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CRISPR-Guided DNA Polymerases for Targeted Genomic
Diversification in Mammalian Cells

by

Juan Eduardo Hurtado Koolik

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Joint Doctor of Philosophy
with the University of California, San Francisco

in

Bioengineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor John E. Dueber, Co-Chair
Professor David V. Schaffer, Co-Chair
Professor David F. Savage
Professor Seth Shipman

Summer 2024

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Abstract

CRISPR-Guided DNA Polymerases for Targeted Genomic Diversification in Mammalian Cells

by

Juan Eduardo Hurtado Koolik

Joint Doctor of Philosophy in Bioengineering with the University of California, San Francisco
and University of California, Berkeley

Professor John E. Dueber, Co-Chair; Professor David V. Schaffer, Co-Chair

Directed evolution has long been impactfully leveraged to engineer therapeutics, industrial enzymes and other revolutionary biotechnologies. Unfavorably, the application of directed evolution towards engineering complex phenotypes in mammalian cells, such as kinase signaling cascades or antitumor T cell cytotoxicity for adoptive cell therapies has been limited by a lack of appropriate biomolecular tools for continuous targeted genomic diversification in mammalian cell genomes. Most critically, existing gene editing modalities for continuous diversification in mammalian cells rely exclusively on targeting deaminases to target loci to generate transition mutations (A to G, G to A, C to T, T to C), limiting the nucleotide substitutions that can be generated to a small minority of possible missense mutations. To enable the full diversification of all four nucleotides within native mammalian genomic loci for the first time, I engineered and characterized the use of RNA-guided error-prone DNA polymerases (EvolvR) in mammalian cells. Using a cell-based fluorescent reporter fluctuation assay and next-generation sequencing, I demonstrated that EvolvR can diversify all four nucleotides throughout a gRNA spacer to generate both transition and transversion substitutions. Then, I implement EvolvR to diversify a native genomic locus and select for previously unidentified selumetinib-resistant MAP2K1 variants in melanoma-derived A375 cells. Additionally, I elucidate the role of nickase fidelity and gRNA length as critical features determining the diversity of EvolvR's editing outcomes and expand the EvolvR's ease of use by identifying a compatible compact, PAM-flexible nuclease the cDNA for which can be packaged into a lentiviral vector to initiate directed evolution in human cell lines with a single transduction. Together, my data show that RNA-guided DNA polymerases can access all 12 nucleotide substitutions to identify gain of function missense mutations not reachable by deaminase-based diversifiers in mammalian cells and establish a novel state-of-the-art modality for genetically engineering mammalian cells via directed evolution.

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Help along the way has come from too many sources to count. Below is my attempt to enumerate the many contributions to this dissertation over the years.

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Thank you especially to John Dueber for countless lively conversations at the bench and for always being game to vigorously challenge me both scientifically and philosophically, which has without a doubt helped me grow as a thinker. Your stamina for diving into the nitty gritty on a moment's notice and remaining there for long periods of time taught me the value of frequent, open, and fearless conversations for arriving at mental models of how things work.

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1. Introduction

1.1. Classical directed evolution:

Throughout Earth's natural history, populations of organisms have evolved through a parallel continuous process of mutation and selection pressures favoring the reproduction of fitness-conferring traits, giving rise to all known terrestrial biodiversity. Human exploitation of evolution to engineer useful organisms predates history, having begun with the domestication of canids at least 33,000 years ago via artificial selection applied by feeding and housing cooperative and docile wolves¹. Though plants and animals were evolved by artificial selection prehistorically, it was not until 1965 that Sol Spiegelman first demonstrated the use of artificial selection to evolve a simple biomolecule known as "Spiegelman's Monster", a 218 nucleotide-long RNA chain that emerged throughout 74 replication cycles of Q β bacteriophage's 4.5 kb-long RNA genome in solution with RNA replicase and free nucleotides², elucidating the superior replicative fitness of shorter RNA sequences in Spiegelman's experiments. Later in the 20th century, pioneers began intentionally hypermutating genes of interest and screening populations of genetic variants for proteins with new or enhanced biomolecular function, a method now referred to as directed evolution.

