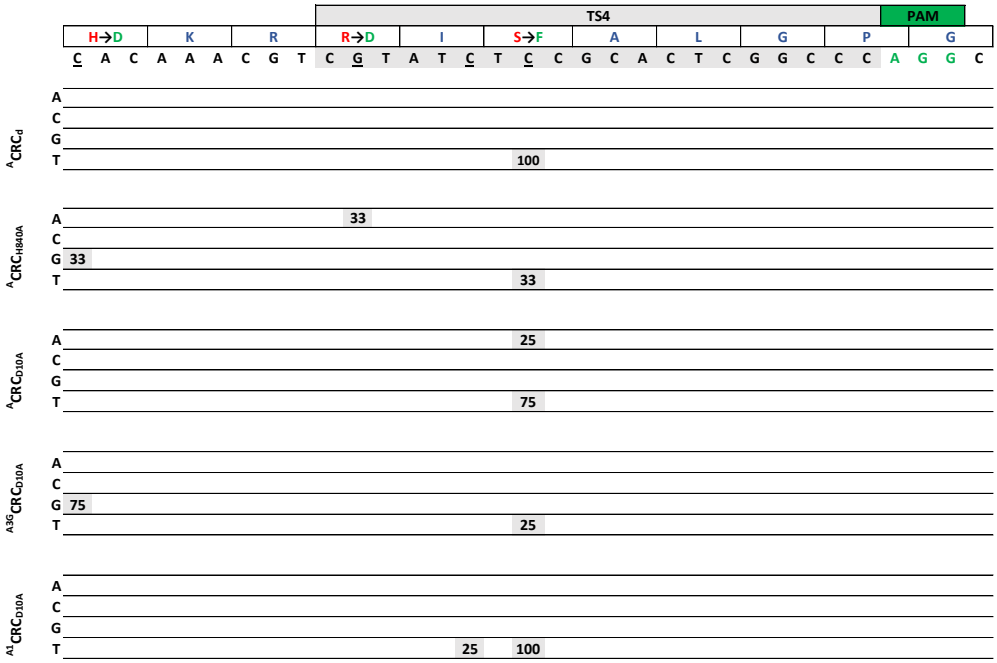
A.



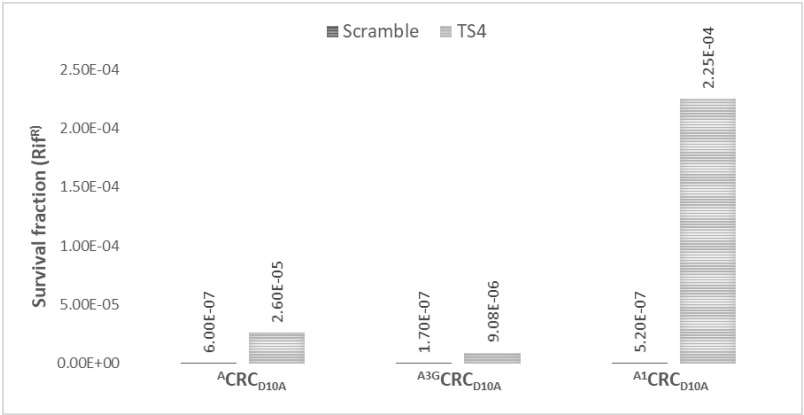
B.


Survival fraction (RifR)
3.50E-05
3.00E-05
2.50E-05
2.00E-05
1.50E-05
1.00E-05
5.00E-06
0.00E+00
3.40E-06
3.17E-05
1.06E-05
5.90E-06
2.61E-05
9.60E-06
2.30E-06
1.49E-05
1.20E-05
3.30E-06
2.77E-05
2.34E-05
3.30E-06
3.27E-05
2.01E-05
1.60E-07
6.00E-07
5.00E-07
L4
L5
L10
L12
L25
Scramble
Effector linker length
dCas9
nCas9_D10A
nCas9_H840A

C.


TS4
PAM
H→D K R R→D I S→F A L G P G
C A C A A A C G T C G T A T C T C C G C A C T C G G C C C A G G C
ACRd
ACRH840A
ACRD10A
A3GCRD10A
A1CRD10A
A C G T
100
33
33
25
75
75
25
25
100

D.


Scramble
TS4
Survival fraction (RifR)
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
6.00E-07
2.60E-05
1.70E-07
9.08E-06
5.20E-07
2.25E-04
ACRD10A
A3GCRD10A
A1CRD10A

E.

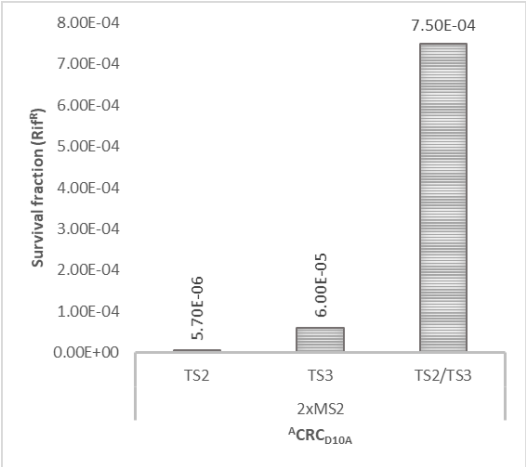


Figure 3. CRC system optimization. A. Efficiency can be increased by using Cas9 nickases ($nCas9_{H840A}$ or $nCas9_{D10A}$) instead of dCas9 as targeting module. Likewise, increasing the number of recruiting motifs from 1xMS2 to 2xMS2 enhances CRC's efficiency. **B.** Effect of linker length variation in effector module. Four linkers of various lengths and flexibilities were testes in these experiments. L4, L5, L10 and L25 are linkers consisting of 4, 5, 10 and 25 amino acids, respectively. **C.** Mutation distribution of clones selected on rifampicin after treatment. Five CRC systems are compared side by side: $^{A}CRC_{d}$, $^{A}CRC_{H840A}$, $^{A}CRC_{D10A}$, $^{A1}CRC_{D10A}$ and $^{A3G}CRC_{D10A}$. rpoB gene from individual clones was PCR amplified and sequenced for genotyping. Numbers represent percentage of clones with a given genotype; n=8 per treatment. **D.** APOEC3G ($^{A3G}CRC_{D10A}$) and APOBEC1 ($^{A1}CRC_{D10A}$) were tested as effectors side by side with the prototype system, $^{A}CRC_{D10A}$. **E.** Survival fraction of cis double nicking treatment with TS2 and TS3 gRNAs compared to single nicking. Targeting efficiency increased with cis double nicking 130- and 12-fold when compared to single targeting with TS2 and TS3 gRNAs.

There are many cytidine deaminases that can be incorporated into CRC system as effectors.[69] In this study, we tested two proteins related to AID from the APOBEC family of cytidine deaminases: APOBEC1 and APOBEC3G ($^{A1}CRC_{D10A}$ and $^{A3G}CRC_{D10A}$, respectively). $^{A1}CRC_{D10A}$ showed greater conversion efficiency, followed by $^{A}CRC_{D10A}$ and finally $^{A3G}CRC_{D10A}$ with the lowest activity (**Figure 3D**). Sequencing analysis revealed that $^{A1}CRC_{D10A}$ induced a high rate of double mutants (25%, **Figure 3C**). The data suggest that CRC system is amenable for further development, and other effectors, such as

adenosine deaminases, DNA glycosylases and other DNA editing proteins, can be easily incorporated into CRC toolbox.

One potential limitation of CRISPR targeting, especially for base editing, is the availability of the protospacer adjacent motif (PAM), a short DNA sequence immediately downstream of the target region that is required for proper Cas9 binding. This limitation is evidenced by the low survival fraction that resulted from treatments using TS2 and TS3 gRNAs, where the target Cs are outside the activity window, specifically on positions 12 and 14, respectively (**Figure 2A – B**). We reasoned that introducing two single strand nicks in cis (i.e. on the same DNA strand), could potentially alleviate this limitation by transiently exposing a longer stretch of single strand DNA, potentially making the target C more accessible to CRC effector module. Double nicking the same strand with two gRNA does not induce double strand DNA breaks or activation of DSB repair pathways.[31] To test this hypothesis, we multiplexed CRC targeting by delivering TS2 and TS3 simultaneously along with $^{A}CRC_{D10A}$ constructs and compare the results to single targeting using either of the gRNAs. The results show that cis double nicking dramatically increased CRC targeting efficiency in a synergistic fashion (**Figure 3E**).

Discussion

We have shown that base editing is feasible in prokaryotic cells using an RNA-aptamer based recruitment mechanism to recruit an effector protein, in this case AID, APOBEC1 and APBEC3G (**Figure 1, 2, 3**). These developments broaden the scope of current base editing systems, by allowing a more flexible configuration compared to direct fusions found in base editors. For example, to form active editosomes, APOBEC1 requires dimerization to become catalytically active.[64] Therefore, CRC's aptamer-based recruitment provides a modular platform where effector multimerization can potentially be achieved more easily, owed to the smaller size of the effector fusion protein.

Several system modifications were tested, highlighting the engineering potential that CRC system can offer. By switching the Cas9 module from dCas9 ($^{A}CRC_{d}$) to $nCas9_{D10A}$ ($^{A}CRC_{D10A}$), targeting efficiency goes from 70- to 325-fold higher than the untreated background (**Figure 3A**). In this configuration we are simultaneously introducing two well tolerated DNA lesions: nicksases introduce single strand breaks (SSB) on the DNA, while AID generates a mismatch. SSBs are among the most common DNA lesions,[35] however this type of DNA damage can become clastogenic if left unrepaired.[36,70] In this way, by introducing these mild insults to target DNA, cells can be biased to repair the most threatening lesion, the DNA nick, thus fixing the single nucleotide editing introduced by the effector module.

Additional effective engineering interventions included increasing the number of recruiting RNA motifs, by which the efficiency went over 1000-fold greater than untreated cells (**Figure 3A**). We also tested variations of the spatial configuration of the effector module. To this end, we engineered the effector module to include linkers of various lengths and sequences shown in **Table 2**. Again, the highest efficiency was observed after $^{A}CRC_{D10A}$ across different treatments (**Figure 3B**). Although the difference was small, the greatest efficiency was achieved with L25 linker, a flexible peptide derived from the hinge region of Immunoglobin gamma 3 protein (**Figure 3B**), and this linker was selected for further development. Finally, we tested different effector proteins in addition to AID ($^{A}CRC_{D10A}$), specifically APOBEC1 ($^{A1}CRC_{D10A}$) and APOBEC3G ($^{A3G}CRC_{D10A}$) (**Figure 3D**). $^{A1}CRC_{D10A}$ showed the highest activity in terms of survival fraction (**Figure 3D**). However, mutational analysis revealed that APOBEC1 is somewhat promiscuous, introducing two simultaneous mutations at high frequency (**Figure 3C**). This could be potentially deleterious, but because bacterial cells have different mechanisms to cope with DNA insults, $^{A1}CRC_{D10A}$ will be revisited in mammalian system.

The nature of RNA-aptamer recruitment also allows for orthogonal engineering. In gene expression regulation[41] and genomic labeling[71] experiments, it is possible to simultaneously recruit two orthogonal RNA binding proteins by tethering heterologous RNA scaffolds to the gRNA. In this way, more complex interventions could be designed

if heteromeric effectors need to be assembled at the target site, or if additional proteins with desirable features (e.g. DNA protection) are incorporated in the system. Taken together, the data show that RNA-aptamer mediated base editing is a feasible approach, and that CRC's modular configuration allows for flexible engineering.

Materials and Methods

Strains: *E. coli* DH5α competent cells were purchased from Thermo Fisher (Cat. No. 18265017) and were used for general cloning purposes. *E. coli* MG1655 strain, used for rpoB gene targeting, was a kind gift from Dr. Stanley Qi (Stanford University). MG1655 cells were made competent using a standard CaCl2 protocol.

Plasmids: pgRNA-bacteria (pUC19, ampicillin resistant; Addgene plasmid # 44251) was engineered to include two offset BbsI restriction sites for guiding sequence cloning, as well as 1 or 2 MS2 stem loop sequences at the 3' end. These modifications were introduced using standard gene synthesis services (Genewiz). The synthesized cassettes were cloned into pUC19 backbone using SpeI and HindIII restriction sites. The effector modules (AID-linker-MCP) were cloned into a pCDFDuet empty vector (DF13, streptomycin resistant; Addgene plasmid # 49796) using BglII and BamHI restriction sites. dCas9-bacteria plasmid (p15A, chloramphenicol resistant; Addgene # 44249), and pwtCas9-bacteria (p15A; Addgene # 44250) were used to generate $nCas9_{D10A}$ and $nCas9_{H840A}$ nickases by swapping portions of the wild type HNH and RuvC active sites,

respectively, from pwtCas9 to dCas9. HNH domain was cloned using Acc65I and BamHI restriction sites. RuvC domain was cloned using XbaI and NheI restriction sites. Cas9 and effector constructs are under the control of a tetracycline inducible promoter. Plasmid sequences are detailed in **Appendix** (Bacterial Expression Plasmid Sequences section).

gRNA design: rpoB targeting gRNAs were designed manually on SnapGene Viewer (GSL Biotech), on or near the rifampicin resistance determining region (RRDR) of *E. coli*'s rpoB gene.[67] gRNA sequences and PAMs are summarized in **Table 1**. Guiding sequences were designed to have 5' overhangs compatible to the overhangs left by BbsI digestion (i.e. Fwd 5'-CTAGN_{20}-3', Rev 5'-AAACN_{20}-3', where N_{20} is the programmable guiding sequence and must be complementary between Fwd and Rev oligos).

Treatments: Chemically competent *E. coli* MG1655 cells were transformed with 9 ng of a 1:1:1 combination of the appropriate plasmids encoding for specific gRNA (ampicillin), AID_MCP (streptomycin) and Cas9 (chloramphenicol) constructs. After transformation, cells were selected overnight in liquid LB media containing working concentrations of ampicillin, streptomycin and chloramphenicol. The day after, cells are diluted in selective media supplemented with 3 µM tetracycline to induce expression of the protein coding modules. After overnight growth, OD is measured, and serial dilutions are performed to plate 10^8-10^3 cells in rifampicin containing LB agar. Plates are incubated at 37° C and monitored for 48h. Surviving fraction is calculated by counting the surviving colonies divided by the number of cells plated.

Mutational analysis: DNA from 8 to 12 colonies from appropriate experiments was extracted. The target region of the rpoB gene (i.e. RRDR region) was PCR amplified using the following primers: Fwd 5'-TTGGCGAAATGGCGGAAAACC-3' and Rev 5'-CACCGACGGATACCACCTGCTG-3'. PCR products were sequenced using Sanger chemistry at Genewiz.

CHAPTER III

Construction of CRC System for Mammalian Expression

With the advent of gene editing systems, for the first time in history humans are able to rationally modify genomes at will. These developments have become instrumental research tools for biotechnology and biological research in general, and have generated hope for the development of targeted therapies for genetic diseases, cancer, HIV, etc. However, gene editing technologies have important limitations that need to be addressed before moving these technologies to the clinic. First, canonical gene editing systems rely on the generation of double strand breaks, which could potentially have deleterious consequences, especially if unintended off-target activity is high.[72,73] Although the development of strategies such as paired nickases[31], catalytically inactive Cas9 fused to dimeric nucleases[27,33] or high-fidelity CRISPR systems[25,26] are thought to mitigate these adverse effects, limitations in on- and off-target mutagenesis detection methods may underestimate the actual effects of gene editing interventions. Importantly, it was recently shown that CRISPR systems induce previously unnoticed deletions and rearrangements that span several kilobases at on-target sites.[74] Likewise, insertional mutagenesis has been observed in experiments using purified Cas9/sgRNA ribonucleoprotein complexes (RNP), a method thought to enhance targeting specificity.[75] Second, in order to introduce precise modifications, it is necessary that the target cells express homology dependent DNA double strand break repair (HDR).[6,10]

Somatic, terminally differentiated cells, however, do not express HDR and instead express the error prone non-homologous end joining (NHEJ) pathway.[76] These findings highlight the limitations of current gene editing systems to develop therapeutics and the need for novel, more accurate ways to modify genomes avoiding potentially pathogenic effects and introducing the right modifications to the right cells.

As mentioned in Chapter II, we and others have developed innovative ways to edit target nucleotides in the genome, bypassing the introduction of DSB by combining the DNA targeting capabilities of CRISPR and the catalytical activity of non-nuclease DNA editing enzymes (e.g. cytidine deaminases). This strategy has been named base editing.[48] One of the most appealing applications for base editing technologies is the development of novel therapeutic tools to tackle the root cause of human genetic diseases. With the information collected from our prokaryotic experiments we sought to construct a mammalian version of our CRC system. In this Chapter, I describe the construction of the first-generation CRC system for mammalian expression, and its application in correcting a loss-of-function mutation in a reporter system. This represents an exciting proof of concept of a therapeutic application, where the function of an inactive gene product is restored with high efficiency by correcting the deficiency-causing mutation, with undetectable off-target effects at exome-wide level.

Results

CRC system in mammalian cells

We recapitulated the $^{A}CRC_{D10A}$ system (for simplicity hereafter called ^{A}CRCn) for mammalian expression by generating a multicistronic vector expressing $nCas9_{D10A}$ and AID_MCP fusion separated by a self-cleavable 2A peptide, under the control of a UbC promoter (**Figure 4A**, top). In order to increase the targeting space, we included a Cas9_VQR variant ($nCas9_{VQR/D10A}$) that recognizes NGA instead of NGG as PAM sequence used by *S. pyogenes* CRISPR/Cas9 system[77] (**Figure 4A**, top). This construct is noted as ^{A}CRCvn. The gRNA vector used in this Chapter have similar architecture than the ones used in the prokaryotic experiments, with 2 MS2 loops at the 3' end of the gRNA scaffold (2xMS2; **Figure 4A**, bottom). To test CRC's base editing activity in mammalian cells, we designed a GFP reporter that harbors an A→G point mutation along the chromophore sequence (**Figure 4B**). This mutation, Y66C, renders the protein non-fluorescent (nfEGFP), thus mimicking a loss of function (LOF) mutation. We designed three gRNAs targeting the non-template strand (NT) around the mutation region (nfEGFP_NT1, NT2 and $NT3^{VQR}$; **Figure 4B**, **Table 3**).

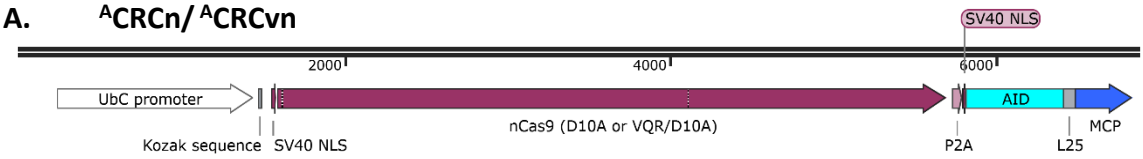
Table 3. nfEGFP targeting gRNA sequences

Name	Target strand	Sequence	PAM
NT1	Non-template	CGCAGGTCAGGGTGGTCACG	AGG
NT2	Non-template	CTGCACGCCGCAGGTCAGGG	TGG
$NT3^{VQR}$	Non-template	GCCGCAGGTCAGGGTGGTCA	CGA

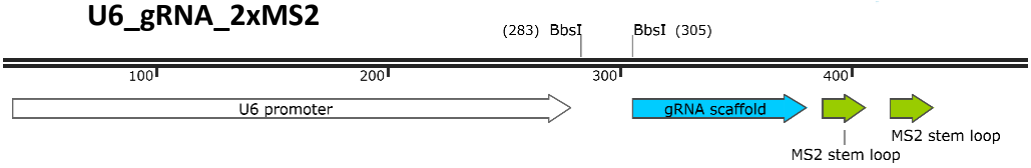
Note: Target C is underlined

First, we sought to correct the LOF mutation in extrachromosomal DNA. To this end, we transiently expressed the target construct in HEK 293 cells together with ^{A}CRCn or ^{A}CRCvn components and the indicated gRNAs (**Figure 4C**). nfEGFP_NT1 treated cells yielded higher GFP conversion than nfEGFP_NT2 treated cells (**Figure 4C**). This result was expected because of the relative position of the target C, which in NT2 lays outside the activity window (position 11) whereas in NT1 the target is more accessible to CRC's effector module (position 3) (**Figure 4B**, **C**). These observations confirmed the notion that the relative position of the target nucleotide within the protospacer sequence is important for efficient conversion, similar to what we observed in our prokaryotic experiments (**Figure 2B**, compare TS3 to TS4). Although ^{A}CRCvn/nfEGFP_NT3^{VQR} treatment showed relatively lower activity than ^{A}CRCn/nfEGFP_NT1 (**Figure 4C**), it demonstrates that CRC base editing activity could be expanded to NGA PAM sequences, thus increasing its targeting space. Finally, for comparison, we tested base editor third generation (BE3)[48] side-by-side with ^{A}CRCn targeted with nfEGFP_NT1. Higher GFP conversion was observed in CRC than in BE3 treated cells. Quantitation by flow cytometry showed 35% GFP positive (GFP+) after ^{A}CRCn/nfEGFP_NT1 treatment, whereas BE3/nfEGFP_NT1 treatment resulted in 19% GFP+ cells (**Figure 4D**). These results confirm that base editing is feasible when treating extrachromosomal DNA in mammalian cells, and that CRC is an efficient alternative to base editors.