Directed evolution classically begins with the diversification of genes encoding biomolecules of interest in vitro, typically by using error-prone PCR³, DNA shuffling⁴, or saturation mutagenesis⁵. These genes are stably installed within cells or viruses, as replicating extrachromosomal elements or genomically-integrated transgenes, such that a given variant's cDNA sequence is necessarily coupled to the host's expression of that variant. Upon expression of the genetic library, researchers apply selective pressures to the host population that enrich the population for genetic variants with improved biomolecular functionality, either by negative selections that kill or eliminate dysfunctional variants or by positive selections that enhance the persistence or reproduction of functional variants to the degree that they execute the desired functionality. Such functionalities famously include enhanced or novel catalytic activity in enzymes for biomanufacturing⁶, specific and high-affinity protein-ligand^{7,8}, protein-protein⁹, and protein-nucleic acid¹⁰ binding for research and therapeutic applications, and improved infectivity, tissue-specific tropism and titers of engineered viral vectors for the delivery of gene therapies to target cells^{11,12,13}.

Advantageously, iterative cycles of diversification and selection for desired functionalities can be executed without prior knowledge of the mechanisms underlying these functionalities. As such, directed evolution is a powerful technique in its capacity to enable the engineering and fine-tuning of incompletely understood biological properties, such as viral tropism and intracellular trafficking.

1.2. In vivo continuous directed evolution

Unlike natural evolution, directed evolution is executed in cycles comprising discrete steps of library diversification, delivery of the library into a host population, selection, and library rescue whereby the genetic library is harvested from the host for sequencing or additional rounds of diversification. The discontinuity between each step introduces bottlenecks in the evolutionary

search process, limiting the diversity of evolutionary paths that can be explored via directed evolution. One such bottleneck is the delivery of the library to genetic hosts, where the diversity of variants that can be tested is limited by the number of host cells that can be transformed. Additionally, rescuing the library from the host population can stochastically remove potentially valuable neutral mutations from the gene pool, as the diversity of variants that can be tested in following rounds is limited to the number of variants that remain after bulk library extraction from the genetic host, reintroduction of the library into expression cassettes, and reamplification of the library through transformation of a cloning host (e.g. *E. coli*). Finally, multiple rounds of directed evolution are labor-intensive, limiting both the number of genes of interest for which directed evolution can be simultaneously performed and the practical duration of directed evolution campaigns.

To eliminate diversity bottlenecks related to the transformation of genetic hosts or library rescue and to improve the scalability of directed evolution campaigns, researchers developed technologies enabling *in vivo* continuous evolution, whereby target DNA is hypermutated directly in host cells as they await or actively undergo selection¹⁴. *In vivo* continuous directed evolution campaigns requires generating cell lines containing both target DNA and transgenes for expressing hypermutators consisting of either error-prone DNA replication machinery^{15,16,17,18,19}, or chemical mutagenesis^{20,21,22,23,24,25}.

Though these hypermutators can be overexpressed to simply increase global mutation rates in host cells and nonspecifically diversify genes of interest in the process^{20,26}, the innovation of diversifiers that hypermutate genes in a targeted fashion has afforded multiple advantages. By mitigating increases in genome-wide mutation rates, researchers can engineer increasingly mutagenic diversifiers while minimizing toxicity associated with deleterious mutations throughout the host's genome. Moreover, minimizing the frequency of mutations outside of the gene of interest (GOI) lowers the probability of generating "cheater" variants that acquire fitness advantages via unintended mechanisms due to mutations outside of the GOI. To date, error-prone DNA synthesis has been targeted to genes of interest using orthogonal error-prone polymerases (OrthoRep, AdPol, VEGAS)^{16,18,19} and CRISPR-guided error-prone DNA polymerases (EvolvR)¹⁷, while nucleobase deamination has been targeted to genes of interest using CRISPR-guided deaminases (CRISPR-X, dCas9-AIDx)^{22,23}, T7-RNA polymerase-guided deaminases (MutaT7, eMutaT7, T7-DIVA, TRIDENT, TRACE)^{27,28,29,30,31}, and most recently helicase-guided deaminases (CoMuTER, HACE)^{24,25}.