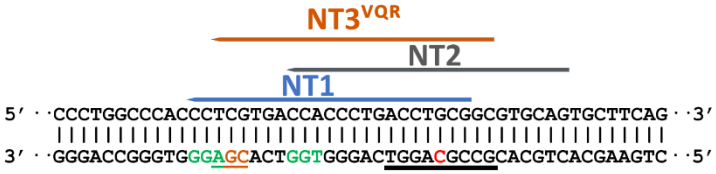
A. ACRCn/ ACRCvn



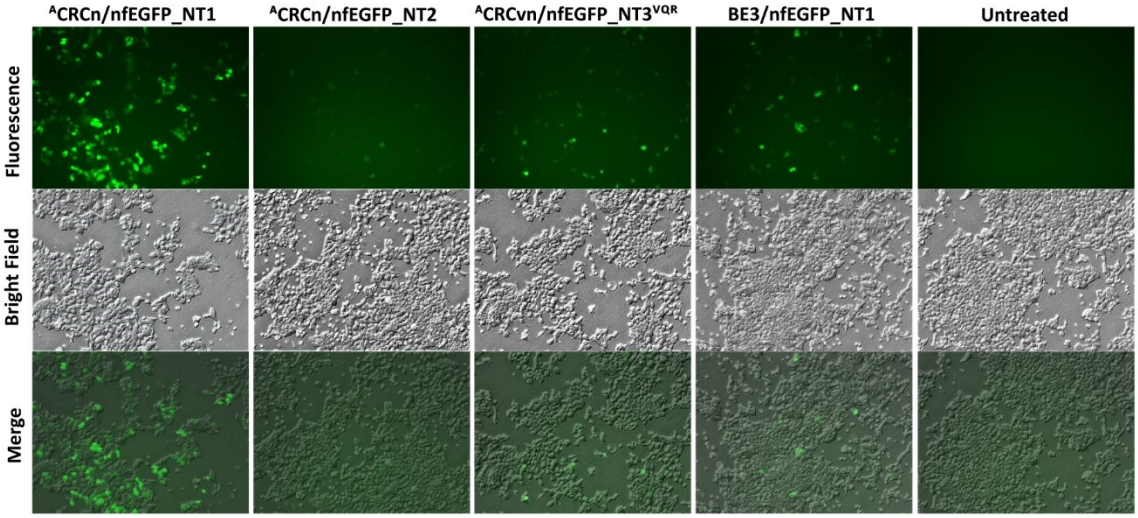
U6_gRNA_2xMS2



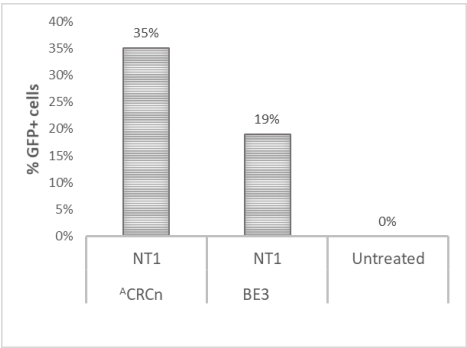
B.



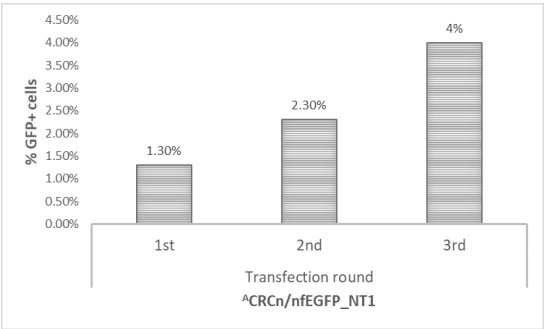
C.



D.



E.



F.

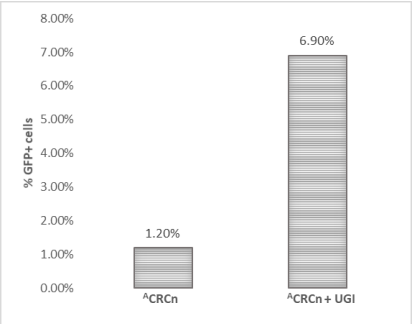


Figure 4. CRC expression and activity in mammalian cells

A. Schematic representation of constructs used in this Chapter. Top: ^{A}CRCn and ^{A}CRCvn are multicistronic constructs expressing AID_L25_MCP fusion protein and nCas9$_{D10A}$ or nCas9$_{VQR/D10A}$, respectively. The two modules are separated by a self-cleavable 2A, and their expression is driven by a UbC promoter. Bottom: gRNA_2xMS2 constructs express from a U6 promoter. Specific guiding sequences are cloned by BbsI digestion **B.** Schematic representation of non-fluorescent EGFP (nfEGFP) target region. A→G LOF mutation occur on the chromophore sequence (underlined in black). Three gRNAs targeting the non-template strand (NT) were designed: NT1, NT2 and NT3^{VQR}. PAM sequences for NT1 and NT2 (NGG) are shown in green, while PAM sequence for NT3^{VQR} (NGA) is orange and green and is underlined with the same colors. **C.** Extrachromosomal treatment. HEK 293 cells were transiently transfected with target DNA containing the nfEGFP mutant together with ^{A}CRCn, ^{A}CRCvu or BE3 components and the indicated gRNAs. **D.** Flow cytometry analysis of cells expressing extrachromosomal nfEGFP gene treated with ^{A}CRCnu and BE3 targeted with nfEGFP_NT1. **E.** nf2.16 cells were subjected to three consecutive treatments with ^{A}CRCn/nfEGFP_NT1, and GFP conversion was measured by flow cytometry. Percentage of GFP positive cells went from 1.3% after a single treatment, to 4% after the third consecutive transfection. **F.** Addition of UGI module to CRC construct enhances GFP conversion efficiency.

CRC Corrects Loss of Function Mutation in Genomic DNA

To examine whether the system can correct loss of function mutations in mammalian genome, we stably integrated the mutant nfEGFP gene into HEK 293 genome (nf2.16 cells). Cells treated with ^{A}CRCn showed 1.3% correction efficiency after a single

transfection (**Figure 4E**). To further enhance base editing efficiency, we hypothesized that chronic treatment would build up the effect. This would mimic chronic treatments in patient's tissues or cells. To this end, nf2.16 cells were subjected to three rounds of treatment with ^{A}CRCn/nfEGFP_NT1. Consecutive treatments resulted in increased GFP conversion, from 1.3% with a single transfection to 4% after three rounds (**Figure 4E**). This approach suggests that if needed, cells can undergo multiple treatment rounds until the desired efficiency is reached.

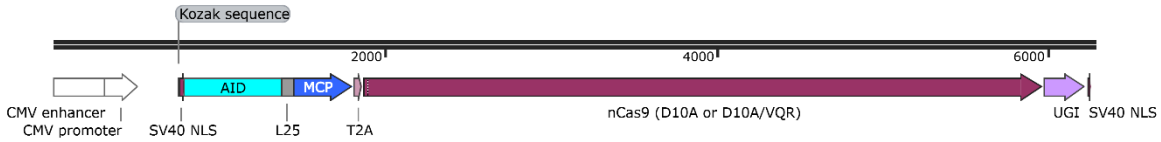
Ideally, however, higher efficiency with a single treatment would be preferable, to avoid accumulation of off-target effects. The observed conversion efficiency could be a balance between target nucleotide deamination and its repair by endogenous DNA repair pathways. In cells, uracil DNA glycosylase (UNG) initiates the repair of U:G mismatches induced by cytidine deamination.[58,78,79] To enhance nucleotide conversion efficiency at the target sites, we fused a bacterial UNG inhibitor peptide (UGI)[80] to Cas9, thus eliciting local UNG inhibition. After a single treatment with this construct guided by nfEGFP_NT1, GFP conversion efficiency reached over 6% (**Figure 4F**). The data show that CRC system is suitable for further engineering and optimization.

We decided to continue the optimization process by restructuring construct architecture and expression. It has been shown that constitutive expression of UbC promoter is inferior to CMV.[81] Therefore, we incorporated the latter into our CRC

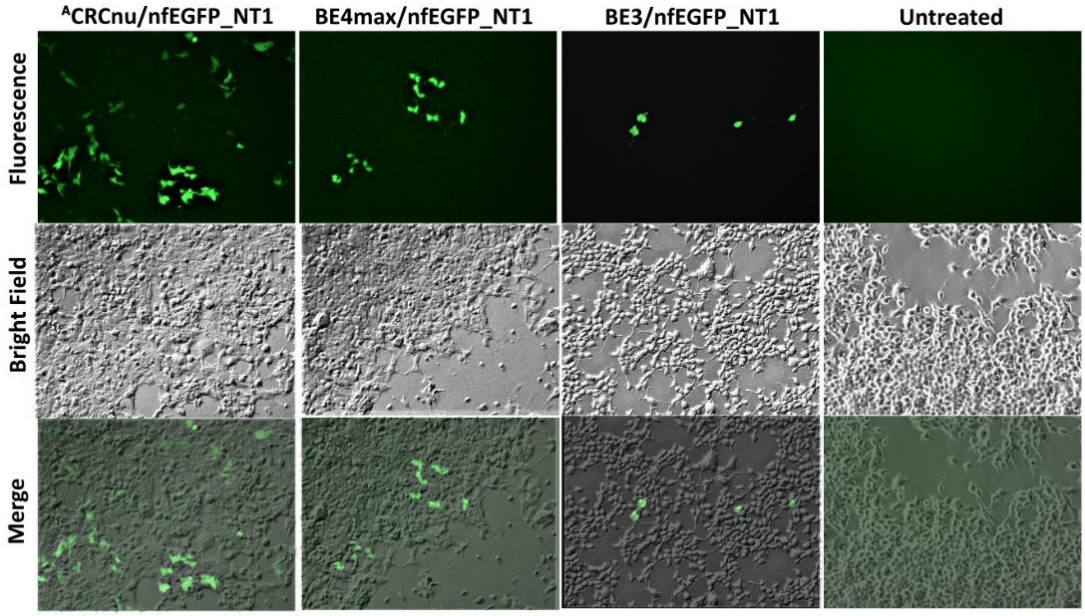
vectors. Next, because Cas9 is a bacterial gene and AID is a human gene, we restructured the multicistronic constructs to enhance expression, cloning AID_MCP fusion upstream of Cas9 (**Figure 5A**), thus potentially improving transcription and translation in HEK 293 cells. The nomenclature for this generation of CRC constructs is: ^{A}CRCnu or ^{A}CRCvnu (A=AID; n=nCas9$_{D10A}$; U=UGI; V=VQR).

To test the latest generation of constructs, cells were treated with ^{A}CRCnu, BE3 and the recently published optimized base editor, BE4max.[62] For this set of experiments, all the base editing systems were targeted with nfEGFP_NT1. Fluorescence was restored at high efficiency after ^{A}CRCnu treatment, with nearly 10% of GFP+ cells after a single transfection (**Figure 5B, C**). As in the extrachromosomal targeting, BE3 treatment resulted in lower GFP conversion, yielding 0.3% of GFP positive cells. BE4max also resulted in modest GFP correction, with 2.7% GFP positive cells (**Figure 5B**, **C**). These results indicate that CRC configuration has greater efficiency than the latest generation of base editors for this target. To visualize the effect of ^{A}CRCnu on genomic nfEGFP gene, we sorted the GFP positive cells by fluorescent activated cell sorting analysis (FACS) followed by sanger sequencing. The results confirmed the G→A conversion at the target position, restoring the wild type sequence (**Figure 5D**). Together, the data demonstrate that CRC mediated base editing is feasible and efficient in mammalian genome, confirming our observations in prokaryotic cells.

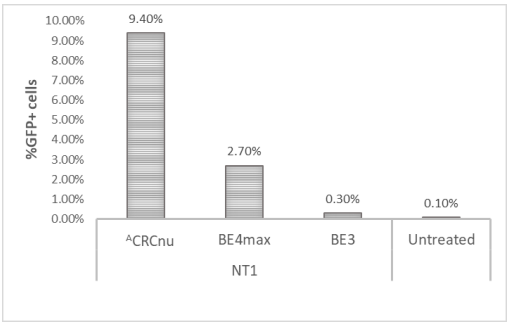
A. **ACRCnu / ACRCvnu**



B.



C.



D.

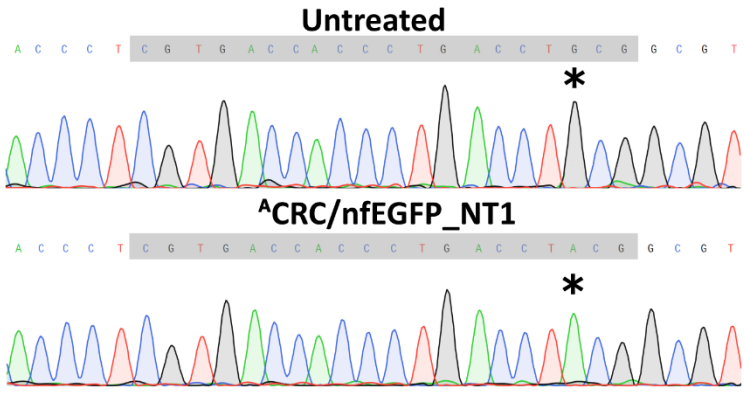


Figure 5. Treating a loss of function mutation in mammalian genome

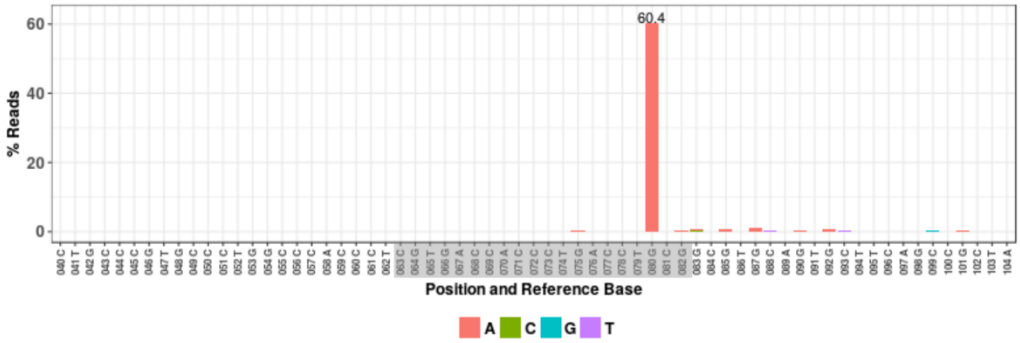
A. Schematic representation of ^{A}CRCnu and ^{A}CRCvnu. **B.** HEK 293 cell stably expressing the non-fluorescent EGFP mutant gene (nf2.16 cells) were treated with ^{A}CRCnu, BE4max and BE3 guided by nfEGFP_NT1 gRNA. **C.** Flow cytometry analysis of cells from **B**. **D.** nf2.16 cell treated with ^{A}CRCnu/nfEGFP_NT1 were subjected to FACS. Sequencing of sorted cells showed specific G→A conversion of the target nucleotide. Highlighted in gray is the targeted region. Black asterisks show the edited position.

Next Generation Sequencing Analysis Reveals High On-Target Base Editing Efficiency and Undetectable Off-Target Effects

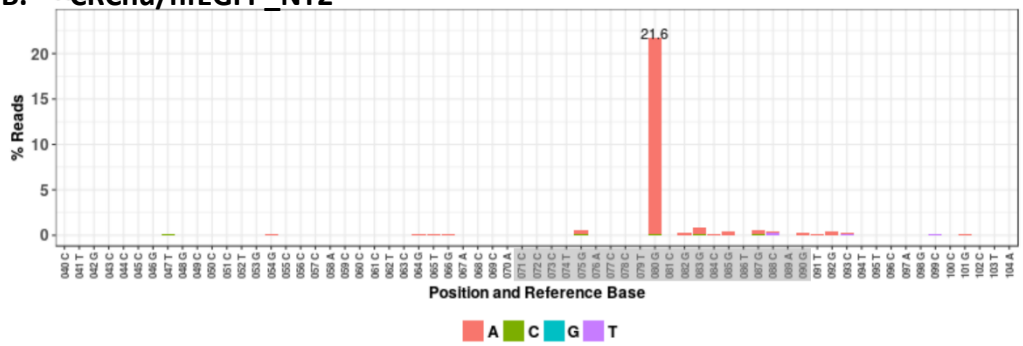
To examine the on–target efficiency of ^{A}CRCnu guided by nfEGFP_NT1, NT2 and NT3^{VQR}, target DNA from treated and untreated cells was amplified by PCR and subjected to high-throughput sequencing analysis. Strikingly, ^{A}CRCnu/nfEGFP_NT1 treatment resulted in over 60% of G→A editing, restoring EGFP wild type sequence, with low bystander effect (**Figure 6A**). Although lower, ^{A}CRCnu/nfEGFP_NT2 and ^{A}CRCvnu/nfEGFP_NT3^{VQR} treatment also resulted in efficient G→A editing, with 21% and 25% conversion efficiency, respectively (**Figure 6B**, **C**). Untreated cells showed background mutations at the target region (**Figure 6G**). In the untreated control,

however, there is a peak at the target nucleotide of approximately 1.4%. This may be due to spontaneous mutation or genetic heterogeneity and could potentially explain the detection of 0.1% of GFP positive cells by flow cytometry (**Figure 5C**, Untreated) that was not observed in the extrachromosomal experiment (**Figure 4D**, Untreated). Contamination was ruled out since no mutation was found in the plasmid encoding nfEGFP, which was amplified and sequenced in parallel with the untreated control (**Figure 6E**). These results confirm that CRC system is efficient, highly specific and suitable for treating loss of function mutations in mammalian genome.

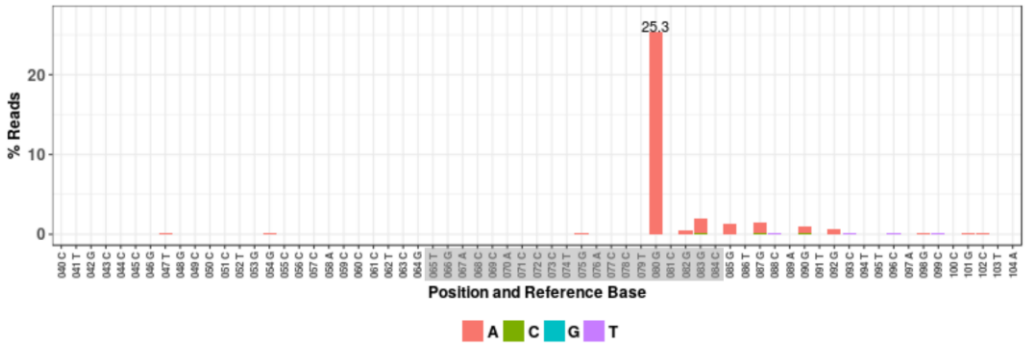
A. ^{A}CRCnu/nfEGFP_NT1



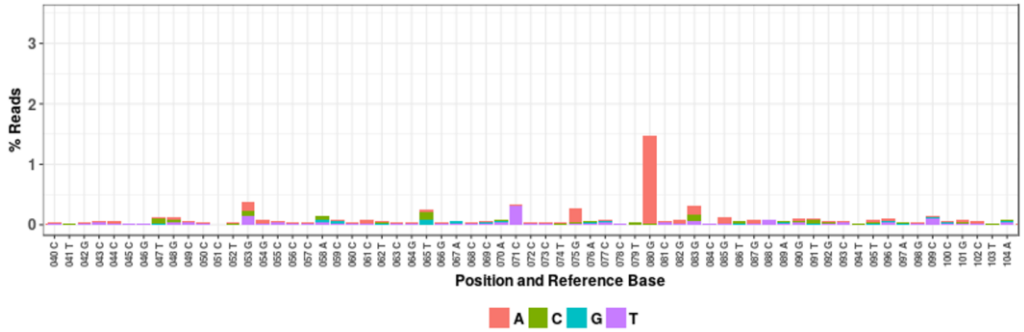
B. ^{A}CRCnu/nfEGFP_NT2



C. ^{A}CRCvnu/nfEGFP_NT3^{VQR}



D. Untreated (genomic)



E. Untreated (plasmid)

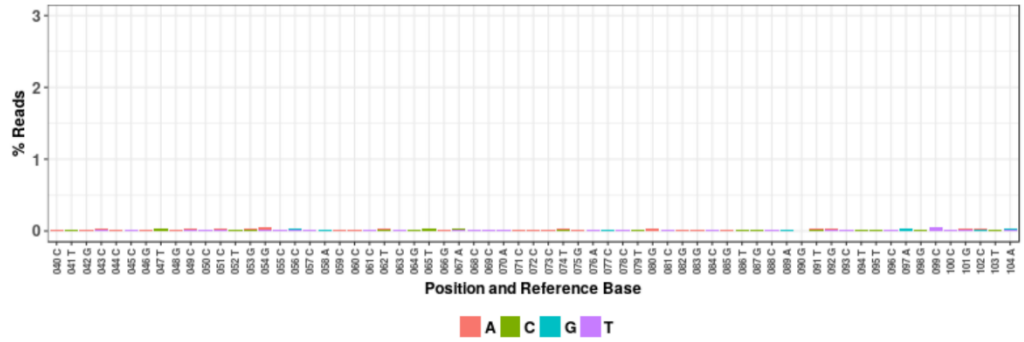
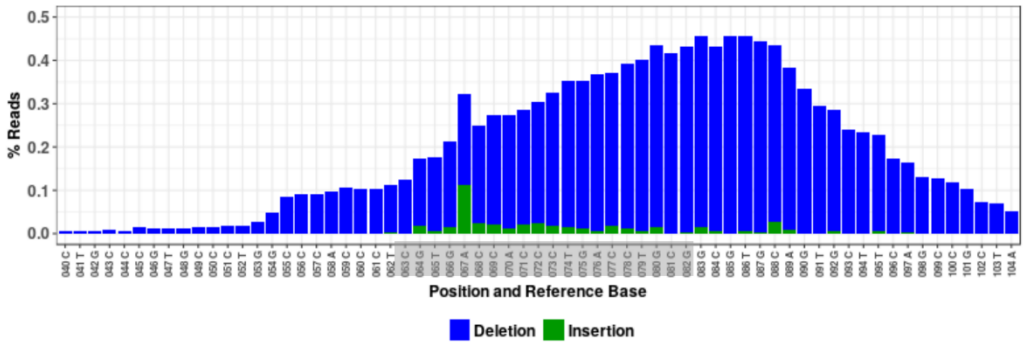


Figure 6. High throughput sequencing analysis of on-target effects. A – D. High-throughput sequencing analysis of target region of stably integrated nfEGFP gene. nf2.16 cells were treated with ^{A}CRCnu/nfEGFP_NT1 (**A**), ^{A}CRCnu/nfEGFP_NT2 (**B**),

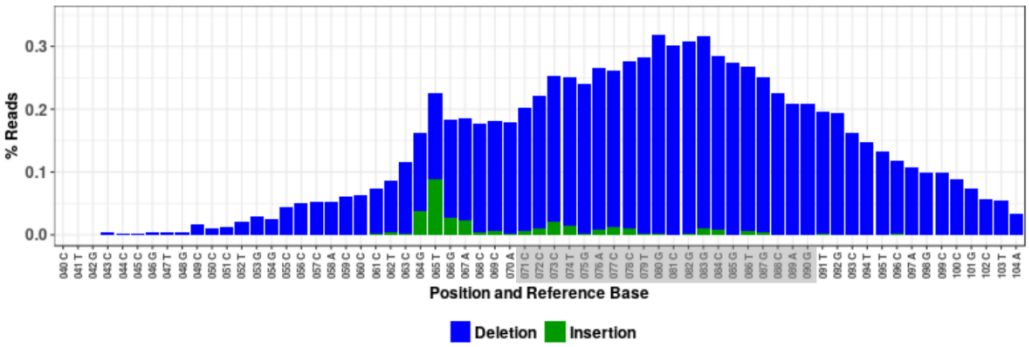
^{A}CRCvnu/nfEGFP_NT3^{VQR} (**C**), or untreated (**D**). In **A – D**, gRNA target sites are highlighted in gray. **E.** Plasmid DNA encoding nfEGFP was amplified and sequenced in parallel to rule out any potential contamination of untreated control (**D**). Numbers below sequences represent the base position within the sequenced amplicon.