Because the target DNA either exists natively in the host cell's genome or is genetically engineered into the host cell line prior to beginning evolution, diversity is generated continuously throughout the entire cell population and can, in principle, be limited only by the number of cells being cultured. Additionally, continuous diversification and selection allows early "hits" to seamlessly gain additional gain-of-function mutations, allowing unperturbed exploration of arbitrarily distant fitness peaks for as long as the host cells can be propagated.

1.3. Problem statement and motivation

The directed evolution of new biomolecular properties requires selective pressure that faithfully interrogates these properties. Though biological properties such as protein-protein binding and enzyme catalysis may depend relatively little on the host cell expressing it, other

biomolecular properties of great biotechnological interest can only be interrogated under species and cell type-specific biological contexts. Examples of functionality relying on specific biological contexts include the signaling kinetics of transmembrane receptors such as GPCRs and synthetic T cell receptors^{32,33}, and novel orthogonal receptor-ligand signaling³⁴.

To reap the advantages of *in vivo* continuous evolution for engineering human cell-specific biomolecular functionality, researchers have invented continuous diversifiers to enable targeted hypermutation in human cell lines. Existing targeted hypermutators for *in vivo* continuous evolution in mammalian cells consist of either orthogonal viral polymerases to diversify genes of interest in viral genomes or CRISPR-guided nucleobase deaminases. Disadvantageously, neither of these modalities are completely suitable for diversifying all four nucleotides in mammalian genomic loci.

Orthogonal viral error-prone polymerase-based diversifiers (AdPol, VEGAS)^{18,19}, which work by coupling a gene of interest's function to viral replication of adenovirus or alphavirus Sindbis rely on orthogonal viral error-prone polymerases to diversify all four nucleotides within genes of interest that are integrated within viral genomes. Unfavorably, the necessary use of viral replication as a means of selection limits the use of AdPol and VEGAS to applications in which the gene of interest's functionality can be coupled to viral replication and enables cheater variants to confound directed evolution campaigns³⁵. Moreover, diversifying genes within viral genomes relinquishes the advantages afforded by diversifying human genes of interest within their native loci. These advantages include the capacity to assess functional variants under transcriptionally-normalized and clinically-relevant expression strengths, in addition to the potential for investigating functional variation arising from the perturbation of splicing and regulatory elements²⁵. Finally, the use of viruses as the unit of selection could be perturbative in directed evolution campaigns in immunologically sensitive cell lines, as interactions with toll-like receptors and other components of innate immunity could undesirably affect the functionality of variants that rely on specific transcriptional states (e.g. differentiation in primary T cells).

In contrast to viral directed evolution systems, CRISPR-targeted nucleobase deaminases have the capacity to diversify native genomic loci in mammalian cells (CRISPR-X, dCas9-AIDx, HACE)^{22,23,25} by recruiting cytosine or adenosine deaminases to mutate target loci. However, the reliance of these tools on deaminases critically limits the diversity of substitutions that can be efficiently generated to transition mutations (C to T, A to G, G to A, T to C), forfeiting access to the vast majority of missense mutations (Figure 1).

Therefore, a technology enabling the installation of all 4 nucleotides to generate all 12 substitutions would enable unprecedented access to previously untapped diversity spaces during *in vivo* continuous directed evolution campaigns in mammalian cells.