Cas9 nickases are largely considered safe because single strand breaks in DNA are well tolerated and efficiently repaired in cells.[35-37] However, it has been shown that nickases can induce mutagenesis[33] and may generate insertion/deletion of bases (indels), though at low rates.[40] Moreover, researchers have found that base editors induce indels at the target site,[48,62] suggesting that these interventions may generate DSBs in a fraction of chromosomes. To determine the extent of indel formation after CRC treatment, we analyzed our data to estimate the frequency of these events in treated and untreated cells. Expectedly, CRC targeting induced indels at low frequencies with all the gRNAs tested, whereas untreated cells showed only background levels of indels (**Figure 7A – D**). Note that the distribution and frequency of indels in treated cells correlates with gRNA target sites. To our knowledge, this is the first report of such effect by Cas9 nickases on target DNA.

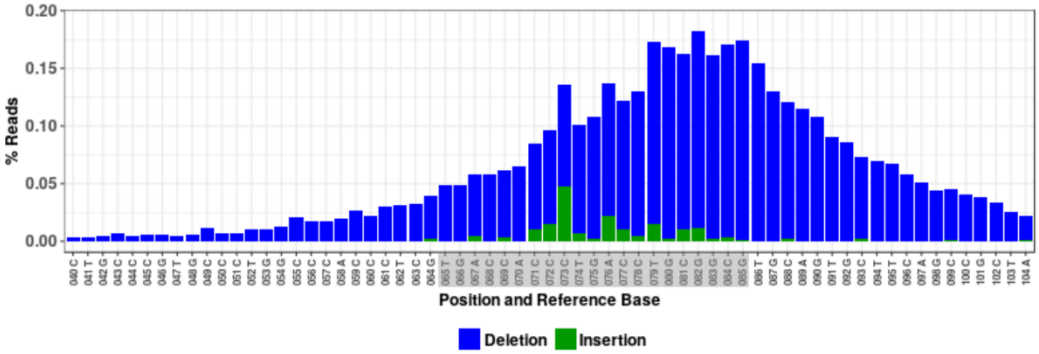
A. ^{A}CRCnu/nfEGFP_NT1



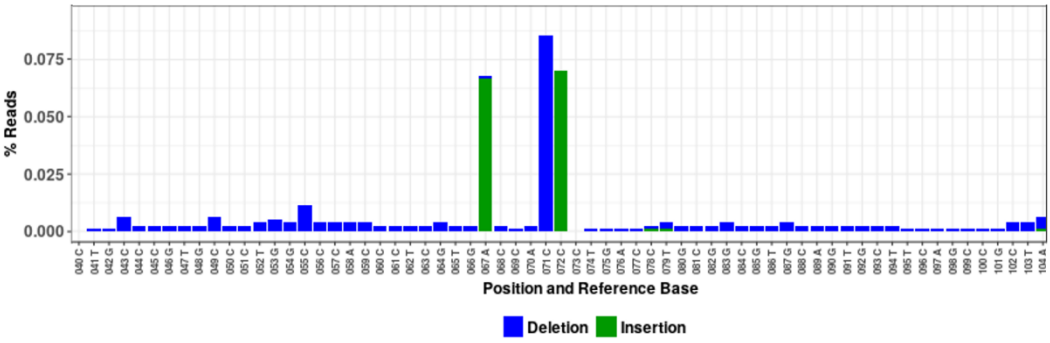
B. ^{A}CRCnu/nfEGFP_NT2



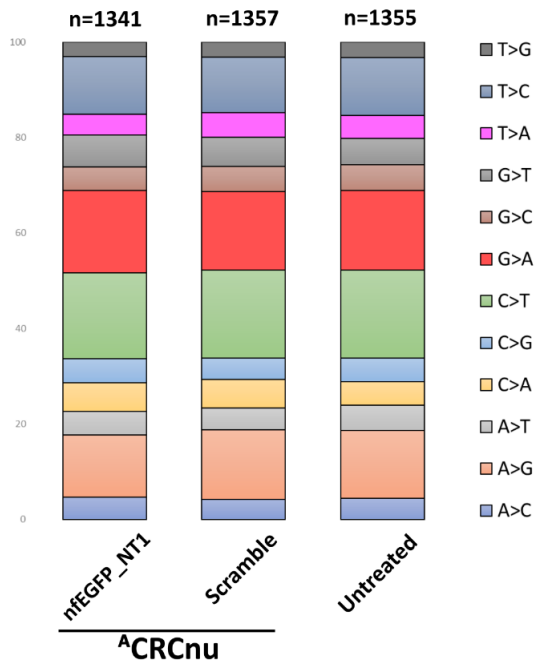
C. ^{A}CRCvnu/nfEGFP_NT3^{VQR}



D. Untreated



E.



F.

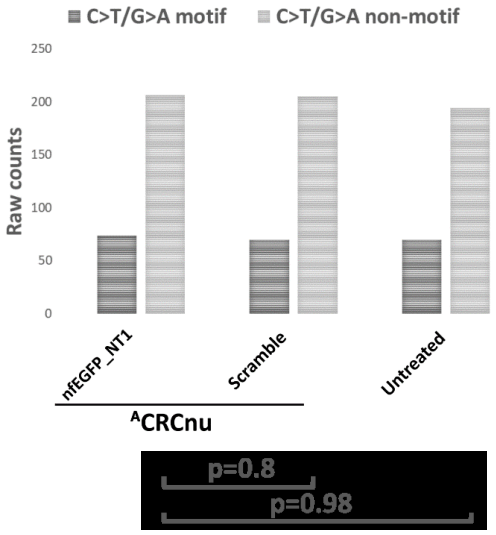


Figure 7. Analysis of on-target mutagenesis and exome-wide off-target effects
A – D. Analysis of indel formation after CRC treatment guided by nfEGFP_NT1 (**A**), nfEGFP_NT2 (**B**), nfEGFP_NT3^{VQR} (**C**) or untreated (**D**). Numbers below sequences represent the base position within the sequenced amplicon. **E.** nf2.16 cells were treated with ^{A}CRCnu/nfEGFP_NT-1, ^{A}CRCnu/Scramble or untreated. Genomic DNA was isolated and subjected to whole exome sequencing. The figure shows the global distribution of single nucleotide polymorphisms of the three samples compared to the human reference genome (hg38), including AID signature mutations C→T/ G→A (green and red,

respectively). The data show no effect of CRC system at exome-wide level. **F.** Using hg38 exome as a reference, occurrences of C>T and G>A events in "AID motif" (WRCH/DGYW, dark gray bars) versus "non-motif" (NNCN/NGNN; light gray bars) were tested. Mutations on CpG sites were not counted to avoid overestimation due to higher mutation rates in these sites. p values were calculated using Chi-square test

To assess potential CRC mediated off-target activity at exome-wide level, nf2.16 cells treated with ^{A}CRCnu/nfEGFP_NT1, ^{A}CRCnu/scramble or untreated cells were subjected to whole exome sequencing. Analysis of point mutations showed no increase in global single nucleotide mutations, including AID signature mutations C→T/ G→A, compared to untreated control, suggesting that AID in the system does not have significant off-target activity (**Figure 7E**). Because AID mutates cytosine residues preferentially within WRCH/DGYW motifs (where the underlined C and G are mutable positions; W: A or T; R: A or G; H: A, C or T; D: A, G or T; Y: C or T),[82] we looked at sites where these residues were mutated with respect to reference human genome, and compared to C or G mutations in non-motifs (NNCN/NGNN) across the different treatments. We found no difference between CRC treated and untreated samples, on both motifs and non-motifs, suggesting that the mutations observed are background mutations and (**Figure 7F).** Taken together, the data show that our GE system represents a promising and safe technology for correcting genetic mutations independent DSB and HDR, with potential therapeutic value in non-dividing somatic cells.

Discussion

Here we have presented a compelling proof of principle showing that CRC is not only active in extrachromosomal and chromosomal DNA in mammalian cells, but more efficient than the third[48] and fourth[62] generation of optimized base editors (**Figure 4 C, D** and **Figure 5 B, C**). The enhanced efficiency of CRC in this specific target compared to BE3 and BE4max could be due to at least three reasons, or a combination thereof. First, CRC uses an RNA-aptamer-based recruitment mechanism, by which the effector fused to a small RNA-aptamer-binding protein is recruited to the target site. Cytidine deaminases are known to form dimers or higher order oligomers to become catalytically active.[64,83-87] The formation of such structures may be hindered when the effector is fused to a bulky protein, such as Cas9 (i.e. in base editors). Therefore, dimer formation could be potentially facilitated in CRC configuration. Second, different cytidine deaminases have certain nucleotide motifs where they deaminate cytosines preferentially. For example, APOBEC1 (effector protein in BE3 and BE4max) deaminates cytosines in a TC context,[48,88] while AID has a more relaxed sequence motif, preferentially deaminating WR<u>C</u>H/D<u>G</u>YW sequences.[82] Third, because of the different effectors and recruitment mechanisms between CRC and BE, the systems may have diverse targeting requirements in terms of the activity window (i.e. the accessible portion of the unpaired strand where effectors can deaminate their target cytosines). Consequently, CRC system increases the base editing tool repertoire, potentially expanding its targets and applications.

In **Chapter II**, we show that CRC's modular design is amenable to optimization by simple changes in vector configuration and treatment modification (**Figure 3A, 3D, 3E**). Here, we decided to take a similar approach to optimize the system for mammalian expression. Functional correction efficiency was dramatically enhanced by using the following strategies: (i) sequential treatment mimicking a multidose therapy (**Figure 4E**); (ii) incorporating a potent UNG inhibitor (UGI) on the Cas9 module (**Figure 4F**); (iii) switching the UbC promoter for CMV and (iv) by restructuring the architecture of the multicistronic construct (**Figure 5B**, **5C**). These simple steps demonstrate the versatility and adaptability of CRC design, and confirm that the system is suitable for further engineering.

We report editing and functional correction of a loss of function mutation in an ectopic EGFP gene, in both extrachromosomal and chromosomal DNA (**Figure 4, 5, 6**). Functional correction of chromosomal GFP reached over 9% efficiency after a single treatment with our improved ^{A}CRCnu guided by nfEGFP_NT1 (**Figure 5B**). Interestingly, we observed that at genomic level, target nucleotide editing was detected in over 60% of reads (**Figure 6A**). These results indicate that CRC is a powerful base editing system with high efficiency in correcting a LOF mutation, highlighting its therapeutic potential.

High base editing efficiency was also observed ^{A}CRCnu/nfEGFP_NT2 (21.6%, **Figure 6B**), although the relative position of the target base is farther from the 5' end compared to

NT1 (**Figure 4B**). We also show that incorporating Cas9 variants with altered PAM recognition requirements[77] (i.e. Cas9_VQR, ^{A}CRCvnu) is feasible and efficient (25%, **Figure 6C**). This is important, because for base editing PAM availability is a major limiting factor since the target base requires to be on the unpaired strand of the CRISPR R-loop, and its position relative to the PAM also determine the editing efficiency. Therefore, adding Cas9 variants that recognize various PAMs could alleviate such limitation, increasing the scope of CRC editing.

Finally, a major concern in gene editing are the unintended effects at on- and off-target sites. It was recently reported that nuclease-dependent CRISPR systems introduce previously unidentified mutagenicity around on-target sites, inducing deletions spanning several kilobases and also triggering complex genomic rearrangements.[74] Moreover, even approaches generally thought to improve editing precision, such as the use of RNPs, have been shown to introduce insertional mutagenesis at on-target sites.[75] Since base editing does not employ nucleases, and therefore it does not directly introduce DSB, this effects should be mitigated. However, indel formation has been observed in base editing experiments,[48] suggesting that these systems introduce unintended DNA breaks. With this in mind, we decided to take a look at indel formation after CRC treatments. As expected, indels were detected in CRC treated cells, although at very low frequencies (**Figure 7A – D**). Additionally, potential off-target effects were analyzed using high-throughput whole exome sequencing. No significant differences were detected in SNPs between treated and untreated cells (**Figure 7E**, **F**), indicating that CRC

has no detectable off-target activity. Taken together, the data show that CRC system can correct a LOF mutation in mammalian genome with high on-target efficiency, and low on- and off-target mutagenic effects, highlighting its therapeutic potential.

Materials and Methods

Plasmids: ^{A}CRCn plasmid was engineered from pLV hUbC-dCas9-T2A-GFP (Addgene plasmid # 53191).[89] P2A-AID_MCP was cloned downstream of Cas9. EGFP was deleted from this construct before performing nfEGFP base editing experiments. nCas9$_{D10A}$ was generated by site directed mutagenesis using 5'-gactacgacgtggatcatatcgtgccccagtc-3' and 5'-gactggggcacgatatgatccacgtcgtagtc-3' as mutagenic primers. To generate ^{A}CRCnu and ^{A}CRCvnu multicistronic constructs, AID_MCP fusion was amplified from ^{A}CRCn and cloned into pCMV_Sports6, followed by nCas9$_{D10A}$ or nCas9$_{VQR/D10A}$ respectively. The two modules are separated by a self-cleavable T2A peptide. To generate gRNA_2xMS2 vector, the gRNA scaffold fused to 2 MS2 loops was synthesized (Genewiz) and cloned into phU6_gRNA (Addgene plasmid # 53188).[89] nfEGFP gene harbors an A→G mutation at nucleotide 200 of the GFP gene. It was synthesized (Genewiz) and cloned into pCMV_Sports6 vector. All plasmid sequences are detailed in **Appendix** (Mammalian Expression Plasmid Sequences section).

gRNA design: nfEGFP targeting gRNAs were designed manually on SnapGene Viewer (GSL Biotech). All gRNAs cover the mutation region (**Figure 4B**) and are listed in **Table 3**.

Cell culture: HEK 293T cells were purchased from ATCC (CRL-3216). Mutant nfEGFP gene was inserted in HEK 293T genome by standard lentiviral transduction and selected by puromycin. Cells expressing mutant nfEGFP were obtained by limiting dilution, generating cell line nf2.16. Cells were grown and maintained at 37° C in Dubelco's Modified Eagle Medium (DMEM, ThermoFisher), supplemented with 10% fetal bovine serum, 1x Glutamine (ThermoFisher) and 1 x Antibiotic-Antimycotic (ThermoFisher).

Transfections: HEK 293T or its derivative nf2.16 cells were plated in 6-well plates the day before experiments. Transfections were performed on cells 75-85% confluent, with a total of 2 µg of combination of DNA from CRC and gRNA constructs in a 3:1 ratio, respectively. Lipofectamine 2000 (ThermoFisher) was used as transfection reagent, following manufacturer's procedure. 72 hours after transfection, fluorescent pictures were taken, GFP signal was quantified by flow cytometry in a Gallios Flow Cytometer instrument (Beckman Coulter) and DNA was purified for downstream analysis using DNeasy Blood and Tissue Kit (Qiagen).

FACS analysis: nf2.16 cells were treated with ^{A}CRCnu/nfEGFP_NT1. 72 hours after transfection, cells were detached from wells and GFP positive cells were sorted on a Beckman Coulter MoFlo XDP Cell Sorter instrument following manufacturer instructions. Sorted cells expressing wild type GFP were cultured, DNA was harvested, and the target region was amplified by PCR followed by Sanger sequencing at Genewiz. Primers used

for PCR were the same as the ones used for high throughput sequencing analysis (see below).

Whole-exome sequencing analysis: WES was carried out by Genewiz (New Jersey, USA). The WES libraries were constructed using Agilent SureSelect Human All Exon (V6 r2) library prep kit and sequenced using Illumina HiSeq with the pair-end 2x150bp format. To estimate potential CRC off-target activity, raw data was analyzed as follows:

- **Variant calling and alternative reference construction**

  WES raw reads were aligned to the human reference genome (hg38) with bwa (version 0.7.15). Variants were identified using Genome Analysis Tool Kit (GATK) version 3.8 roughly following the GATK best practices (REF). Briefly, duplicate reads were first marked with Picard MarkDuplicates. BaseRecalibrator was used to recalibrate base quality, and HaplotypeCaller was then used to call variants on each sample followed by joint genotyping with GenotypeGVCFs. The detected variants in the resulting VCF file were further recalibrated with VariantRecalibrator.

  In the downstream analysis, we only focused on the exonic regions as defined in "SureSelect Human All Exon V6 r2". In the analysis, the overlapping regions were merged using function bedtools merge.

  To construct alternate reference based on the parental cell line T6, we extracted all variants that are genotyped in T6. GATK3.8 FastaAlternateReferenceMaker

was used with default options to construct alternate reference sequence in exonic regions specified in the merged exon-target file.

- **Motif definition and mutation analysis**

AID “WRCH” binding motifs represent a product of ['AT','AG','C','ACT'], and coordinates for any such four consecutive nucleotides were stored. We used python to identify and extract genomic locations of WRCH motifs within reference fasta sequence (either hg38 or alternate reference). Reference fasta sequence was also scanned for sequences complementary to “WRCH”, i.e. “DGYW”, and given by the product of ['AGT','G','CT','AT']. We defined non-WRCH-motif as a four-nucleotide sequence with a C on the third position, which is not WRCH. Similarly, non-DGYW-motif is any four-nucleotide sequence with a G on the second position and not DGYW. In total, there are 12 possible WRCH motifs, 12 DGYW motifs, 52 non-WRCH-motifs, and 52 non-DGYW-motifs. In the mutation analysis we look at WRCH and DGYW categories separately. When looking for potential AID-derived mutated sites a C>T change is categorized as a WRCH motif mutation or a non-WRCH-motif mutation based on its surrounding bases. Similarly, G>A changes are categorized as a DGYW motif mutation or a non-DGYW-motif mutation based on their surrounding bases.

- **Putative CRISPR off-target regions**

Reference genome hg38 was scanned for the putative loci of CRISPR gRNA targeting using CCTop (https://crispr.cos.uni-heidelberg.de/) and CRISPRDesign

(http://crispr.mit.edu/). Together we obtained 54 putative off target regions and variants within these regions were extracted.

High-throughput sequencing analysis: Target region of nfEGFP from treated and untreated samples was amplified using high fidelity Phusion Hot Start Flex DNA polymerase (New England Biolabs) with the following primers: 5'-ggcaagctgaccctgaagttc-3' and 5'-catggcggacttgaagaagtc-3'. PCR products were purified with QIAquick PCR Purification Kit (Qiagen) and submitted to Genewiz for high-throughput sequencing analysis (Aplicon-EZ NGS service). Data analysis, specifically frequency of single nucleotide polymorphisms (SNPs) and insertions/deletions (indels), was performed by Genewiz personnel using a proprietary pipeline.

CHAPTER IV

Targeting Endogenous Loci in the Human Genome

Therapeutic genome editing requires efficient on-target outcomes with reduced off-target effects.[10] Despite the limitations that these technologies currently face for clinical implementation, there are several clinical trials underway, testing safety, efficacy and delivery of genome editing systems in humans.[12,90] Because of its relative ease of use and scalability, CRISPR is poised to dominate the therapeutic landscape, making it the most attractive technology to develop novel applications with therapeutic value. However, most of ongoing clinical trials exploit NHEJ to knock out genes involved in disease by targeted gene deletion.[90] Precise genome modifications, on the other hand, are more difficult to achieve, since NHEJ is the predominant DSB repair pathway, even in dividing cells.[12,91] Moreover, current clinical efforts are aimed to edit cells ex vivo, limiting genome editing interventions to tissues that can be cultured in laboratory settings and amenable of autografts. Therefore, diseases that impact somatic tissues, such as liver, lung, muscle, etc., would not benefit from current genome editing therapeutic applications.

Base editing is a revolutionary genome editing technology that edits single bases without inducing DSB at high rates.[92] These technologies could relieve the limitations that standard CRISPR systems face. . In **Chapters II** and **III**, extensive proof of principle of

the effectiveness and specificity of CRC system was presented. The therapeutic applications of base editing could include correction of disease-causing point mutations, introduction of second-site suppressor mutations, introduction of premature stop codons to knock out genes without breaking the chromosome, deletion of intron-exon junctions for exon skipping approaches. The aim of this Chapter is to explore the capabilities of the CRC system in modifying human genome, including targeting of endogenous genomic sites and an exon skipping proof of principle application, by targeting the human DMD gene, involved in Duchenne muscular dystrophy.

Results

CRC is able to edit target Cs in humane genomic loci

To determine CRC's ability to modify endogenous loci in the human genome, we targeted regions that have been extensively studied for standard nuclease-dependent CRISPR[93,94] as well as base editing applications[48]. These sites and their targeting gRNAs are listed in **Table 4**. As with nfEGFP transgene targeting, CRC treatment resulted in significant C→T conversion, with high purity (i.e. low transversion frequency) and low, but detectable, indel formation (**Figure 8**). These observations further confirm that CRC is a suitable base editing platform, capable of targeting endogenous human genomic sequences.