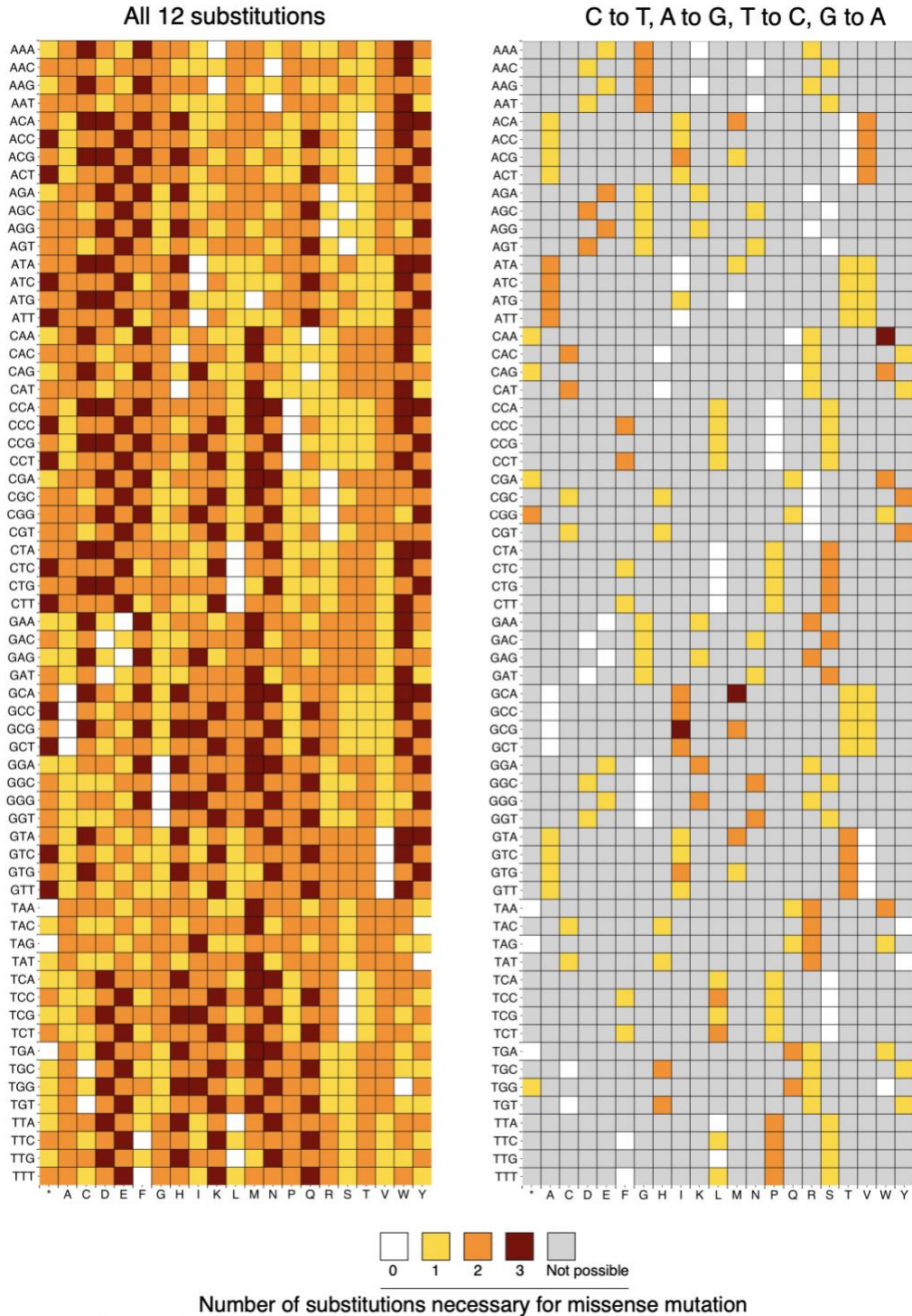


Figure 1. This heatmap shows the minimum number of substitutions necessary for converting each of 64 codons to encode any of the other 19 amino acids or stop codons. The left heatmap represents missense mutations accessible with all 12 substitution. The right heatmap represents missense mutations accessible using only transition mutations.

1.4. Approach and summary of work completed

The ideal diversifier enables continuous, unbiased substitution of nucleotides at user-defined loci, within a controllable window and at controllable mutation rates.