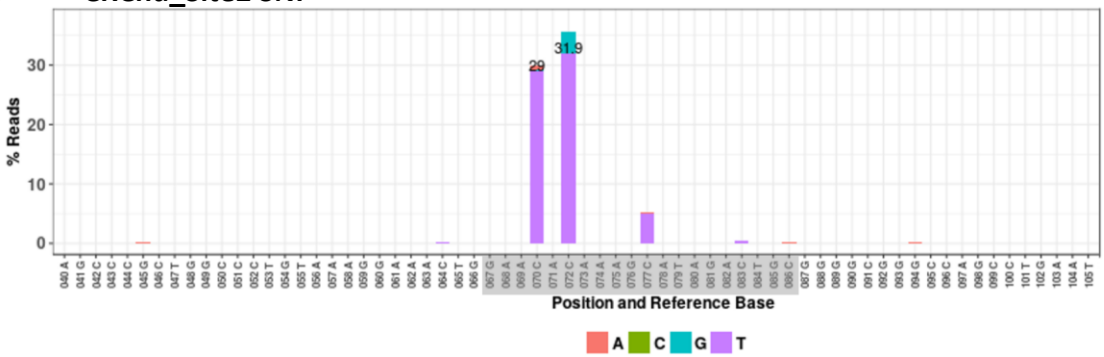
Table 4. Target sequence and genomic locations of endogenous HEK293 loci

Site	Target sequence	PAM	Genomic coordinates (GRCh38/hg38)
2	GAACACAAAGCATAGACTGC	GGG	chr5:87,944,780-87,944,799
3	GGCCCAGACTGAGCACGTGA	TGG	chr9:107,422,339-107,422,358
4	GGCACTGCGGCTGGAGGTGG	GGG	chr20:32,761,950-32,761,969

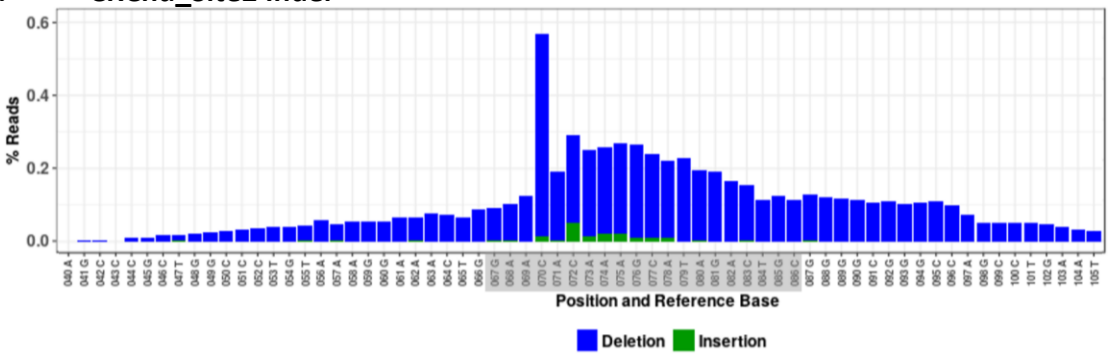
In the case of HEK293 site2 (**Figure 8A**), higher nucleotide conversion was achieved with ^{A}CRCnu treatment compared to the reported BE3 performance (i.e. 29% for C4 and 32% for C6 after CRC treatment, compared to 18% and 15% for the same positions following BE3 treatments).[48] This observation highlights the importance of alternative base editing platforms that may have different targeting performances in specific genomic loci. Similarly, ^{A}CRCnu treatment at site3 and site4 resulted in efficient nucleotide conversion (**Figure 8E, I**). Indel formation followed a similar pattern as observed in nfEGFP targeting (**Figure 7A – E**), which distributions seem to be related to some extent to the gRNA binding sites (**Figure 8B, D, F, H, J**). In Site3 we observed a G→A mutation with high frequency downstream of the target site, however it was also detected in the untreated control (**Figure 8E**, **G**), meaning the this mutation was present in the cell line before the experiments were performed, ruling out any potential unintended bystander effect induced by CRC activity. It is worth noting that, for these targets, CRC seems to have a broader activity window than base editors. In ^{A}CRCnu treatments, inner Cs are being targeted with relatively high efficiency (C11 in Site2, C9 in Site3 and C8 in Site4, **Figure 8A**, **E**, **I**, respectively), whereas BE3 reportedly does not reach these positions.[48] Because base editing is greatly constrained by PAM availability and the relative position of the

target nucleotide within the protospacer, it could be advantageous to have systems with differences in activity window width.

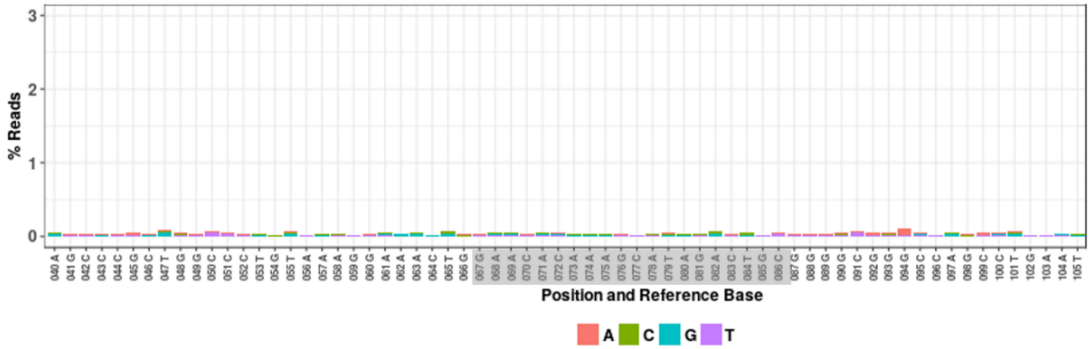
A. **^{A}CRCnu_Site2 SNP**



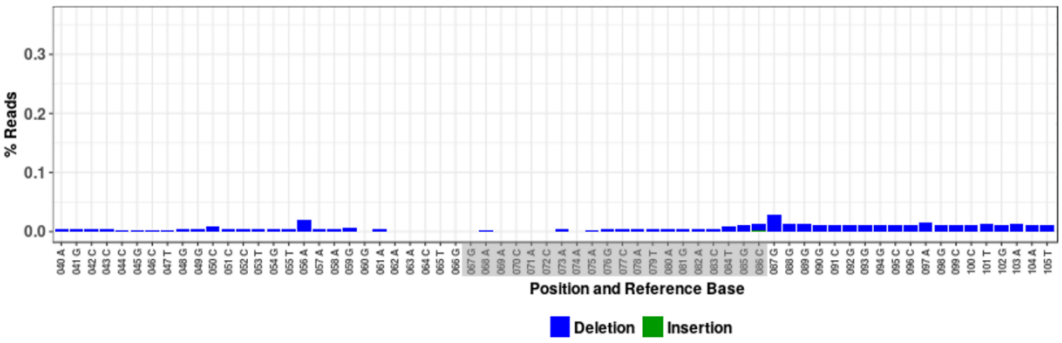
B. **^{A}CRCnu_Site2 Indel**



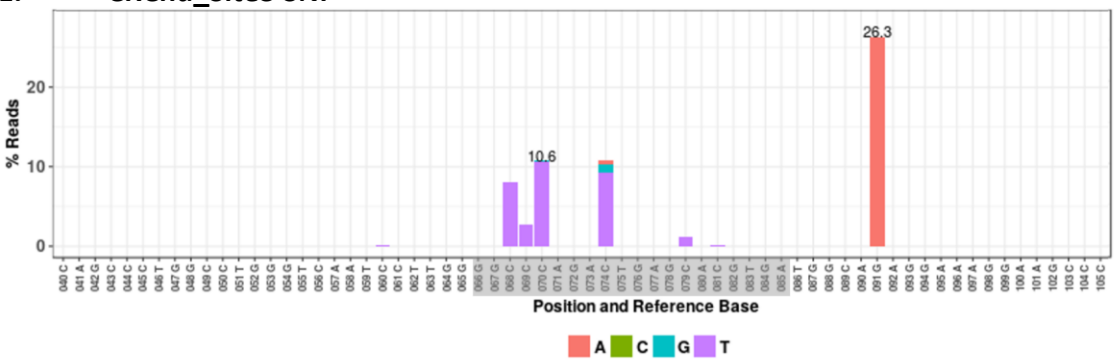
C. **Untreated Site2 SNP**



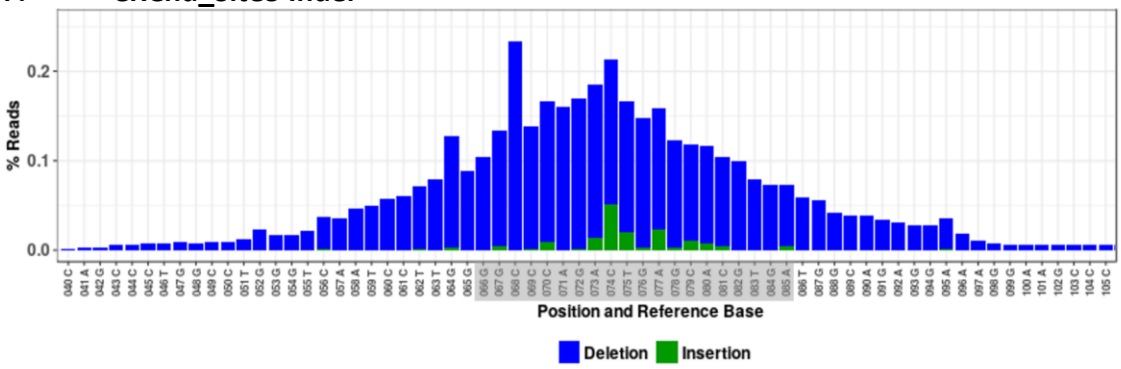
D. Untreated Site2 Indel



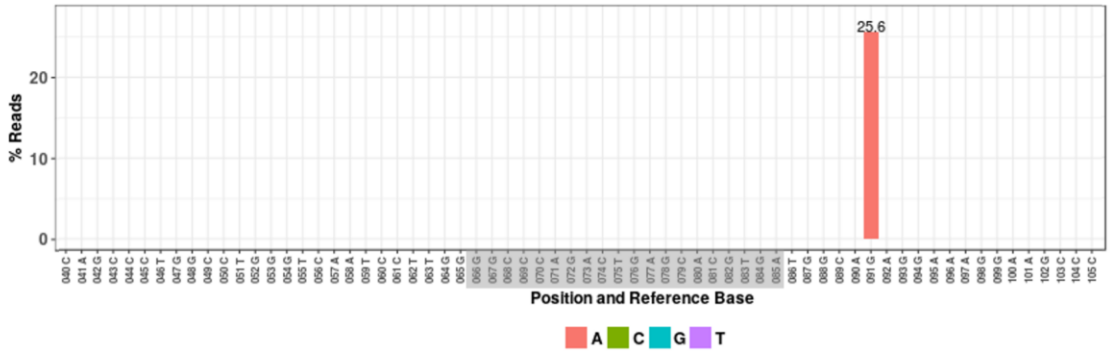
E. ACRCnu_Site3 SNP



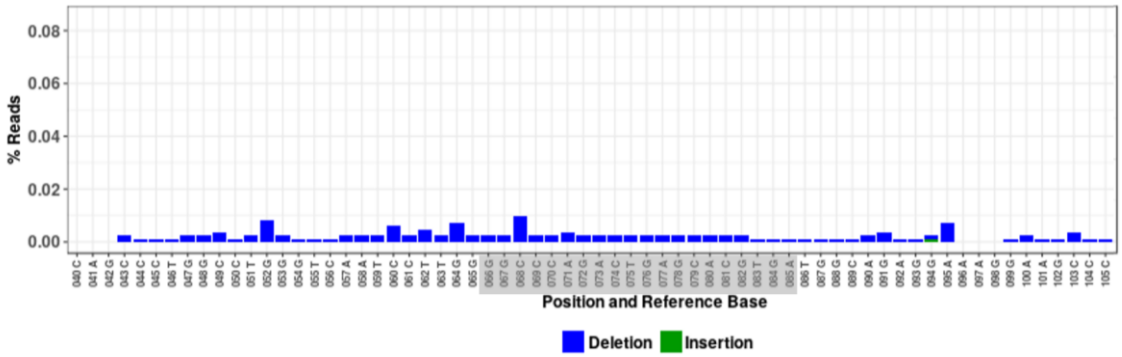
F. ACRCnu_Site3 Indel



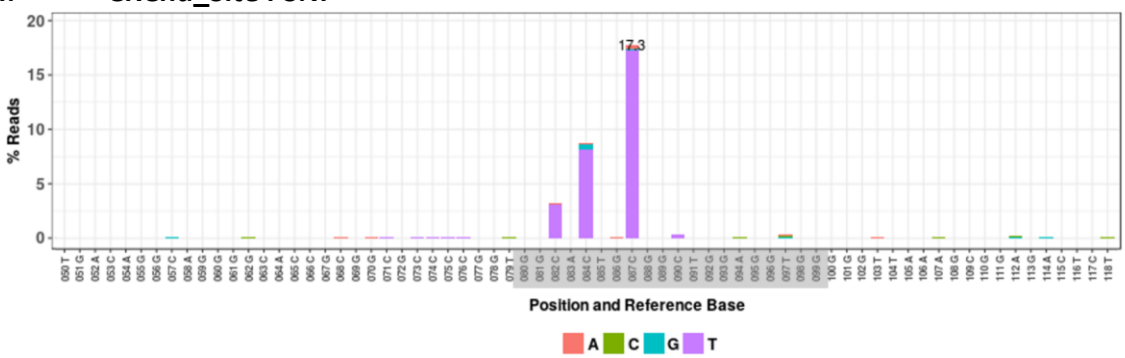
G. Untreated Site3 SNP



H. Untreated Site3 Indel



I. ACRCnu_Site4 SNP



J. ACRCnu_Site4 Indel

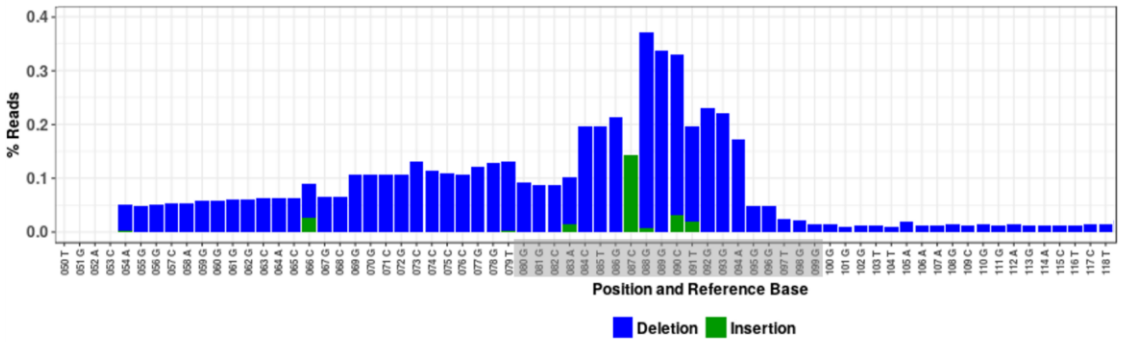


Figure 8. ACRCnu edits endogenous targets in human genome

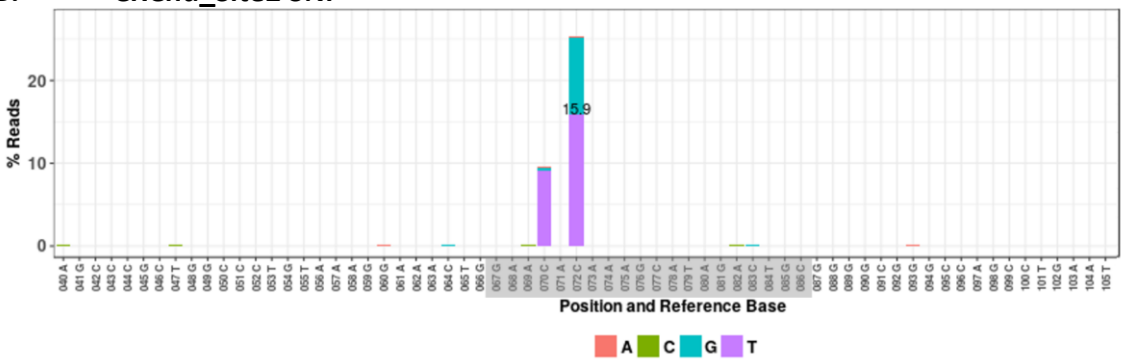
A – J. Base editing outcomes and indel formation after targeting endogenous human loci with ACRCnu and the indicated gRNAs. Numbers below sequences represent the base position within the sequenced amplicon.

APOBEC1 as CRC effector has lower efficiency and purity than AID

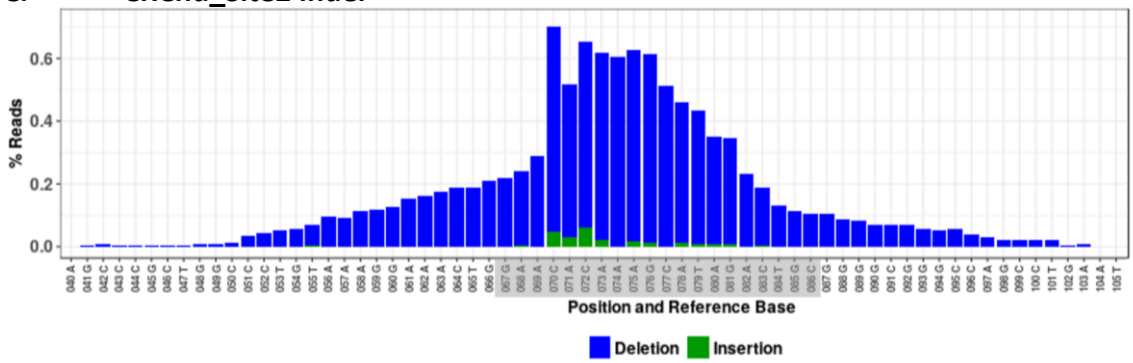
To test if activity window is determined by the effector protein, we decided to construct a CRC system with APOBEC1 as effector (^{A1}CRCnu), following the same general architecture of its AID expressing counterpart (**Figure 9A**). We used the same gRNAs targeting Site2, Site3 and Site4 (**Figure 9B – G**). The results suggest that indeed, ^{A1}CRCnu has a narrower activity widow than ^{A}CRCnu, with internal Cs being edited at much lower frequencies. However, the overall efficiency and base editing purity are lower in ^{A1}CRCnu (**Figure 9B, D, F**). Indel formation was observed in all experiments, suggesting that this phenomenon is not effector specific (**Figure 9C**, **E**, **G**). Because efficiency was lower in APOBEC1 expressing CRC, we decided to continue using ^{A}CRCnu for subsequent experiments.

A.

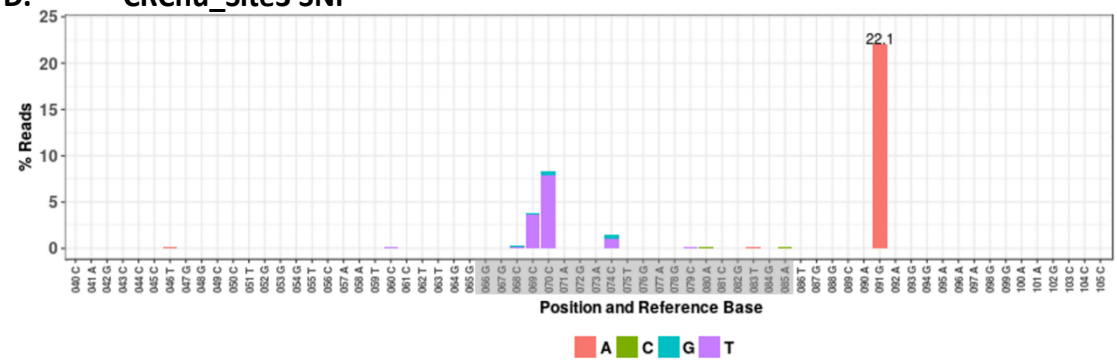
B. **^{A1}CRCnu_Site2 SNP**



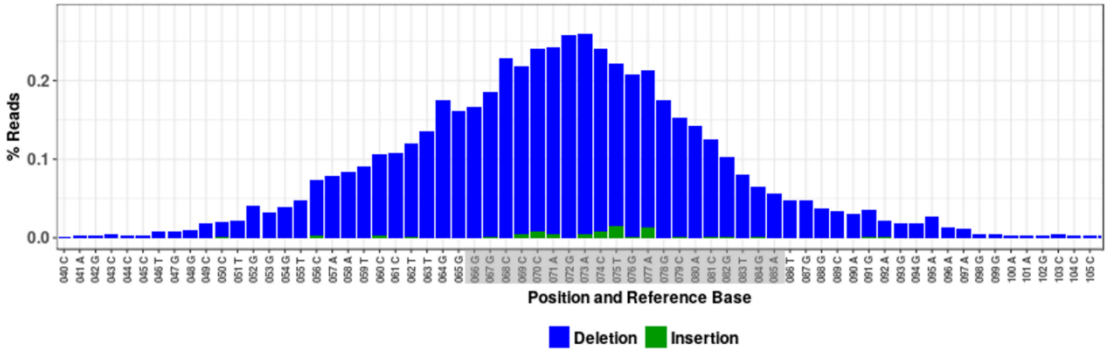
C. **A1CRCnu_Site2 Indel**



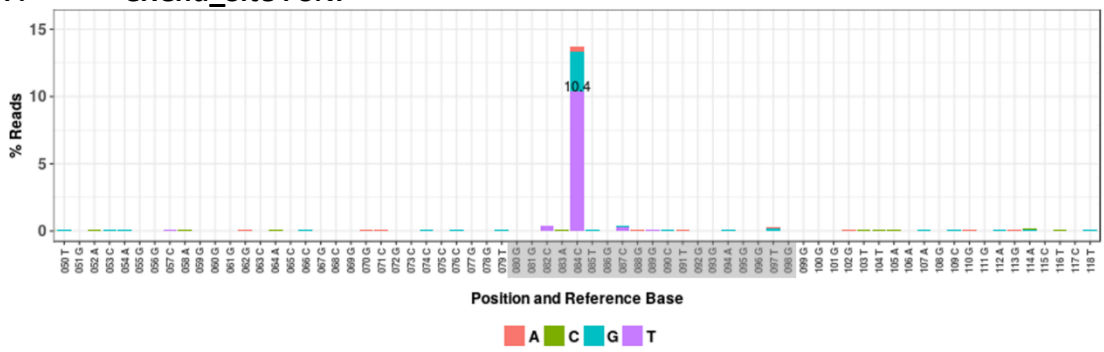
D. **A1CRCnu_Site3 SNP**



E. **A1CRCnu_Site3 Indel**



F. **A1CRCnu_Site4 SNP**



G. ^{A1}CRCnu_Site4 Indel

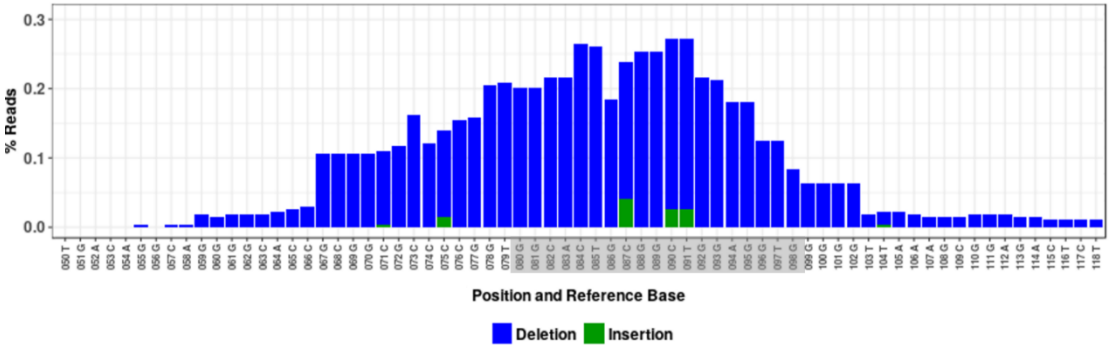


Figure 9. APOBEC1 as CRC effector in the human genome
A – G. Base editing outcomes and indel formation after targeting endogenous human loci with ^{A1}CRCnu and the indicated gRNAs. Numbers below sequences represent the base position within the sequenced amplicon.