CRISPR-guided DNA polymerases, previously described as “EvolvR”¹⁷ localize error-prone DNA synthesis to user-defined loci by generating a priming nick at a target site. Typically, enhanced SpCas9 nickase (eSpnCas9 D10A) is fused to an error-prone *E. coli* PolI and directed by a gRNA to generate a single-stranded break at a target locus. After dissociating, PolI is believed to utilize the nicked strand’s exposed 3’ end as a primer for low-fidelity DNA synthesis, displacing the incumbent strand as it generates substitution mutations (Figure 2). Substitutions are “locked in” upon error-prone DNA repair or cell division, generating new library members. In *E. coli*, EvolvR generates substitution mutations at approximately 10,000-fold the background mutation rate¹⁷ within mutation windows that are tunable between 0-50 base pairs (and up to 347 bp at lower mutation rates) by polymerase engineering.

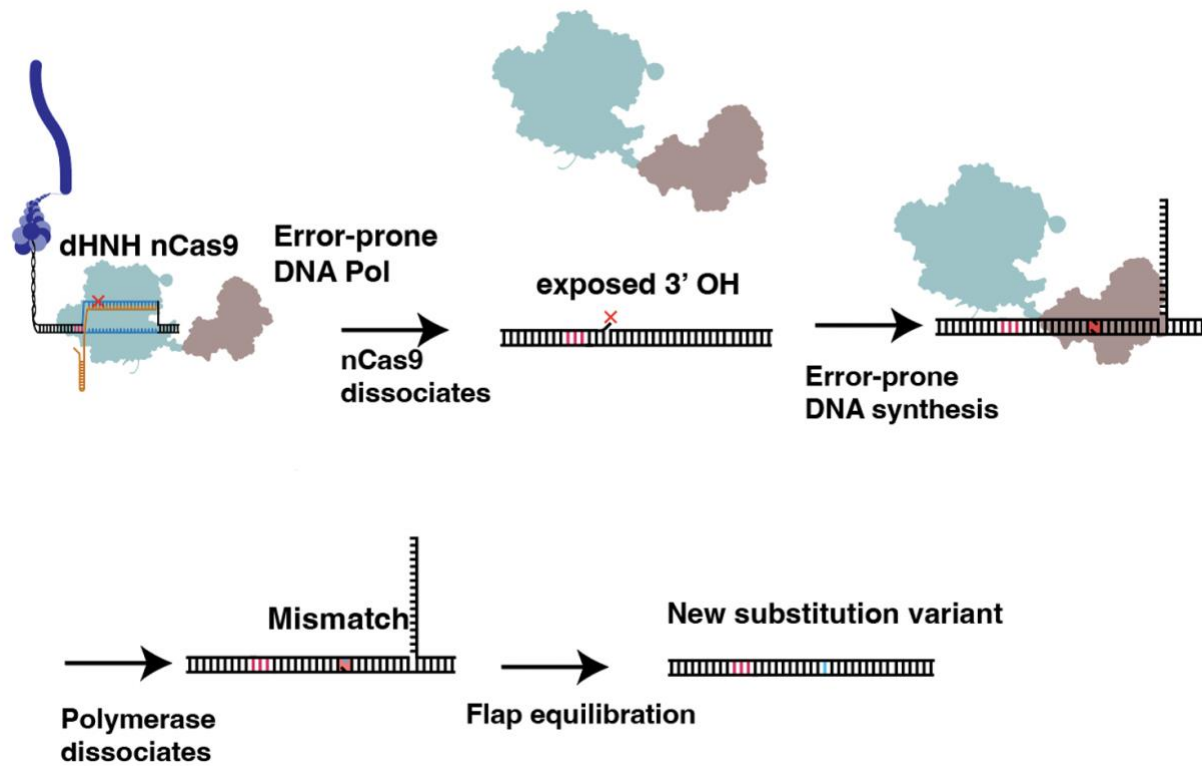


Figure 2. EvolvR consists of a Cas9 with an inactivated HNH domain (nCas9) fused to an error-prone DNA polymerase. nCas9 generates an RNA-guided single-stranded break in the gRNA target strand. After nCas9 dissociation, the error-prone DNA polymerase performs error-prone DNA synthesis, displacing the incumbent strand and generating substitution errors in the process. Pink lines represent the position of the PAM site. Red crosses represent the presence of a Cas9-generated single-stranded break.