CRC induces abrogation of 3' splice acceptor site in intron 50 of human DMD

Duchenne muscular dystrophy (DMD; OMIM 310200) is an X-linked genetic disorder that produces progressive muscular degeneration in affected males.[95] This disease arises from mutations in the *DMD* gene, which encodes for an important muscle cell-specific protein, dystrophin. There is a wide range of mutations involved in DMD, including large deletions of entire exons. For example, 13% of patients present deletions of exon 50, which in turn disrupts the open reading frame (ORF) of the transcript introducing a premature stop codon in exon 51, thereby truncating the protein.[49] Exon 51 skipping restores the ORF and produces a truncated yet functional dystrophin protein, similar to a milder related disease called Becker muscular dystrophy.[50,51] There is an FDA approved antisense oligonucleotide drug based on morpholino chemistry (Eteplirsen, commercially known as Exondys 51), which induces exon 51 skipping, with relatively

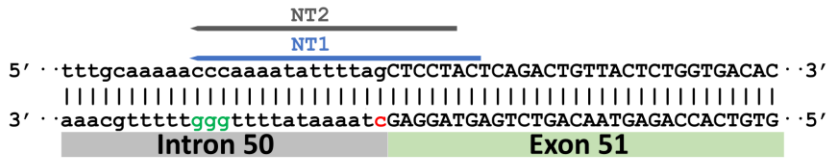
low efficiency (less than 1% improvement in dystrophin expression).[96] Thus, a more efficient and lasting method to induce exon 51 skipping would be beneficial for DMD patients with this particular genetic background. Although nuclease-dependent gene editing has proved effective in achieving this outcome in animal models,[97,98] an approach that avoids introduction of DSB could potentially expedite its clinical application.

As mentioned earlier in this Chapter, a tantalizing application for base editing is to induce therapeutic exon skipping by destroying the 3' splice acceptor site in a target intron – exon junction. The consensus sequence that signal the 3' intron-exon boundary is almost invariably AG in human genes,[99,100] making the G in this motif potentially targetable by CRC, effectively editing the sequence to AA, thereby deleting this regulatory sequence. To test this approach, we designed two gRNAs targeting the intron-exon boundary between intron 50 and exon 51 of the human DMD gene in HEK293 cells (**Figure 10A**). Because there was a single PAM sequence available at the target site, the gRNAs were designed to use the same PAM, varying in length between them (i.e. DMD_NT1 is 19-mer gRNA, DMD_NT2 is 17-mer gRNA; **Figure 10A**). Treatment with both ^{A}CRCnu/DMD_NT1 and NT2 resulted G→A conversion at the target site, with no bystander effect. Although treatment with both gRNAs resulted in similar low efficiencies (around 0.5%; **Figure 10B**, **D**, **F**), this constitutes an exciting proof of concept of the use base editing to treat DMD, considering that the approved antisense oligonucleotide drug has less than 1% of efficiency in restoring dystrophin expression.[96]

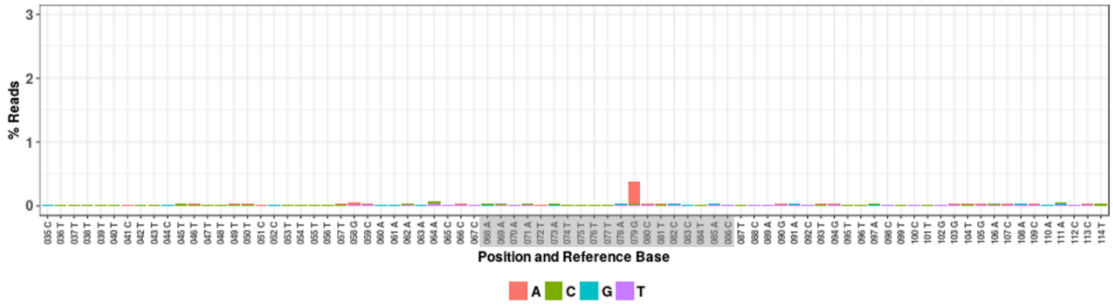
However, higher base editing efficiency is pivotal to develop an effective therapeutic alternative to antisense oligonucleotide-based treatments.

CRC targeting efficiency can be enhanced in multiple ways. For example, chronic treatment could build up the effect, as shown in our GFP reverse mutation assay (**Figure 4E**). Alternatively, by using a novel Cas9 variant with simplified PAM sequence requirement (NG),[101] we could extend the gRNA length to strengthen target binding and to improve the relative position of the target C within the protospacer. Another way to improve targeting efficiency is to optimize the constructs themselves. We have shown that the system is suitable for optimization through vector engineering (**Figure 3, 4F, 5A – B)**. A simple method to enhance construct performance is to optimize the codon usage according to the host cell preferences.[102] Furthermore, codon optimization has been shown to be an effective approach for improving vector expression and editing efficiency in base editing systems.[62,103] This strategy will be discussed in "**Future Directions: CRC Optimization for Improved Expression in Human Cells**" section, in **Chapter V**. Lastly, a possible additional explanation for the observed low conversion efficiency could stem from the cell line used. *DMD* is a muscle-specific gene and these proof of concept experiments were performed in HEK 293 cells, which are kidney-derived, hence these cells do not express *DMD*. Heterochromatin environment present in silenced genes can negatively affect CRISPR targeting,[104-106] therefore a muscle derived cell line with active *DMD* expression, ideally patient-derived, would be a better model for these experiments.

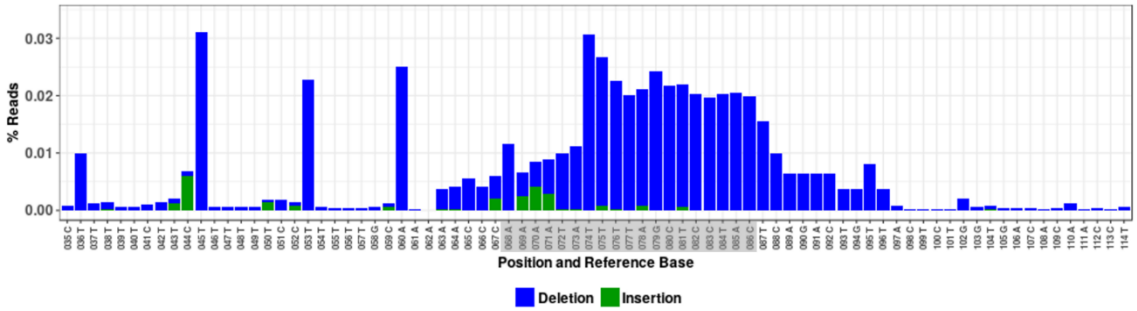
A.



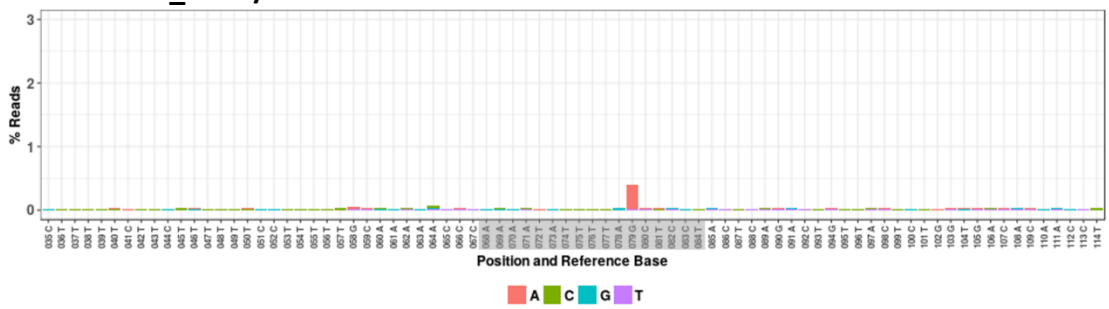
B. **ACRCnu_DMD/NT1 SNP**



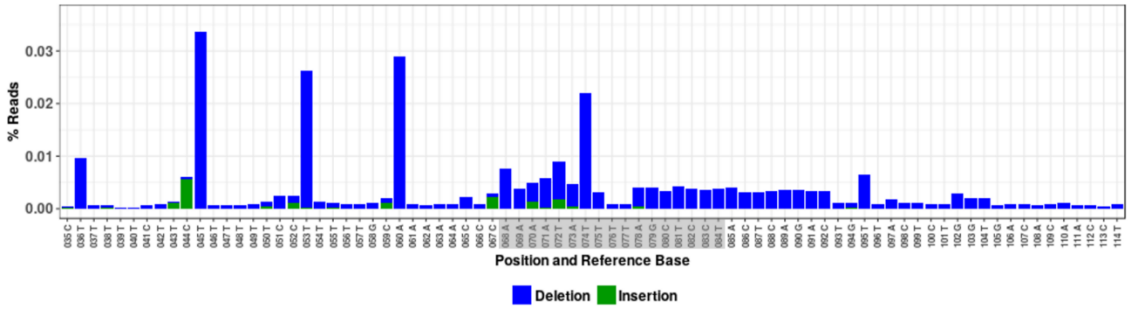
C. **ACRCnu_DMD/NT1 Indel**



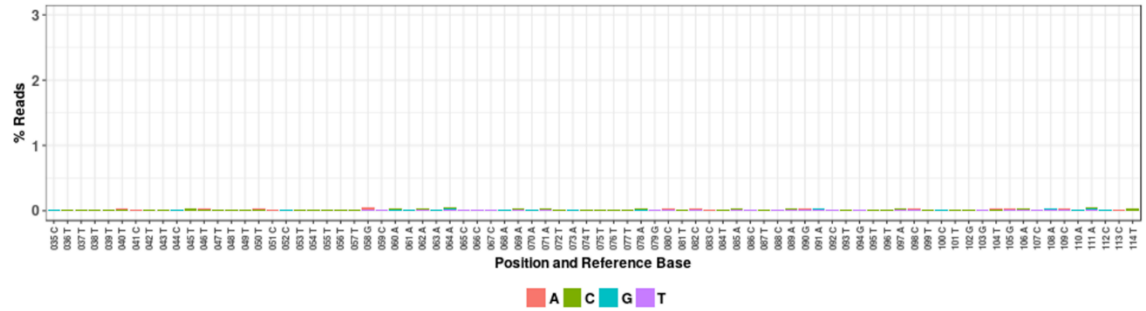
D. **ACRCnu_DMD/NT2 SNP**



E. **^{A}CRCnu_DMD/NT2 Indel**



F. **Untreated DMD In50-Ex51 SNP**



G. **Untreated DMD In50-Ex51 Indel**

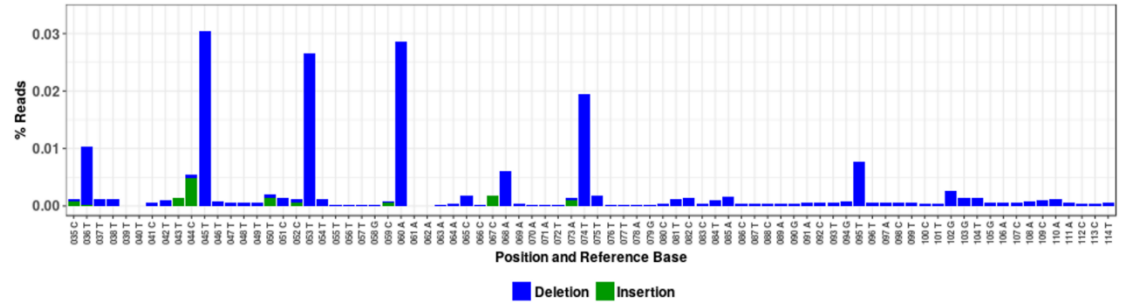


Figure 10. CRC induced abrogation of 3' splice acceptor site in intron 50 of human DMD.

A. Schematic representation of the target site at the intron-exon boundary of intron 50 and exon 51 of the human *DMD* gene. Two gRNAs were designed to target the single PAM motif available at the target site (shown in green). DMD_NT1 and NT2 are 19-mer and 17-mer gRNAs, respectively. In red is shown the target C on the complementary stand of the 3' splice acceptor site (AG). **B – G.** Base editing outcomes and indel formation after targeting endogenous human loci with ^{A}CRCnu and the indicated gRNAs. Note that NT2 treatment (17-mer gRNA) induces lower indel frequency than NT1 (19-mer gRNA), almost at background level (**C**, **E**, **G**), while maintaining the same on-target

base editing efficiency (**B**, **D**, **F**). Numbers below sequences represent the base position within the sequenced amplicon.

Interestingly, the shorter gRNA (DMD_NT2) induced less indels than DMD_NT1 (**Figure 10C**, **E**, **G**). Truncated gRNAs (tru-gRNAs) of 17 nucleotides in length are known to increase specificity by weakening off-target binding while maintaining on-target performance.[30] This could explain the similar on-target efficiency observed with both gRNAs (**Figure 10B**, **D**), while NT2 (a tru-gRNA) reduced indel frequency almost to background levels (**Figure 10C**, **E**, **G**). This suggests that tru-gRNAs could be used to guide base editing platforms in order to reduce unintended on-target mutagenesis by mitigating indel formation, in addition to reducing off-target activity. Note that although indel distribution follows a similar pattern in DMD_NT1 treatment as previously observed, the overall indel frequency, is 10-20-fold lower than those observed in our earlier experiments (compare **Figure 10C**, **E** to **Figure 7A – C** and **Figure 8B**, **F**, **J**). This lower indel formation could be correlated with the possible lack of access to this genomic locus in HEK 293 cells, since these cells do not express *DMD*. Taken together, the data show that therapeutic exon skipping in DMD is a feasible application for CRC-mediated base editing. Nonetheless, the system requires optimization to increase efficiency. Some strategies to improve CRC system will be discussed in **Chapter V**.

Discussion

To test CRC's ability to modify human genomic sites, we targeted three well characterized endogenous loci[93] (**Table 4**). ^{A}CRCnu-mediated base editing was observed in all the targeted sequences tested (**Figure 8**), showing that our base editing system can efficiently modify the human genome. Indel formation was detected around the target sites (**Figure 8B**, **F**, **J**), with a distribution apparently related to gRNA binding, confirming our prior observations in our GFP reporter experiments (**Figure 7 A – D**). Although indel frequencies are relatively low after CRC treatment, and have been observed in other base editing systems,[48] it is necessary to address this phenomenon and find ways to mitigate it. Strategies such as titrating the delivered constructs to find optimal dose, or using truncated gRNAs (**Figure 10E**, see further discussion below) could reduce indels. Additionally, adding DNA binding peptides such as Gam protein has been shown to also reduce base editing-related indels.[60] It would be simple to incorporate such protein into CRC configuration by using heterologous recruiting aptamers (e.g. PP7 or Com stem loops) in the gRNA module and fusing Gam to their respective RNA-binding proteins (e.g. PCP and Com peptides, respectively). In this way, gRNAs fused to heterologous aptamers could simultaneously recruit the effector module as well as Gam fusion protein.

We previously showed that $^{A1}CRC_{D10A}$, where APOBEC1 is the effector protein, has high activity in prokaryotic cells, even inducing double mutations as observed in our

rifampicin negative selection assay (**Figure 3C, D**). Thus, in addition to AID as effector protein in our human experiments, we decided to test the related protein APOBEC1 (^{A1}CRCnu) targeted to the same genomic sites. The result show that ^{A1}CRCnu was able to edit target bases, with a narrower activity widow than ^{A}CRCnu, though the overall base editing efficiency and product purity was lower in ^{A1}CRCnu. Additionally, ^{A1}CRCnu induced similar frequency of indel formation than ^{A}CRCnu, suggesting that this effect is not effector specific, but rather might be the result of the cellular response to the DNA lesions that CRC introduce. ^{A1}CRCnu will be revisited for the second-generation CRC constructs, described in "**Future Directions: CRC Optimization for Improved Expression in Human Cells**" section in **Chapter V**.

To continue testing CRC's editing capabilities in a clinically relevant target, we aimed the system to the human *DMD* gene. Mutations in this gene are involved in the development of the most common deadly inherited disorder, Duchenne muscular dystrophy (DMD).[51] Around 13% of patients suffering from this disease have large deletions in the *DMD* gene that disrupt the ORF and truncate the protein by introducing a premature stop codon in exon 51.[49] The only approved drug designed to restore the reading frame in this genetic background, the antisense oligonucleotide Eteplirsen, induces skipping of exon 51 generating a truncated though functional dystrophin protein,[50,51] with less than 1% of efficiency.[96] One of the downsides of this strategy is that it targets the *DMD* transcript and not the gene, meaning that the effect is transient and patients need to take this drug for life. Thus, a treatment with a more lasting

therapeutic effect could dramatically improve the lives of these patients. Motivated by this, we targeted ^{A}CRCnu to the intron 50 – exon 51 boundary using two gRNAs varying in length (i.e. 19-mer and 17-mer guides) to eliminate the AG consensus motif at the 3' splice acceptor site (**Figure 10A**). Both gRNAs used in this experiment resulted in low yet detectable target editing, with approximately 0.5% of the reads showing AG→AA editing outcome. It is important to note that these experiments were performed in HEK 293 cells, which do not express *DMD*, therefore the chromatin environment around the target site may interfere with targeting efficiency. Efforts to increase the efficiency in this target are already underway, and include construct expression optimization, use of patient-derived muscle cells and non-integrating viral delivery (see **Future Directions: CRC Optimization for Improved Expression in Human Cells** in the next Chapter).

An interesting finding in our *DMD* targeting experiments is the lower indel formation observed with the 17-mer gRNA (DMD_NT2) compared to the 19-mer (DMD_NT1) gRNA (**Figure 10C**, **E**). Truncated gRNAs, also known as Tru-gRNAs, which contain targeting sequences with shortened 5' ends (as short as 17 nucleotides in length), have been shown to improve CRISPR specificity. The shorter binding interface is thought to reduce the energy of gRNA-DNA base pairing, making the gRNA/Cas9 complex more sensitive to mismatches, thus reducing off-target activity. This reduced binding energy at the target site could potentially be behind the lower indel frequency observed in ^{A}CRCnu/DMD_NT2 treatment, however more research in needed to confirm and fully characterize these observations.

One question that remains to be addressed is the extent of CRC-induced off-target effects. In our nfEGFP reverse mutation experiments in **Chapter III**, we showed that CRC treatment does not generate detectable off-target effects at exome-wide level (**Figure 7E**, **F**). However, a more thorough analysis should be performed. To this end, known off-target sites of HEK293 sites 2, 3 and 4 will be analyzed by deep sequencing in CRC treated and untreated samples. Likewise, potential off-target sites for the *DMD* targeting gRNAs have been identified using the CRISPR target prediction tool CCTop,[107] and these sites will also be subjected to high throughput sequencing analysis to assess off-target activity.

Together, the data presented in this Chapter clearly show that CRC is a versatile base editing system capable of modifying endogenous human loci. However, higher efficiency and reduced on-target mutagenicity need to be addressed. This is particularly important to develop therapeutic applications. To boost efficiency, several efforts are being currently taken, such as improving vector expression by codon optimization. More research is needed to better understand the mechanisms involved in editing, the cellular response to CRC treatment, the influence of chromatin environment in base editing outcomes, etc.

Materials and Methods

All methods used in this Chapter are described in the **Material and Methods** section of **Chapter III**.

High throughput sequencing: High throughput sequencing analysis was performed as described in **Chapter III**. To generate the amplicons, the primers described below were used. HEK 293 site2 was amplified with 5'- CCTGGCTGAGCTAACTGTGACAG-3' and 5'-GTCAAACTGTGCGTATGACATCATCAG-3'. HEK 293 site3 was amplified with 5'-GCATGCATTTGTAGGCTTGATGC-3' and 5'-GCCCCTGTCTAGGAAAAGCTGTC-3'. HEK 293 site4 was amplified with 5'-CTGGGTGGAAGGAAGGGAGGAAG-3' and 5'-TCAACCCGAACGGAGACACACAC-3'. Human *DMD* intron 50 – exon 51 boundary was amplified with 5'-GAAATTGGCTCTTTAGCTTGTGTTTC and 5'-GCATTTCTAGTTTGGAGATGGCAG-3'. All PCR amplification were performed with high fidelity Phusion Hot Start DNA Polymerase (New England Biolabs), as per manufacturer's instructions.

CHAPTER V

Final Discussion and Future Directions

Here we present a modular base editing system built by a combination of an engineered *S. pyogenes* CRISPR system and RNA-aptamer based recruitment of non-nuclease DNA editing enzymes, such as cytidine deaminases, expanding the scope of base editing systems. Our platform is called CRC (CRISPR/RNA-Aptamer-Mediated Correction and Modification System; **Figure 1A**). We systematically tested CRC's editing capabilities, starting with a bacterial model. Here we aimed the system to modify the bacterial *rpoB* gene, which encodes the β subunit of RNA polymerase. Mutants of this gene, particularly in a region called RRDR (rifampicin resistance determining region), can survive in the presence of rifampicin, a potent antibiotic that binds and inhibits the bacterial RNA polymerase protein, shutting down global transcription.[67] Thus, it is possible to estimate base editing efficiency by calculating the survival fraction after treatment.

By the time this project was conceived, nothing was known about base editing or its underlying mechanisms, consequently we designed our gRNAs manually, using the available PAM sequences in RRDR near critical residues involved in rifampicin resistance that could be edited by cytidine deamination (**Figure 2A**). Efficient base editing was achieved when the gRNA was targeted to the complementary strand of the target

cytosine, with higher efficiency if the latter is positioned closer to the 5' end of the protospacer (i.e. in the PAM distal region of the unpaired strand left by the CRISPR R-loop; compare rpoB_TS3 and TS4 in **Figure 2B**, **C**). Importantly, efficiency can be enhanced by simple engineering processes, such as using Cas9 nickase instead of catalytically dead Cas9, increasing the number of recruiting stem loops, using alternative effector protein or introducing double nicks in cis for difficult targets (**Figure 3**). The data show not only that CRC-mediated base editing is feasible in the prokaryotic genome, but importantly that the system is suitable for enhancement to improve base editing outcomes.