EvolvR's functionality affords numerous potential advantages as a tool for forward genetics and directed evolution within mammalian cells. By directly diversifying genomic loci, EvolvR eliminates screening bottlenecks related to inefficiencies in library delivery. Furthermore, EvolvR's multiplexability allows continuous mutagenesis for as long as at least one gRNA target region is sufficiently intact for its corresponding gRNA to allow nicking, enabling multiple rounds of diversification and the screening of combinatorial libraries. EvolvR's short mutation window per target site also enables the construction of domain-specific libraries, which may be desirable for saturating mutagenesis efforts in known functional protein domains. Finally, unlike targeted somatic hypermutators, EvolvR relies on error-prone DNA synthesis for mutagenesis and is less biased than deaminase-based technologies in the substitution space it can traverse.

Here, I engineer EvolvR to generate diversity in mammalian cells with comparable efficiency and window lengths per gRNA as those reported in *E. coli*. I use EvolvR to identify previously undescribed G>A, G<T, G>C, T>A, and A>C substitution-derived selumetinib resistance MAP2K1 variants in A375 melanoma cells. Then, I examine the effects of gRNA length and nickase variant fidelity on EvolvR's mutation window and positional substitution biases, using this knowledge to draw new insight on EvolvR's mechanism of action. In parallel, I show that EvolvR can be assembled using PAM-flexible Cas9 orthologs, drastically increasing the number of gRNAs that can be used to diversify a given nucleotide.

My work presents the first tool for diversifying all four nucleotides efficiently within native genomic loci in mammalian cells and improves substantially upon EvolvR's original design by enabling PAM-flexible (NNG) targeting of EvolvR via constitutive expression, increasing the availability of potential target loci and setting the groundwork for initiating long-term directed evolution campaigns in mammalian cells.

2. Characterizing EvolvR's mutagenesis in mammalian cells

2.1. First iteration of EvolvR design for expression in mammalian cells

To test whether CRISPR-guided DNA polymerases can diversify targeted genomic loci in human cells, I designed a modified version of the eSpnCas9-PolI5M construct originally described¹⁷, fusing two nuclear localization sequences along with an mCherry fluorescent reporter tag. Additionally, aiming to reduce the length of EvolvR's cDNA for packaging into lentiviral vectors, I excluded the N-terminal flap endonuclease domain on PolI5M to determine whether the remaining Klenow fragment alone is sufficient for EvolvR-mediated mutagenesis (Figure 3).

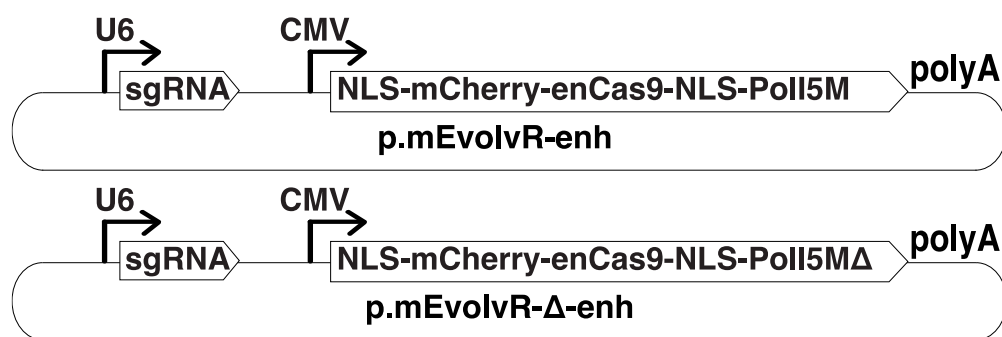


Figure 3. Plasmids encoding EvolvR for expression in mammalian cells was constructed as an mCherry-tagged enhanced nicking Cas9 (Cas9 D10A, K848A, K1003A, R1060A) flanked by two nuclear localization sequences and fused to PolI5M's Klenow fragment. PolI5M Δ indicates the deletion of the first 325 amino acids from PolI5M.