With the knowledge gathered in our bacterial experiments, we reconstructed the system for eukaryotic expression. First, as a proof of principle, we designed a GFP-based reverse mutation assay. GFP fluorescence was abolished by introducing a point A→G point mutation destroying the chromophore sequence (generating a non-fluorescent GFP, nfEGFP), mimicking a loss-of-function mutation. Various gRNAs covering the target region were designed to restore the wild-type sequence, thus functionally recovering GFP fluorescence (**Figure 4A**). Extrachromosomal and chromosomal targeting resulted significant GFP signal detection, with a functional correction of 35% and 9.4%, respectively (**Figure 4C**, **D** and **Figure 5B**, **C**). Interestingly, CRC treatment resulted in higher efficiency than third-[48] and optimized fourth-generation[62] base editors (0.3% and 2.7% GFP positive cells for BE3 and BE4max, respectively; **Figure 4C**, **D** and **Figure 5B**, **C**). Besides regular *S. pyogenes* Cas9, we also tested a Cas9^{VQR} variant in CRC configuration

(^{A}CRCvnu), which recognizes NGA instead of the standard NGG PAM sequence.[77] Although functional correction of GFP after ^{A}CRCvnu was relatively lower than ^{A}CRCnu (**Figure 4B**), these results show that the targeting space can be expanded by incorporating novel Cas9 variants into the CRC toolbox. On-target effect analysis by high-throughput sequencing revealed highly efficient editing of the target nucleotide, up to 60% with ^{A}CRCnu/nfEGFP_NT1 and 25% with ^{A}CRCvnu/nfEGFP_NT3^{VQR} treatments (**Figure 6**), while no off-target effects were detected at exome-wide range (**Figure 7E**, **F**), suggesting that CRC is a safe and efficient base editing system. Together, these results show strong evidence that CRC can perform base editing tasks efficiently in human cells, expanding the available gene editing tools.

Encouraged by the results in our GFP reporter experiments, we probed endogenous sites in the human genome. We show that both ^{A}CRCnu and ^{A1}CRCnu edit these sites, though they show differences in efficiency, activity window width and product purity. ^{A}CRCnu had higher base editing efficiency and better editing purity (i.e. most sequencing reads showed C→T conversion, while a fraction of them were transversions) than ^{A1}CRCnu (**Figure 8** and **Figure 9**, respectively). However, the base editing window appears to be wider in ^{A}CRCnu treated cells than in ^{A1}CRCnu. This observation suggests that the activity window might be determined by the specific effector protein, rather than system architecture, since the activity window of ^{A1}CRCnu is similar to reports in BE3 and BE4max (where effector protein is also APOBEC1).[48,62] Exploring more effectors

and incorporating them in CRC could further improve and expand the base editing scope.

Recently, reports showed that Cas9-mediated DSB induced previously unnoticed on-target mutagenicity that could be potentially deleterious.[74,75] Base editing is designed to avoid DNA breaks altogether. However, we and others[48] have found that base editing interventions still generate indels at low frequencies in human DNA, suggesting that DNA breaks still occur, probably as a result of cellular response to the DNA damage induced by these systems rather than by their intrinsic gene editing mechanism. Interestingly, the distribution and frequency of indels in CRC treated DNA seem to relate to gRNA binding site, with the highest frequency toward the center in most cases (**Figure 7A – D**; **Figure 8B**, **F**, **J**; **Figure 9C**, **E**, **G**). As we note in our *DMD* targeting experiments, using shorter gRNAs could be an appealing strategy to reduce indels at on-target sites (**Figure 10**). Additionally, incorporating the bacteriophage double strand DNA binding protein Gam to base editing systems mitigates indel formation.[60] Therefore, it would be worth exploring other proteins and tactics that could potentially protect the DNA from breaking after base editing treatments to improve the safety profile of these systems.

To continue testing CRC base editing performance, we decided to aim the system to a clinically relevant target: *DMD*, a gene involved in Duchenne muscular dystrophy (DMD), the most common fatal genetic disease.[51] We show that CRC is able to delete the 3'

splice acceptor site of intron 50, which would induce exon 51 skipping. This approach is relevant for 13% of patients who harbor deletions that introduce a premature stop codon in exon 51. Antisense oligonucleotide therapy has been approved to treat DMD patients by inducing exon skipping, however the effect is modest, with less than 1% of dystrophin recovery.[96] Although the base editing outcome after CRC treatment in HEK 293 cells resulted in merely 0.5% G→A conversion at the 3' splice AG motif, this is exciting evidence showing that base editing could be used to induce therapeutic exon skipping in DMD patients, with longer lasting effects than antisense oligonucleotide therapeutics. The low efficiency observed in these is most likely due to target accessibility in HEK 293 cells, since chromatin environment in silenced genes can prevent CRISPR targeting, probably by physically impeding access to target DNA.[104-106] To confirm these results in a more relevant model, future experiments will be performed in patient-derived immortalized myofibroblast cells. In addition, to determine the universality of CRC-based exon skipping treatment, other in-frame exons (i.e. exons multiples of 3 to avoid nonsense mediated mRNA decay) in highly expressed genes should be targeted.

A key feature of CRC is its modularity. Both in bacteria and human experiments we have shown that the system can be revamped by engineering individual modules. Strategies like increasing the number of recruiting RNA-aptamers; incorporating different Cas9 variants for enhanced efficiency, improved specificity or different PAM recognition; testing several effector proteins; vector expression optimization; construct delivery;

etc., can improve CRC's efficiency and increase its targeting scope. This highlights the need for continuing the engineering efforts to tailor the system to specific targets, a desirable attribute for genome editing systems aimed toward personalized medicine applications. Some of these strategies are currently in progress and are described in the following section.

Future Directions: CRC Optimization for Improved Expression in Human Cells

Expression optimization through optimal codon usage has been shown to improve construct expression and base editing outcomes.[62,103] To generate the second-generation CRC system (CRCnu.2), we designed several new constructs, all of them codon optimized for human expression, using GeneScript and Genewiz online codon optimization tools for the effector module, while Cas9_UGI module was derived from the already optimized BE4max construct.[62] The main difference between the novel Cas9_UGI module and the one present in CRCnu constructs, besides codon optimization, is that the former contains two UGI genes and two nuclear localization sequences (NLS), whereas the latter has only one of each. CRCnu.2 constructs are summarized in **Figure 11**.

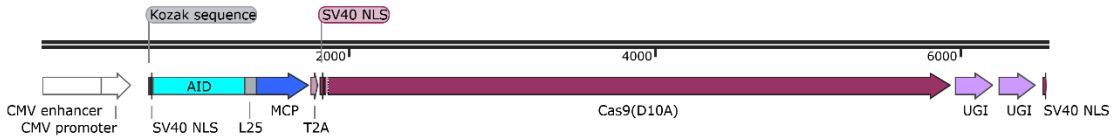
We first recapitulated a basic ^{A}CRCnu construct, generating ^{A}CRCnu.2 (**Figure 11A**). Next, to enhance nuclear presence of the AID_MCP effector module, we incorporated an

additional NLS downstream of MCP (^{2NA}CRCnu.2, **Figure 11B**). AID has an intrinsic nuclear export signal (NES) that could limit its dwelling time in the nucleus[108,109], thus an alternative strategy to accumulate AID_MCP in the nucleus is to delete the C-terminal NES from AID ($^{N\Delta A}$CRCnu.2, **Figure 11C**). Because AID and other deaminases from the APOBEC family work as multimers,[64,83-87] we designed an effector module expressing two non-homologous AID sequences fused through a flexible 32 amino acid linker (L32) to facilitate oligomerization at the site of action (^{2xA}CRCnu.2, **Figure 11D**). Finally, to further expand the CRC toolbox, we engineered an optimized APOBEC1-containing effector module (^{A1}CRCnu.2, **Figure 11E**).

In addition to improvements to the effector and Cas9 modules, we decided to revisit our gRNA design. Engineered gRNAs containing multiple MS2 sequences reduce signal-to-noise ratio in genomic labeling experiments.[53] Thus, we incorporated these scaffolds into our system. The first vector contains six MS2 sequences in tandem at the 3' end of the gRNA[53] (6xMS2_gRNA, **Figure 11F**). The second gRNA harbors two MS2 scaffolds within the gRNA sequence itself, specifically at the tetraloop and stem loop 2, plus four MS2 sequences at the 3' end[53] (2+4xMS2_gRNA, **Figure 11G**).

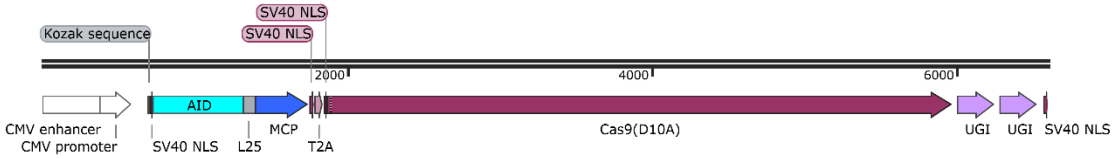
A.

^{A}CRCnu.2



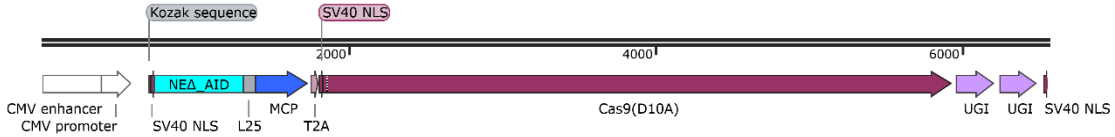
B.

^{2NA}CRCnu.2



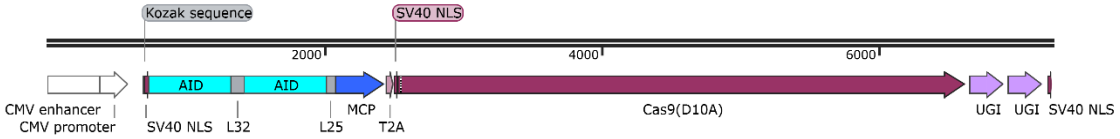
C.

$^{N\Delta A}$CRCnu.2



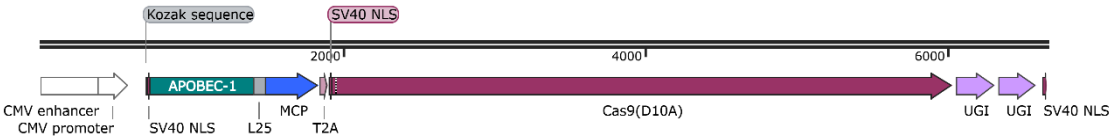
D.

^{2xA}CRCnu.2



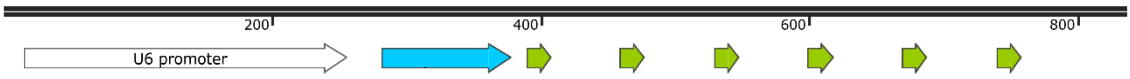
E.

^{A1}CRCnu.2



F.

6xMS2_gRNA



G.

2+4xMS2_gRNA

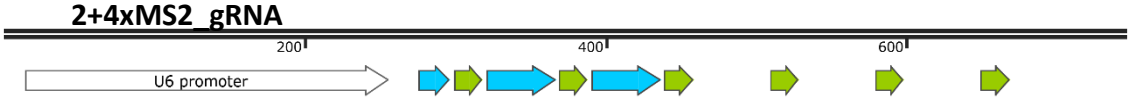


Figure 11. Design of second-generation CRC constructs
A – G. Schematic maps of optimized, second-generation CRC constructs. All protein coding constructs (**A – E**) were codon optimized for efficient expression in human cells; the Cas9 module contains two UGI copies in tandem (purple) and has two NLS at N- and C-terminus. **A.** ^{A}CRCnu.2 expresses standard AID_MCP fusion protein, recapitulating ^{A}CRCnu. **B - C.** To enhance effector module presence and activity in the nucleus, an additional NLS was fused downstream of MCP generating ^{2NA}CRCnu.2 (**B**), or the intrinsic NES sequence was deleted form the C-terminal domain of AID generating $^{N\Delta A}$CRCnu.2 (**C**). **D.** To facilitate the formation of dimers or other oligomers, two copies of AID were fused in tandem to MCP generating ^{2xA}CRCnu.2. **E.** To expand CRC's effector repertoire, an optimized APOBEC1 was fused to MCP generating ^{A1}CRCnu.2. **F - G** Engineered gRNA scaffolds were designed to express 6 MS2 stem loops at the 3' end (6xMS2_gRNA, **F**) or 2 MS2 loops within the gRNA sequence, followed by 4 MS2 sequences at the 3' end (2+4xMS2_gRNA, **G**).

The construction of the second-generation CRC constructs in currently in progress. These optimized systems will be tested on the following targets: nfEGFP, HEK293 site2, site3, site4 and *DMD*, among others. For *DMD* targeting we have obtained an immortalized patient-derived myofibroblast cell line harboring a deletion of exons 45 to 50. This genetic background can be treated by exon 51 skipping, as described earlier, restoring the ORF. To reduce potential cytotoxicity associated with nucleofection of large amounts of DNA in sensitive cell lines, the constructs that show best performance will be packaged in AAV vectors.