2.2. Validating a BFP to GFP fluorescent reporter assay for measuring the frequency of variants generated by EvolvR in HEK293 cells

To rapidly and sensitively measure the frequency of single substitution variants generated by EvolvR, I used a fluorescent reporter for gene editing in HEK293 cells expressing a genomically-integrated, single-copy of a blue fluorescent protein (BFP) gene with a plasmid encoding strong expression of EvolvR (Figures 3 and 4) along with a 20 nt-long gRNA (henceforth referred to as “gBFP15”) directing a nick 15 bp upstream of a T which, upon undergoing a C->T H42Y missense mutation, results in a transition to green fluorescence (Figure 5). 6 days after transfection, I measured the frequency of GFP positive cells by flow cytometry to compare the performance of different versions of EvolvR to various controls (Figure 6). As expected, conditions in which enCas9 and PolI5M were not fused and targeted to the BFP locus did not significantly increase the frequency of GFP + cells when compared to cells expressing mCherry alone (Figure 6). Additionally, EvolvR constructed with wildtype nCas9 fused to PolI5M did not consistently generate GFP positive cells. In contrast, all three EvolvR variants consisting of enCas9 fused to either PolI3M, PolI5M, or PolI5M Δ consistently generated GFP positive cells in three out of three replicates. Notably, EvolvR lacking a flap endonuclease domain generated the highest mean frequency of GFP cells in the final population, though no differences between enCas9-PolI3M, enCas9-PolI5M, or enCas9-PolI5M Δ were statistically significant. These data suggest that PolI’s flap endonuclease domain is unessential for EvolvR’s targeted mutagenicity in mammalian cells, potentially due to redundancy with native flap exonucleases in the nucleus.

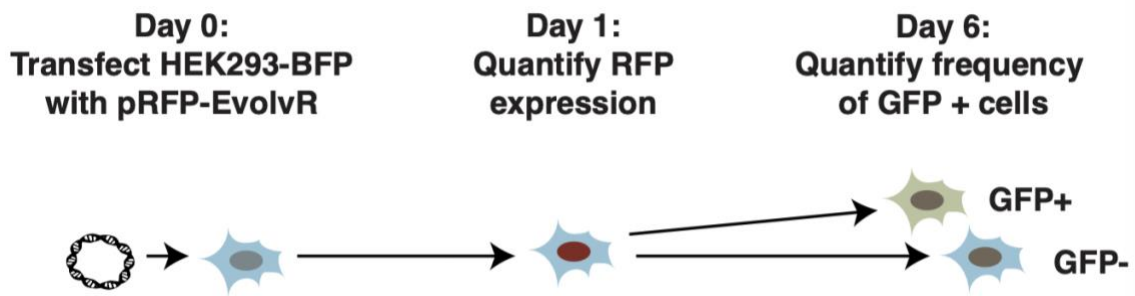


Figure 4. HEK293 cells expressing BFP are transfected with a plasmid encoding strong expression of mCherry-tagged EvolvR. Transfection efficiency is quantified the following day to enable normalization of the final frequency of GFP positive cells by the frequency of cells expressing EvolvR on day 1. On day 6, the cells are harvested and analyzed by flow cytometry to quantify the frequency of GFP + cells.



Figure 5. The diagram depicts a fluorescent reporter gene for EvolvR mutagenesis using an example gRNA generating a nick in the sense strand 15 bp in the 5' direction from the codon encoding residue H67. Substitution of a T for a C in H67 yields expression of GFP instead of BFP. The gRNA sequence is written in green. The PAM sequence is written in gold.