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APPENDIX: SEQUENCES OF PLASMIDS DEVELOPED IN THIS STUDY

Bacterial Expression Plasmid Sequences

1. pTet_dCas9-bacteria/p15A

```
LOCUS       Exported                6705 bp ds-DNA     circular SYN 11-APR-2015
DEFINITION  pTet_dCas9/p15A; encodes for nuclease dead Cas9 protein; CRC
       targeting module
ACCESSION   .
VERSION     pTet_dCas9/p15A; CRC targeting module
KEYWORDS    .
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 6705)
  AUTHORS   .
  TITLE     Direct Submission
  JOURNAL   Exported Sep 29, 2018 from SnapGene Viewer 4.2.5
            http://www.snapgene.com
FEATURES             Location/Qualifiers
     source          1..6705
                     /organism="synthetic DNA construct"
                     /mol_type="other DNA"
     CDS             complement(7..630)
                     /codon_start=1
                     /gene="tetR from transposon Tn10"
                     /product="tetracycline repressor TetR"
                     /label=TetR
                     /note="TetR binds to the tetracycline operator tetO to
                     inhibit transcription. This inhibition can be relieved by
                     adding tetracycline or doxycycline."

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PTEKQYETLENQLAFLCQQGFSLENALYALSAVGHFTLGCVLEDQEHQVAKEERETPTT
                     DSMPPLLRQAIELFDHQGAEPAFLFGLELIICGLEKQLKCESGS"
     promoter        649..704
                     /gene="tetR"
                     /label=tetR/tetA promoters
                     /note="overlapping promoters for bacterial tetR and tetA"
     protein_bind    685..703
                     /gene="tetO"
                     /label=tet operator
                     /bound_moiety="tetracycline repressor TetR"
                     /note="bacterial operator O2 for the tetR and tetA genes"
     CDS             740..4846
                     /codon_start=1
                     /product="catalytically dead mutant of the Cas9
                     endonuclease from the Streptococcus pyogenes Type II
                     CRISPR/Cas system"
                     /label=dCas9
                     /note="RNA-guided DNA-binding protein that lacks
                     endonuclease activity due to the D10 mutation in the
                     RuvC-like domain and the H840A mutation in the HNH domain"
```

```
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FRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRL
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TIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNE
LALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILAD
ANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVL
                      DATLIHQSITGLYETRIDLSQLGGD"
     terminator       4870..4941
                      /label=rrnB T1 terminator
                      /note="transcription terminator T1 from the E. coli rrnB
                      gene"
     terminator       4957..4984
                      /label=T7Te terminator
                      /note="phage T7 early transcription terminator"
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                      /direction=LEFT
                      /label=p15A ori
                      /note="Plasmids containing the medium-copy-number p15A
                      origin of replication can be propagated in E. coli cells
                      that contain a second plasmid with the ColE1 origin."
     terminator       5805..5899
                      /label=lambda t0 terminator
                      /note="transcription terminator from phage lambda"
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                     /product="chloramphenicol acetyltransferase"
                     /label=CmR
                     /note="confers resistance to chloramphenicol"

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LWSEYHDDFRQFLHIYSQDVACYGENLAYFPKGFIENMFFVSANPWVSFTSFDLNVANM
                     DNFFAPVFTMGKYYTQGDKVLMPLAIQVHHAVCDGFHVGRMLNELQQYCDEWQGGA"
     promoter        complement(6580..6682)
                     /label=cat promoter
                     /note="promoter of the E. coli cat gene encoding
                     chloramphenicol acetyltransferase"
ORIGIN
        1 gacgtcttaa gacccacttt cacatttaag ttgtttttct aatccgcata tgatcaattc
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       protein; CRC targeting module
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VERSION     .
KEYWORDS    pTet_nCas9H840A/p15A; CRC targeting module
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 6705)
  AUTHORS   .
  TITLE     Direct Submission
  JOURNAL   Exported Sep 29, 2018 from SnapGene Viewer 4.2.5
            http://www.snapgene.com
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3. pTet_nCas9D10A-bacteria/p15A

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VERSION     .
KEYWORDS    .
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 6705)
  AUTHORS   .
  TITLE     Direct Submission
  JOURNAL   Exported Sep 29, 2018 from SnapGene Viewer 4.2.5
            http://www.snapgene.com
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                     to the D10 mutation in the RuvC-like catalytic domain"
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4. pTet_AID_MCP/DF13

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DEFINITION  pTet_AID_MCP/DF13, encodes for AID_MCP fusion protein, CRC Effector
            Module
ACCESSION   .
VERSION     .
KEYWORDS    .pTet_AID_MCP/DF13; CRC Effector Module
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 5533)
  AUTHORS   .Collantes, JC
  TITLE     Direct Submission
  JOURNAL   Exported Sep 29, 2018 from SnapGene Viewer 4.2.5
            http://www.snapgene.com
FEATURES             Location/Qualifiers
     source          1..5533
                     /organism="synthetic DNA construct"
                     /mol_type="other DNA"
     source          2732..2754
                     /organism="Enterobacteria phage T7"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:10760"
     promoter        complement(558..635)
                     /gene="lacI"
                     /label=lacI promoter
     CDS             complement(737..1360)
                     /codon_start=1
                     /gene="tetR from transposon Tn10"
                     /product="tetracycline repressor TetR"
                     /label=TetR
                     /note="TetR binds to the tetracycline operator tetO to
                     inhibit transcription. This inhibition can be relieved by
                     adding tetracycline or doxycycline."

/translation="MSRLDKSKVINSALELLNEVGIEGLTTRKLAQKLGVEQPTLYWHV

KNKRALLDALAIEMLDRHHTHFCPLEGESWQDFLRNNAKSFRCALLSHRDGAKVHLGTR

PTEKQYETLENQLAFLCQQGFSLENALYALSAVGHFTLGCVLEDQEHQVAKEERETPTT
                     DSMPPLLRQAIELFDHQGAEPAFLFGLELIICGLEKQLKCESGS"
     promoter        1379..1434
                     /gene="tetR"
                     /label=tetR/tetA promoters
                     /note="overlapping promoters for bacterial tetR and tetA"
     protein_bind    1415..1433
                     /gene="tetO"
                     /label=tet operator
                     /bound_moiety="tetracycline repressor TetR"
                     /note="bacterial operator O2 for the tetR and tetA genes"
     RBS             1452..1463
                     /note="strong bacterial ribosome binding site (Elowitz and
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                     Leibler, 2000)"
     CDS             1470..2063
                     /codon_start=1
                     /gene="AICDA"
                     /product="human activation-induced cytidine deaminase
                     (Dickerson et al., 2003)"
                     /label=AID

/translation="MDSLLMNRRKFLYQFKNVRWAKGRRETYLCYVVKRRDSATSFSLD

FGYLRNKNGCHVELLFLRYISDWDLDPGRCYRVTWFTSWSPCYDCARHVADFLRGNPNL

SLRIFTARLYFCEDRKAEPEGLRRLHRAGVQIAIMTFKDYFYCWNTFVENHERTFKAWE
                     GLHENSVRLSRQLRRILLPLYEVDDLRDAFRTLGL"
     CDS             2064..2138
                     /codon_start=1
                     /label=L25
                     /translation="ELKTPLGDTTHTSPPCPAPELLGGP"
     CDS             2139..2495
                     /codon_start=1
                     /product="MCP"
                     /label=MCP

/translation="MASNFTQFVLVDNGGTGDVTVAPSNFANGIAEWISSNSRSQAYKV

TCSVRQSSAQNRKYTIKVEVPKGAWRSYLNMELTIPIFATNSDCELIVKAMQGLLKDGN
                     PIPSAIAANSGIY*"
     terminator      2519..2590
                     /label=rrnB T1 terminator
                     /note="transcription terminator T1 from the E. coli rrnB
                     gene"
     terminator      2606..2633
                     /label=T7Te terminator
                     /note="phage T7 early transcription terminator"
     promoter        2674..2692
                     /label=T7 promoter
                     /note="promoter for bacteriophage T7 RNA polymerase"
     protein_bind    2693..2717
                     /label=lac operator
                     /bound_moiety="lac repressor encoded by lacI"
                     /note="The lac repressor binds to the lac operator to
                     inhibit transcription in E. coli. This inhibition can be
                     relieved by adding lactose or
                     isopropyl-beta-D-thiogalactopyranoside (IPTG)."
     RBS             2732..2754
                     /note="efficient ribosome binding site from bacteriophage
                     T7 gene 10 (Olins and Rangwala, 1989)"
     CDS             2828..2872
                     /codon_start=1
                     /product="affinity and epitope tag derived from pancreatic
                     ribonuclease A"
                     /label=S-Tag
                     /translation="KETAAAKFERQHMDS"
     terminator      2925..2972
                     /label=T7 terminator
                     /note="transcription terminator for bacteriophage T7 RNA
                     polymerase"
     CDS             complement(3129..3920)
                     /codon_start=1
                     /gene="aadA"
                     /product="aminoglycoside adenylyltransferase (Murphy,
                     1985)"
                     /label=SmR
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                     /note="confers resistance to spectinomycin and
                     streptomycin"

/translation="MREAVIAEVSTQLSEVVGVIERHLEPTLLAVHLYGSAVDGGLKPH

SDIDLLVTVTVRLDETTRRALINDLLETSASPGESEILRAVEVTIVVHDDIIPWRYPAK

RELQFGEWQRNDILAGIFEPATIDIDLAILLTKAREHSVALVGPAAEELFDPVPEQDLF

EALNETLTLWNSPPDWAGDERNVVLTLSRIWYSAVTGKIAPKDVAADWAMERLPAQYQP
                     VILEARQAYLGQEEDRLASRADQLEEFVHYVKGEITKVVGK"
     promoter        complement(3921..4012)
                     /gene="bla"
                     /label=AmpR promoter
     rep_origin      complement(4060..4798)
                     /direction=LEFT
                     /label=CloDF13 ori
                     /note="Plasmids containing the CloDF13 (CDF) origin of
                     replication can be propagated in E. coli cells that
contain
                     additional plasmids with compatible origins."
     CDS             complement(join(5008..5533,1..557))
                     /codon_start=1
                     /gene="lacI"
                     /product="lac repressor"
                     /label=lacI
                     /note="The lac repressor binds to the lac operator to
                     inhibit transcription in E. coli. This inhibition can be
                     relieved by adding lactose or
                     isopropyl-beta-D-thiogalactopyranoside (IPTG)."

/translation="MKPVTLYDVAEYAGVSYQTVSRVVNQASHVSAKTREKVEAAMAEL

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EACKAAVHNLLAQRVSGLIINYPLDDQDAIAVEAACTNVPALFLDVSDQTPINSIIFSH

EDGTRLGVEHLVALGHQQIALLAGPLSSVSARLRLAGWHKYLTRNQIQPIAEREGDWSA

MSGFQQTMQMLNEGIVPTAMLVANDQMALGAMRAITESGLRVGADISVVGYDDTEDSSC

YIPPLTTIKQDFRLLGQTSVDRLLQLSQGQAVKGNQLLPVSLVKRKTTLAPNTQTASPR
                     ALADSLMQLARQVSRLESGQ"
ORIGIN
        1 gctaacagcg cgatttgctg gtgacccaat gcgaccagat gctccacgcc cagtcgcgta
       61 ccgtcttcat gggagaaaat aatactgttg atgggtgtct ggtcagagac atcaagaaat
      121 aacgccggaa cattagtgca ggcagcttcc acagcaatgg catcctggtc atccagcgga
      181 tagttaatga tcagcccact gacgcgttgc gcgagaagat tgtgcaccgc cgctttacag
      241 gcttcgacgc cgcttcgttc taccatcgac accaccacgc tggcacccag ttgatcggcg
      301 cgagatttaa tcgccgcgac aatttgcgac ggcgcgtgca gggccagact ggaggtggca
      361 acgccaatca gcaacgactg tttgcccgcc agttgttgtg ccacgcggtt gggaatgtaa
      421 ttcagctccg ccatcgccgc ttccactttt tcccgcgttt tcgcagaaac gtggctggcc
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      541 aacgttactg gtttcacatt caccaccctg aattgactct cttccgggcg ctatcatgcc
      601 ataccgcgaa aggttttgcg ccattcgatg gtgtccggga tctcgacgct ctcccttatg
      661 cgactcctgc attaggaagc agcccagtag taggttgagg ccgttgagca ccgccgccgc
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      781 tgatcaattc aaggccgaat aagaaggctg gctctgcacc ttggtgatca aataattcga
      841 tagcttgtcg taataatggc ggcatactat cagtagtagg tgtttccctt tcttctttag
      901 cgacttgatg ctcttgatct tccaatacgc aacctaaagt aaaatgcccc acagcgctga
      961 gtgcatataa tgcattctct agtgaaaaac cttgttggca taaaaaggct aattgatttt
     1021 cgagagtttc atactgtttt tctgtaggcc gtgtacctaa atgtactttt gctccatcgc
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1261 gcttctgggc gagtttacgg gttgttaaac cttcgattcc gacctcatta agcagctcta
1321 atgcgctgtt aatcacttta cttttatcta atctagacat cattaattcc taatttttgt
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1441 tcaaaagatc taaagaggag aaaggatcta tggacagcct cttgatgaac cggaggaagt
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2281 gcgttcgtca gagctctgcg cagaatcgca aatacaccat caaagtcgag gtgcctaaag
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2401 actgcgagct tattgttaag gcaatgcaag gtctcctaaa agatggaaac ccgattccct
2461 cagcaatcgc agcaaactcc ggcatctact gatgactcga gtaaggatct ccaggcatca
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2581 gaacgctctc tactagagtc acactggctc accttcgggt gggcctttct gcgtttatac
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2761 tatggcagat ctcaattgga tatcggccgg ccacgcgatc gctgacgtcg gtaccctcga
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4561 ggagttcgca gaggatttgt ttagctaaac acgcggttgc tcttgaagtg tgcgccaaag
4621 tccggctaca ctggaaggac agatttggtt gctgtgctct gcgaaagcca gttaccacgg
4681 ttaagcagtt ccccaactga cttaaccttc gatcaaacca cctccccagg tggtttttc
4741 gtttacaggg caaaagatta cgcgcagaaa aaaaggatct caagaagatc ctttgatctt
4801 ttctactgaa ccgctctaga tttcagtgca atttatctct tcaaatgtag cacctgaagt
4861 cagccccata cgatataagt tgtaattctc atgttagtca tgccccgcgc ccaccggaag

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  5041 tgccagctgc attaatgaat cggccaacgc gcggggagag gcggtttgcg tattgggcgc
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  5341 cgccatctga tcgttggcaa ccagcatcgc agtgggaacg atgccctcat tcagcatttg
  5401 catggtttgt tgaaaaccgg acatggcact ccagtcgcct tcccgttccg ctatcggctg
  5461 aatttgattg cgagtgagat atttatgcca gccagccaga cgcagacgcg ccgagacaga
  5521 acttaatggg ccc
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5. sgRNA_MS2-bacteria/pUC19

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LOCUS       Exported                2606 bp ds-DNA     circular SYN 29-SEP-
2018
DEFINITION  pgRNA_MS2-bacteria/pUC19, synthetic circular DNA
ACCESSION   .
VERSION     .
KEYWORDS    .
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 2606)
  AUTHORS   .
  TITLE     Direct Submission
  JOURNAL   Exported Sep 29, 2018 from SnapGene Viewer 4.2.5
            http://www.snapgene.com
FEATURES             Location/Qualifiers
     source          1..2606
                     /organism="synthetic DNA construct"
                     /mol_type="other DNA"
     promoter        16..50
                     /label=J23119(SpeI) promoter
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Biological
                     Parts BBa_J23119) modified to end with an SpeI site"
     misc_RNA        68..143
                     /label=gRNA scaffold
                     /note="guide RNA scaffold for the CRISPR/Cas9 system"
     misc_RNA        148..166
                     /label=MS2 stem loop
                     /note="stem loop that binds the bacteriophage MS2 coat
                     protein"
     CDS             189..218
                     /codon_start=1
                     /product="Myc (human c-Myc oncogene) epitope tag"
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                     /translation="EQKLISEEDL"
     CDS             234..251
                     /codon_start=1
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                     /label=6xHis
                     /translation="HHHHHH"
     terminator      477..523
                     /label=rrnB T1 terminator
                     /note="transcription terminator T1 from the E. coli
rrnB
                     gene"
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     terminator      477..522
                     /label=rrnB T1 terminator
                     /note="transcription terminator T1 from the E. coli
rrnB
                     gene"
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                     /label=rrnB T1 terminator
                     /note="transcription terminator T1 from the E. coli
rrnB
                     gene"
     rep_origin      complement(747..1335)
                     /direction=LEFT
                     /label=ori
                     /note="high-copy-number ColE1/pMB1/pBR322/pUC origin of
                     replication"
     CDS             complement(1506..2366)
                     /codon_start=1
                     /gene="bla"
                     /product="beta-lactamase"
                     /label=AmpR
                     /note="confers resistance to ampicillin, carbenicillin,
and
                     related antibiotics"

/translation="MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYI

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EPELNEAIPNDERDTTMPVAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSA

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                     /label=AmpR promoter
     terminator      2561..2604
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                     /note="putative bacterial transcription terminator"
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        1561 tccatagttg cctgactccc cgtcgtgtag ataactacga tacgggaggg cttaccatct
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        1741 atccagtcta ttaattgttg ccgggaagct agagtaagta gttcgccagt taatagtttg
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        1861 tcattcagct ccggttccca acgatcaagg cgagttacat gatcccccat gttgtgcaaa
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        1981 tcactcatgg ttatggcagc actgcataat tctcttactg tcatgccatc cgtaagatgc
        2041 ttttctgtga ctggtgagta ctcaaccaag tcattctgag aatagtgtat gcggcgaccg
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        2461 ggggttccgc gcacatttcc ccgaaaagtg ccacctgacg tctaagaaac cattattatc
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6. sgRNA_2xMS2-bacteria/pUC19

```
LOCUS       Exported                2648 bp ds-DNA     circular SYN 08-AUG-2016
DEFINITION  pgRNA_2xMS2-bacteria/pUC19, synthetic circular DNA
ACCESSION   .
VERSION     .
KEYWORDS    .
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 2648)
  AUTHORS   .
  TITLE     Direct Submission
  JOURNAL   Exported Sep 29, 2018 from SnapGene Viewer 4.2.5
            http://www.snapgene.com
FEATURES             Location/Qualifiers
     source          1..2648
                     /organism="synthetic DNA construct"
                     /mol_type="other DNA"
     promoter        16..50
                     /label=J23119(SpeI) promoter
                     /note="bacterial promoter (Registry of Standard Biological
                     Parts BBa_J23119) modified to end with an SpeI site"
     misc_RNA        68..143
                     /label=gRNA scaffold
                     /note="guide RNA scaffold for the CRISPR/Cas9 system"
     misc_RNA        150..168
                     /label=MS2 stem loop
                     /note="stem loop that binds the bacteriophage MS2 coat
                     protein"
     misc_RNA        179..197
                     /label=MS2 stem loop
                     /note="stem loop that binds the bacteriophage MS2 coat
                     protein"
     CDS             231..260
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                     /label=Myc
                     /translation="EQKLISEEDL"
     CDS             231..260
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                     /label=Myc
                     /translation="EQKLISEEDL"
     CDS             276..293
                     /codon_start=1
                     /product="6xHis affinity tag"
                     /label=6xHis
                     /translation="HHHHHH"
     CDS             276..293
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                     /label=6xHis
                     /translation="HHHHHH"
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                     /label=rrnB T1 terminator
                     /note="transcription terminator T1 from the E. coli rrnB
                     gene"
     terminator      519..564
                     /label=rrnB T1 terminator
                     /note="transcription terminator T1 from the E. coli rrnB
                     gene"
     terminator      522..565
                     /gene="Escherichia coli rrnB"
                     /label=rrnB T1 terminator
                     /note="transcription terminator T1 from the E. coli rrnB
                     gene"
     primer_bind     complement(566..586)
                     /label=R
     rep_origin      complement(789..1377)
                     /direction=LEFT
                     /label=ori
                     /note="high-copy-number ColE1/pMB1/pBR322/pUC origin of
                     replication"
     CDS             complement(1548..2408)
                     /codon_start=1
                     /gene="bla"
                     /product="beta-lactamase"
                     /label=AmpR
                     /note="confers resistance to ampicillin, carbenicillin,
and
                     related antibiotics"

/translation="MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYI

ELDLNSGKILESFRPEERFPMMSTFKVLLCGAVLSRIDAGQEQLGRRIHYSQNDLVEYS

PVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRW

EPELNEAIPNDERDTTMPVAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSA

LPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGAS
                     LIKHW"
     promoter        complement(2409..2513)
                     /gene="bla"
                     /label=AmpR promoter
     terminator      2603..2646
                     /label=bacterial terminator
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                     /note="putative bacterial transcription terminator"
     primer_bind     join(2648,1..22)
                     /label=F
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      121 aaaaagtggc accgagtcgg tgcgggagca catgaggatc acccatgtgc cacgagcgac
      181 atgaggatca cccatgtcgc tcgtgttccc tttttttgaa gcttgggccc gaacaaaaac
      241 tcatctcaga agaggatctg aatagcgccg tcgaccatca tcatcatcat cattgagttt
      301 aaacggtctc cagcttggct gttttggcgg atgagagaag attttcagcc tgatacagat
      361 taaatcagaa cgcagaagcg gtctgataaa acagaatttg cctggcggca gtagcgcggt
      421 ggtcccacct gaccccatgc cgaactcaga agtgaaacgc cgtagcgccg atggtagtgt
      481 ggggtctccc catgcgagag tagggaactg ccaggcatca aataaaacga aaggctcagt
      541 cgaaagactg ggcctttcgt tttatctgtt gtttgtcggt gaactggatc cttactcgag
      601 tctagactgc aggcttcctc gctcactgac tcgctgcgct cggtcgttcg gctgcggcga
      661 gcggtatcag ctcactcaaa ggcggtaata cggttatcca cagaatcagg ggataacgca
      721 ggaaagaaca tgtgagcaaa aggccagcaa aaggccagga accgtaaaaa ggccgcgttg
      781 ctggcgtttt tccataggct ccgcccccct gacgagcatc acaaaaatcg acgctcaagt
      841 cagaggtggc gaaacccgac aggactataa agataccagg cgtttccccc tggaagctcc
      901 ctcgtgcgct ctcctgttcc gaccctgccg cttaccggat acctgtccgc ctttctccct
      961 tcgggaagcg tggcgctttc tcatagctca cgctgtaggt atctcagttc ggtgtaggtc
     1021 gttcgctcca agctgggctg tgtgcacgaa ccccccgttc agcccgaccg ctgcgcctta
     1081 tccggtaact atcgtcttga gtccaacccg gtaagacacg acttatcgcc actggcagca
     1141 gccactggta acaggattag cagagcgagg tatgtaggcg gtgctacaga gttcttgaag
     1201 tggtggccta actacggcta cactagaagg acagtatttg gtatctgcgc tctgctgaag
     1261 ccagttacct tcggaaaaag agttggtagc tcttgatccg gcaaacaaac caccgctggt
     1321 agcggtggtt tttttgtttg caagcagcag attacgcgca gaaaaaaagg atctcaagaa
     1381 gatcctttga tcttttctac ggggtctgac gctcagtgga acgaaaactc acgttaaggg
     1441 attttggtca tgagattatc aaaaaggatc ttcacctaga tccttttaaa ttaaaaatga
     1501 agttttaaat caatctaaag tatatatgag taaacttggt ctgacagtta ccaatgctta
     1561 atcagtgagg cacctatctc agcgatctgt ctatttcgtt catccatagt tgcctgactc
     1621 cccgtcgtgt agataactac gatacgggag ggcttaccat ctggccccag tgctgcaatg
     1681 ataccgcgag acccacgctc accggctcca gatttatcag caataaacca gccagccgga
     1741 agggccgagc gcagaagtgg tcctgcaact ttatccgcct ccatccagtc tattaattgt
     1801 tgccgggaag ctagagtaag tagttcgcca gttaatagtt tgcgcaacgt tgttgccatt
     1861 gctacaggca tcgtggtgtc acgctcgtcg tttggtatgg cttcattcag ctccggttcc
     1921 caacgatcaa ggcgagttac atgatccccc atgttgtgca aaaaagcggt tagctccttc
     1981 ggtcctccga tcgttgtcag aagtaagttg gccgcagtgt tatcactcat ggttatggca
     2041 gcactgcata attctcttac tgtcatgcca tccgtaagat gcttttctgt gactggtgag
     2101 tactcaacca agtcattctg agaatagtgt atgcggcgac cgagttgctc ttgcccggcg
     2161 tcaatacggg ataataccgc gccacatagc agaactttaa aagtgctcat cattggaaaa
     2221 cgttcttcgg ggcgaaaact ctcaaggatc ttaccgctgt tgagatccag ttcgatgtaa
     2281 cccactcgtg cacccaactg atcttcagca tcttttactt tcaccagcgt ttctgggtga
     2341 gcaaaaacag gaaggcaaaa tgccgcaaaa aagggaataa gggcgacacg gaaatgttga
     2401 atactcatac tcttccttt tcaatattat tgaagcattt atcagggtta ttgtctcatg
     2461 agcggataca tatttgaatg tatttagaaa aataaacaaa tagggggttcc gcgcacattt
     2521 ccccgaaaag tgccacctga cgtctaagaa accattatta tcatgacatt aacctataaa
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     2641 gatttctg
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Mammalian Expression Plasmid Sequences

1. pCMV_nfEGFP

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LOCUS       Exported                5112 bp ds-DNA     circular SYN 09-OCT-2018
DEFINITION  Non-fluorescent EGFP synthetic circular DNA
ACCESSION   .
VERSION     .
KEYWORDS    .
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 5112)
  AUTHORS   Juan-Carlos Collantes
  TITLE     Direct Submission
  JOURNAL   Exported Oct 9, 2018 from SnapGene Viewer 4.2.6
            http://www.snapgene.com
FEATURES             Location/Qualifiers
     source          1..5112
                     /organism="synthetic DNA construct"
                     /mol_type="other DNA"
     polyA_signal    417..551
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                     /note="SV40 polyadenylation signal"
     rep_origin      677..1132
                     /direction=RIGHT
                     /label=f1 ori
                     /note="f1 bacteriophage origin of replication; arrow
                     indicates direction of (+) strand synthesis"
     CDS             1420..2280
                     /codon_start=1
                     /gene="bla"
                     /product="beta-lactamase"
                     /label=AmpR
                     /note="confers resistance to ampicillin, carbenicillin,
and
                     related antibiotics"

/translation="MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYI

ELDLNSGKILESFRPEERFPMMSTFKVLLCGAVLSRIDAGQEQLGRRIHYSQNDLVEYS

PVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRW

EPELNEAIPNDERDTTMPVAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSA

LPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGAS
                     LIKHW"
     protein_bind    complement(2382..2415)
                     /label=loxP
                     /bound_moiety="Cre recombinase"
                     /note="Cre-mediated recombination occurs in the 8-bp core
                     sequence (GCATACAT)."
     rep_origin      2493..3081
                     /direction=RIGHT
                     /label=ori
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                     /note="high-copy-number ColE1/pMB1/pBR322/pUC origin of
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                     /note="human cytomegalovirus immediate early enhancer"
     promoter        3845..4048
                     /label=CMV promoter
                     /note="human cytomegalovirus (CMV) immediate early
                     promoter"
     primer_bind     4174..4190
                     /label=M13 rev
                     /note="common sequencing primer, one of multiple similar
                     variants"
     promoter        4201..4219
                     /label=SP6 promoter
                     /note="promoter for bacteriophage SP6 RNA polymerase"
     protein_bind    4220..4244
                     /gene="mutant version of attB"
                     /label=attB1
                     /bound_moiety="BP Clonase(TM)"
                     /note="recombination site for the Gateway(R) BP reaction"
     regulatory      4280..4289
                     /regulatory_class="other"
                     /note="vertebrate consensus sequence for strong initiation
                     of translation (Kozak, 1987)"
     CDS             4286..5005
                     /codon_start=1
                     /product="non-fluorescent enhanced GFP"
                     /label=nfEGFP
                     /note="mammalian codon-optimized"

/translation="MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTL

KFICTTGKLPVPWPTLVTTLTCGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDD

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VNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLL
                     EFVTAAGITLGMDELYK"
     protein_bind    complement(5053..5077)
                     /gene="mutant version of attB"
                     /label=attB2
                     /bound_moiety="BP Clonase(TM)"
                     /note="recombination site for the Gateway(R) BP reaction"
     promoter        complement(5079..5097)
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                     /note="promoter for bacteriophage T7 RNA polymerase"
     primer_bind     complement(join(5108..5112,1..12))
                     /label=M13 fwd
                     /note="common sequencing primer, one of multiple similar
                     variants"
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      121 taaatataaa atttttaagt gtataatgtg ttaaactagc tgcatatgct tgctgcttga
      181 gagttttgct tactgagtat gatttatgaa aatattatac acaggagcta gtgattctaa
      241 ttgtttgtgt attttagatt cacagtccca aggctcattt caggcccctc agtcctcaca
      301 gtctgttcat gatcataatc agccatacca catttgtaga ggttttactt gctttaaaaa
      361 acctcccaca cctccccctg aacctgaaac ataaaatgaa tgcaattgtt gttgttaact
      421 tgtttattgc agcttataat ggttacaaat aaagcaatag catcacaaat ttcacaaata
      481 aagcattttt ttcactgcat tctagttgtg gtttgtccaa actcatcaat gtatcttatc
      541 atgtctggat cgatcctgca ttaatgaatc ggccaacgcg cggggagagg cggtttgcgt
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 781 ccttcctttc tcgccacgtt cgccggcttt ccccgtcaag ctctaaatcg ggggctccct
 841 ttagggttcc gatttagtgc tttacggcac ctcgacccca aaaaacttga ttagggtgat
 901 ggttcacgta gtgggccatc gccctgatag acggtttttc gccctttgac gttggagtcc
 961 acgttcttta atagtggact cttgttccaa actggaacaa cactcaaccc tatctcggtc
1021 tattcttttg atttataagg gattttgccg atttcggcct attggttaaa aaatgagctg
1081 atttaacaaa aatttaacgc gaattttaac aaaatattaa cgtttacaat ttcaggtggc
1141 acttttcggg gaaatgtgcg cggaacccct atttgtttat ttttctaaat acattcaaat
1201 atgtatccgc tcatgccagg tcttggactg gtgagaacgg cttgctcggc agcttcgatg
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1381 ccctgataaa tgcttcaata atattgaaaa aggaagagta tgagtattca acatttccgt
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1681 caactcggtc gccgcataca ctattctcag aatgacttgg ttgagtactc accagtcaca
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1861 gctttttgc acaacatggg ggatcatgta actcgccttg atcgttggga accggagctg
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1981 ttgcgcaaac tattaactgg cgaactactt actctagctt cccggcaaca attaatagac
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2161 gggccagatg gtaagccctc ccgtatcgta gttatctaca cgacggggag tcaggcaact
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2281 ctgtcagacc aagtttactc atatatactt tagattgatt taaaacttca tttttaattt
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2581 ctaccaactc tttttccgaa ggtaactggc ttcagcagag cgcagatacc aaatactgtc
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2701 ctcgctctgc taatcctgtt accagtggct gctgccagtg gcgataagtc gtgtcttacc
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3121 tgctggcctt ttgctcacat gttctttcct gcgttatccc ctgattctgt ggataaccgt
3181 attaccgcct ttgagtgagc tgataccgct cgccgcagcc gaacgaccga gcgcagcgag
3241 tcagtgagcg aggaagcgga agagcgccca atacgcaaac cgcctctccc cgcgcgttgg
3301 ccgattcatt aatgcagagc ttgcaattcg cgcgtttttc aatattattg aagcatttat
3361 cagggttatt gtctcatgag cggatacata tttgaatgta tttagaaaaa taaacaaata
3421 ggggttccgc gcacatttcc ccgaaaagtg ccacctgacg tctaagaaac cattattatc
3481 atgacattaa cctataaaaa taggcgtagt acgaggccct ttcactcatt agatgcatgt
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3601 gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc attgacgtca
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3721 aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt atgcccagta
3781 catgacctta tgggactttc ctacttggca gtacatctac gtattagtca tcgctattac
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4081 ccatccacgc tgttttgacc tccatagaag acaccgggac cgatccagcc tccggactct
4141 agcctaggcc gcggagcgga taacaatttc acacaggaaa cagctatgac cattaggcct
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4261 tcccgggata tcgtcgacag ccaccatggt gagcaagggc gaggagctgt tcaccggggt
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4381 cgagggcgag ggcgatgcca cctacggcaa gctgaccctg aagttcatct gcaccaccgg
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     4561 ctacgtccag gagcgcacca tcttcttcaa ggacgacggc aactacaaga cccgcgccga
     4621 ggtgaagttc gagggcgaca ccctggtgaa ccgcatcgag ctgaagggca tcgacttcaa
     4681 ggaggacggc aacatcctgg ggcacaagct ggagtacaac tacaacagcc acaacgtcta
     4741 tatcatggcc gacaagcaga agaacggcat caaggtgaac ttcaagatcc gccacaacat
     4801 cgaggacggc agcgtgcagc tcgccgacca ctaccagcag aacacccccca tcggcgacgg
     4861 ccccgtgctg ctgcccgaca accactacct gagcacccag tccgccctga gcaaagaccc
     4921 caacgagaag cgcgatcaca tggtcctgct ggagttcgtg accgccgccg ggatcactct
     4981 cggcatggac gagctgtaca agtgataagc ggccgctcta gagtatccct cgaggggccc
     5041 aagcttacgc gtacccagct ttcttgtaca aagtggtccc tatagtgagt cgtattataa
     5101 gctaggcact gg
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2. pUbC_ACRCn

```
LOCUS       Exported               15129 bp ds-DNA     circular SYN 08-OCT-2018
DEFINITION  pUbC_ACRCn synthetic circular DNA
ACCESSION   .
VERSION     .
KEYWORDS    .
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 15129)
  AUTHORS   Juan-Carlos Collantes
  TITLE     Direct Submission
  JOURNAL   Exported Oct 8, 2018 from SnapGene Viewer 4.2.6
            http://www.snapgene.com
FEATURES             Location/Qualifiers
     source          1..15129
                     /organism="synthetic DNA construct"
                     /mol_type="other DNA"
     enhancer        238..617
                     /label=CMV enhancer
                     /note="human cytomegalovirus immediate early enhancer"
     promoter        618..821
                     /label=CMV promoter
                     /note="human cytomegalovirus (CMV) immediate early
                     promoter"
     LTR             835..1015
                     /label=5' LTR (truncated)
                     /note="truncated 5' long terminal repeat (LTR) from HIV-1"
     misc_feature    1062..1187
                     /label=HIV-1 Psi
                     /note="packaging signal of human immunodeficiency virus
                     type 1"
     misc_feature    1680..1913
                     /label=RRE
                     /note="The Rev response element (RRE) of HIV-1 allows for
                     Rev-dependent mRNA export from the nucleus to the
                     cytoplasm."
     misc_feature    2440..2557
                     /label=cPPT/CTS
                     /note="central polypurine tract and central termination
                     sequence of HIV-1"
     protein_bind    2602..2635
                     /label=loxP
                     /bound_moiety="Cre recombinase"
                     /note="Cre-mediated recombination occurs in the 8-bp core
                     sequence (GCATACAT)."
     CDS             complement(2622..2948)
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                     /product="LacZ-alpha fragment of beta-galactosidase"
                     /label=lacZ-alpha

/translation="MTMITPSAQLTLTKGNKSWRTSRGGPVPNSPYSESYYARSLAVVL

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                     HNFV"
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                     /label=M13 fwd
                     /note="common sequencing primer, one of multiple similar
                     variants"
     promoter        2839..2857
                     /label=T7 promoter
                     /note="promoter for bacteriophage T7 RNA polymerase"
     promoter        complement(2905..2923)
                     /label=T3 promoter
                     /note="promoter for bacteriophage T3 RNA polymerase"
     primer_bind     complement(2944..2960)
                     /label=M13 rev
                     /note="common sequencing primer, one of multiple similar
                     variants"
     protein_bind    2968..2984
                     /label=lac operator
                     /bound_moiety="lac repressor encoded by lacI"
                     /note="The lac repressor binds to the lac operator to
                     inhibit transcription in E. coli. This inhibition can be
                     relieved by adding lactose or
                     isopropyl-beta-D-thiogalactopyranoside (IPTG)."
     promoter        complement(2992..3022)
                     /label=lac promoter
                     /note="promoter for the E. coli lac operon"
     protein_bind    3037..3058
                     /label=CAP binding site
                     /bound_moiety="E. coli catabolite activator protein"
                     /note="CAP binding activates transcription in the presence
                     of cAMP."
     promoter        3124..4334
                     /label=UbC promoter
                     /note="human ubiquitin C promoter"
     regulatory      4363..4372
                     /regulatory_class="other"
                     /note="vertebrate consensus sequence for strong initiation
                     of translation (Kozak, 1987)"
     CDS             4372..4437
                     /codon_start=1
                     /product="three tandem FLAG(R) epitope tags, followed by
an
                     enterokinase cleavage site"
                     /label=3xFLAG
                     /translation="DYKDHDGDYKDHDIDYKDDDDK"
     CDS             4444..4464
                     /codon_start=1
                     /product="nuclear localization signal of SV40 (simian
virus
                     40) large T antigen"
                     /label=SV40 NLS
                     /translation="PKKKRKV"
     CDS             4474..8577
                     /codon_start=1
                     /product="nickase mutant of the Cas9 endonuclease from the
                     Streptococcus pyogenes Type II CRISPR/Cas system"
```

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                     /label=Cas9(D10A)
                     /note="generates RNA-guided single strand nicks in DNA due
                     to the D10A mutation in the RuvC catalytic domain"

/translation="MDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKK

NLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEES

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FRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRL

ENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQ

IGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVR

QQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLL

RKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARG

NSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEY

FTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFD

SVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEER

LKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFM

QLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGR

HKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLY

LYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVP

SEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKH

VAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYL

NAVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKT

EITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSK

ESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGI

TIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNE

LALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILAD

ANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVL
                     DATLIHQSITGLYETRIDLSQLGGD"
     CDS             8590..8610
                     /codon_start=1
                     /product="nuclear localization signal of SV40 (simian
virus
                     40) large T antigen"
                     /label=SV40 NLS
                     /translation="PKKKRKV"
     CDS             8620..8676
                     /codon_start=1
                     /product="2A peptide from porcine teschovirus-1
                     polyprotein"
                     /label=P2A
                     /note="Eukaryotic ribosomes fail to insert a peptide bond
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                     between the Gly and Pro residues, yielding separate
                     polypeptides."
                     /translation="ATNFSLLKQAGDVEENPGP"
     CDS             8677..8697
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virus
                     40) large T antigen"
                     /label=SV40 NLS
                     /translation="PKKKRKV"
     CDS             8698..9291
                     /codon_start=1
                     /gene="AICDA"
                     /product="human activation-induced cytidine deaminase
                     (Dickerson et al., 2003)"
                     /label=AID

/translation="MDSLLMNRRKFLYQFKNVRWAKGRRETYLCYVVKRRDSATSFSLD

FGYLRNKNGCHVELLFLRYISDWDLDPGRCYRVTWFTSWSPCYDCARHVADFLRGNPNL

SLRIFTARLYFCEDRKAEPEGLRRLHRAGVQIAIMTFKDYFYCWNTFVENHERTFKAWE
                     GLHENSVRLSRQLRRILLPLYEVDDLRDAFRTLGL"
     misc_feature    9292..9366
                     /label=L25
     CDS             9367..9717
                     /codon_start=1
                     /label=MCP

/translation="MASNFTQFVLVDNGGTGDVTVAPSNFANGIAEWISSNSRSQAYKV

TCSVRQSSAQNRKYTIKVEVPKGAWRSYLNMELTIPIFATNSDCELIVKAMQGLLKDGN
                     PIPSAIAANSGIY"
     protein_bind    9763..9796
                     /label=loxP
                     /bound_moiety="Cre recombinase"
                     /note="Cre-mediated recombination occurs in the 8-bp core
                     sequence (GCATACAT)."
     misc_feature    9821..10409
                     /label=WPRE
                     /note="woodchuck hepatitis virus posttranscriptional
                     regulatory element"
     CDS             complement(10292..10303)
                     /codon_start=1
                     /product="Factor Xa recognition and cleavage site"
                     /label=Factor Xa site
                     /translation="IEGR"
     primer_bind     complement(10412..10428)
                     /label=KS primer
                     /note="common sequencing primer, one of multiple similar
                     variants"
     LTR             10934..11114
                     /label=5' LTR (truncated)
                     /note="truncated 5' long terminal repeat (LTR) from HIV-1"
     polyA_signal    11146..11370
                     /label=bGH poly(A) signal
                     /note="bovine growth hormone polyadenylation signal"
     rep_origin      11416..11844
                     /direction=RIGHT
                     /label=f1 ori
                     /note="f1 bacteriophage origin of replication; arrow
                     indicates direction of (+) strand synthesis"
     promoter        11858..12187
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                     /label=SV40 promoter
                     /note="SV40 enhancer and early promoter"
     rep_origin      12038..12173
                     /label=SV40 ori
                     /note="SV40 origin of replication"
     promoter        12235..12282
                     /label=EM7 promoter
                     /note="synthetic bacterial promoter "
     CDS             12301..12675
                     /codon_start=1
                     /gene="Sh ble from Streptoalloteichus hindustanus"
                     /product="antibiotic-binding protein"
                     /label=BleoR
                     /note="confers resistance to bleomycin, phleomycin, and
                     Zeocin(TM)"

/translation="MAKLTSAVPVLTARDVAGAVEFWTDRLGFSRDFVEDDFAGVVRDD

VTLFISAVQDQVVPDNTLAWVWVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGR
                     EFALRDPAGNCVHFVAEEQD"
     polyA_signal    12805..12926
                     /label=SV40 poly(A) signal
                     /note="SV40 polyadenylation signal"
     primer_bind     complement(12975..12991)
                     /label=M13 rev
                     /note="common sequencing primer, one of multiple similar
                     variants"
     protein_bind    12999..13015
                     /label=lac operator
                     /bound_moiety="lac repressor encoded by lacI"
                     /note="The lac repressor binds to the lac operator to
                     inhibit transcription in E. coli. This inhibition can be
                     relieved by adding lactose or
                     isopropyl-beta-D-thiogalactopyranoside (IPTG)."
     promoter        complement(13023..13053)
                     /label=lac promoter
                     /note="promoter for the E. coli lac operon"
     protein_bind    13068..13089
                     /label=CAP binding site
                     /bound_moiety="E. coli catabolite activator protein"
                     /note="CAP binding activates transcription in the presence
                     of cAMP."
     rep_origin      complement(13377..13965)
                     /direction=LEFT
                     /label=ori
                     /note="high-copy-number ColE1/pMB1/pBR322/pUC origin of
                     replication"
     CDS             complement(14136..14996)
                     /codon_start=1
                     /gene="bla"
                     /product="beta-lactamase"
                     /label=AmpR
                     /note="confers resistance to ampicillin, carbenicillin,
and
                     related antibiotics"

/translation="MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYI

ELDLNSGKILESFRPEERFPMMSTFKVLLCGAVLSRIDAGQEQLGRRIHYSQNDLVEYS

PVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRW

EPELNEAIPNDERDTTMPVAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSA
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                     /label=AmpR promoter
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      121 gcgcgagcaa aatttaagct acaacaaggc aaggcttgac cgacaattgc atgaagaatc
      181 tgcttagggt taggcgtttt gcgctgcttc gcgatgtacg ggccagatat acgcgttgac
      241 attgattatt gactagttat taatagtaat caattacggg gtcattagtt catagcccat
      301 atatggagtt ccgcgttaca taacttacgg taaatggccc gcctggctga ccgcccaacg
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      661 ttgactcacg gggatttcca agtctccacc ccattgacgt caatgggagt ttgttttggc
      721 accaaaatca acgggacttt ccaaaatgtc gtaacaactc cgccccattg acgcaaatgg
      781 gcggtaggcg tgtacggtgg gaggtctata taagcagcgc gttttgcctg tactgggtct
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      901 aagcctcaat aaagcttgcc ttgagtgctt caagtagtgt gtgcccgtct gttgtgtgac
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3. pCMV_ACRCnu

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ACCESSION   .
VERSION     .
KEYWORDS    .
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 9890)
  AUTHORS   Juan-Carlos Collantes
  TITLE     Direct Submission
  JOURNAL   Exported Oct 8, 2018 from SnapGene Viewer 4.2.6
            http://www.snapgene.com
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4. pCMV_ACRCvnu

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DEFINITION  pCMV_ACRCvnu synthetic circular DNA
ACCESSION   .
VERSION     .
KEYWORDS    .
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 9890)
  AUTHORS   Juan-Carlos Collantes
  TITLE     Direct Submission
  JOURNAL   Exported Oct 8, 2018 from SnapGene Viewer 4.2.6
            http://www.snapgene.com
FEATURES             Location/Qualifiers
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and
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  TITLE     Direct Submission
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6. pCMV_^{A}CRCnu.2

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VERSION     .
KEYWORDS    .
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  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 9303)
  AUTHORS   Juan-Carlos Collantes
  TITLE     Direct Submission
  JOURNAL   Exported Aug 14, 2018 from SnapGene Viewer 4.2.3
            http://www.snapgene.com
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REFERENCE   1  (bases 1 to 9333)
  AUTHORS   Juan-Carlos Collantes
  TITLE     Direct Submission
  JOURNAL   Exported Aug 14, 2018 from SnapGene Viewer 4.2.3
            http://www.snapgene.com
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VERSION     .
KEYWORDS    .
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 9294)
  AUTHORS   Juan-Carlos Collantes
  TITLE     Direct Submission
  JOURNAL   Exported Aug 14, 2018 from SnapGene Viewer 4.2.3
            http://www.snapgene.com
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VERSION     .
KEYWORDS    .
SOURCE      synthetic DNA construct
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REFERENCE   1  (bases 1 to 9994)
  AUTHORS   Juan-Carlos Collantes
  TITLE     Direct Submission
  JOURNAL   Exported Aug 14, 2018 from SnapGene Viewer 4.2.3
            http://www.snapgene.com
FEATURES             Location/Qualifiers
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10. pCMV_^{A1}CRCnu.2

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ACCESSION   .
VERSION     .
KEYWORDS    .
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 9393)
  AUTHORS   Juan-Carlos Collantes
  TITLE     Direct Submission
  JOURNAL   Exported Aug 14, 2018 from SnapGene Viewer 4.2.3
            http://www.snapgene.com
FEATURES             Location/Qualifiers
     source          1..9393
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                     /note="human cytomegalovirus (CMV) immediate early
                     promoter"
     misc_feature    353..1938
                     /label=Synthetic
     promoter        377..395
                     /label=T7 promoter
                     /note="promoter for bacteriophage T7 RNA polymerase"
     regulatory      445..454
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                     of translation (Kozak, 1987)"
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                     40) large T antigen"
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/translation="SSETGPVAVDPTLRRRIEPHEFEVFFDPRELRKETCLLYEINWGG

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                     /note="The lac repressor binds to the lac operator to
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                     relieved by adding lactose or
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                     of cAMP."
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and
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11. phU6_gRNA_6xMS2

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DEFINITION  synthetic circular DNA
ACCESSION   .
VERSION     .
KEYWORDS    .
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 3949)
  AUTHORS   Juan-Carlos Collantes
  TITLE     Direct Submission
  JOURNAL   Exported Aug 14, 2018 from SnapGene Viewer 4.2.3
            http://www.snapgene.com
FEATURES             Location/Qualifiers
     source          1..3949
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                     /mol_type="other DNA"
     rep_origin      complement(3..458)
                     /direction=LEFT
                     /label=f1 ori
                     /note="f1 bacteriophage origin of replication; arrow
                     indicates direction of (+) strand synthesis"
     primer_bind     600..616
                     /label=M13 fwd
                     /note="common sequencing primer, one of multiple similar
                     variants"
     promoter        626..644
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     promoter        696..936
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     misc_RNA        1070..1088
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     misc_RNA        1140..1158
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     promoter        complement(1528..1546)
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     primer_bind     complement(1567..1583)
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     protein_bind    1591..1607
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                     /bound_moiety="lac repressor encoded by lacI"
                     /note="The lac repressor binds to the lac operator to
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                     relieved by adding lactose or
                     isopropyl-beta-D-thiogalactopyranoside (IPTG)."
     promoter        complement(1615..1645)
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                     /note="promoter for the E. coli lac operon"
     protein_bind    1660..1681
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                     /bound_moiety="E. coli catabolite activator protein"
                     /note="CAP binding activates transcription in the presence
                     of cAMP."
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                     /note="high-copy-number ColE1/pMB1/pBR322/pUC origin of
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     CDS             complement(2634..3428)
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                     /gene="aph(3')-II (or nptII)"
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                     /note="confers resistance to neomycin, kanamycin, and G418
                     (Geneticin(R))"

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                     LATRDIAEELGGEWADRFLVLYGIAAPDSQRIAFYRLLDEFF"
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12. phU6_gRNA_2+4xMS2

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DEFINITION  synthetic circular DNA
ACCESSION   .
VERSION     .
KEYWORDS    phU6_sgRNA2.0_2xBbsI
SOURCE      synthetic DNA construct
  ORGANISM  synthetic DNA construct
REFERENCE   1  (bases 1 to 3859)
  AUTHORS   Juan-Carlos Collantes
  TITLE     Direct Submission
  JOURNAL   Exported Aug 14, 2018 from SnapGene Viewer 4.2.3
            http://www.snapgene.com
FEATURES             Location/Qualifiers
     source          1..3859
                     /organism="synthetic DNA construct"
                     /mol_type="other DNA"
     rep_origin      complement(3..458)
                     /direction=LEFT
                     /label=f1 ori
                     /note="f1 bacteriophage origin of replication; arrow
                     indicates direction of (+) strand synthesis"
     primer_bind     600..616
                     /label=M13 fwd
                     /note="common sequencing primer, one of multiple similar
                     variants"
     promoter        626..644
                     /label=T7 promoter
                     /note="promoter for bacteriophage T7 RNA polymerase"
     promoter        696..936
                     /label=U6 promoter
                     /note="RNA polymerase III promoter for human U6 snRNA"
     misc_feature    866..1434
                     /label=Synthetic
     primer_bind     866..889
                     /label=CRC2_U6sg-F
     misc_RNA        980..998
                     /label=MS2 stem loop
                     /note="stem loop that binds the bacteriophage MS2 coat
                     protein"
     misc_RNA        1050..1068
                     /label=MS2 stem loop
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                     protein"
     misc_RNA        1120..1138
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                     protein"
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     misc_RNA        1190..1208
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     misc_RNA        1330..1348
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     primer_bind     complement(1417..1434)
                     /label=CRC2_U6sg-R
     promoter        complement(1438..1456)
                     /label=T3 promoter
                     /note="promoter for bacteriophage T3 RNA polymerase"
     primer_bind     complement(1477..1493)
                     /label=M13 rev
                     /note="common sequencing primer, one of multiple similar
                     variants"
     protein_bind    1501..1517
                     /label=lac operator
                     /bound_moiety="lac repressor encoded by lacI"
                     /note="The lac repressor binds to the lac operator to
                     inhibit transcription in E. coli. This inhibition can be
                     relieved by adding lactose or
                     isopropyl-beta-D-thiogalactopyranoside (IPTG)."
     promoter        complement(1525..1555)
                     /label=lac promoter
                     /note="promoter for the E. coli lac operon"
     protein_bind    1570..1591
                     /label=CAP binding site
                     /bound_moiety="E. coli catabolite activator protein"
                     /note="CAP binding activates transcription in the presence
                     of cAMP."
     rep_origin      complement(1879..2467)
                     /direction=LEFT
                     /label=ori
                     /note="high-copy-number ColE1/pMB1/pBR322/pUC origin of
                     replication"
     CDS             complement(2544..3338)
                     /codon_start=1
                     /gene="aph(3')-II (or nptII)"
                     /product="aminoglycoside phosphotransferase from Tn5"
                     /label=NeoR/KanR
                     /note="confers resistance to neomycin, kanamycin, and G418
                     (Geneticin(R))"

/translation="MIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRP

VLFVKTDLSGALNELQDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLS

SHLAPAEKVSIMADAMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQ

GLAPAELFARLKASMPDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIA
                     LATRDIAEELGGEWADRFLVLYGIAAPDSQRIAFYRLLDEFF"
     promoter        complement(3373..3730)
                     /label=SV40 promoter
                     /note="SV40 enhancer and early promoter"
     rep_origin      3387..3522
                     /label=SV40 ori
                     /note="SV40 origin of replication"
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     promoter        complement(3732..3836)
                     /gene="bla"
                     /label=AmpR promoter
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      121 gatagggttg agtgttgttc cagtttggaa caagagtcca ctattaaaga acgtggactc
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      961 ctgtttaaga gctaggccaa catgaggatc acccatgtct gcagggccta gcaagtttaa
     1021 ataaggctag tccgttatca acttggccaa catgaggatc acccatgtct gcagggccaa
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     1981 tggaagctcc ctcgtgcgct ctcctgttcc gaccctgccg cttaccggat acctgtccgc
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     2821 ctggcgaaca gttcggctgg cgcgagcccc tgatgctctt cgtccagatc atcctgatcg
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     3181 aggtcggtct tgacaaaaag aaccgggcgc ccctgcgctg acagccggaa cacggcggca
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     3781 atgagcggat acatatttga atgtatttag aaaaataaac aaataggggt tccgcgcaca
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//
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