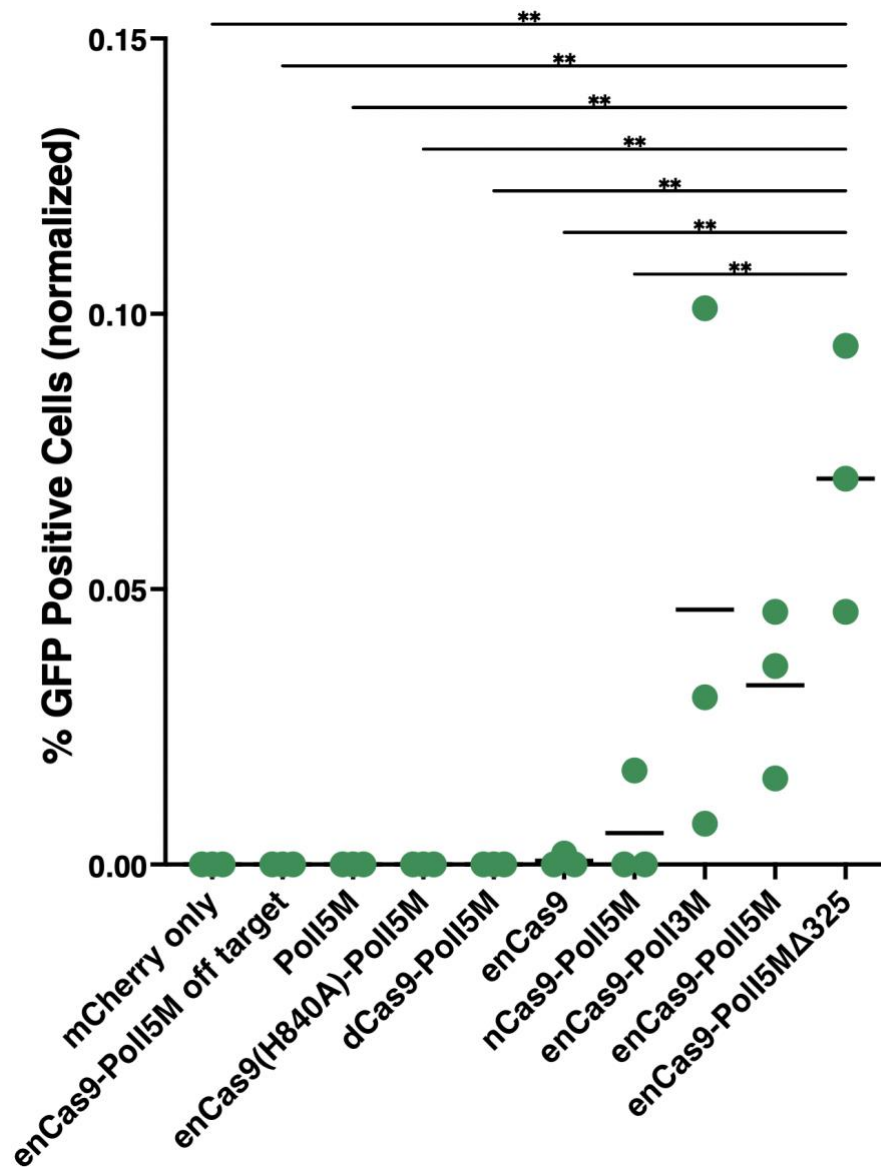


Figure 6. Both enCas9 and error-prone PolII are required for increasing the frequency of H67Y BFP to GFP mutations at the target locus. The percentage of GFP positive cells generated by each construct was measured by flow cytometry and normalized by transfection efficiency. Green dots represent separate biological replicates. Black lines represent mean % GFP + cells. Double asterisks (**) denote statistical significance with p values less than 0.01 as calculated using a one way ANOVA followed by a Tukey's HSD test. "Off target" indicates the expression of a gRNA targeting safe harbor locus AAVS1. All other constructs were co-expressed with a gRNA targeting a nick 15 bp upstream of the codon encoding H67.

2.3. Quantifying EvolvR's substitution window and positional substitution biases using deep amplicon sequencing.

Having demonstrated that EvolvR elevates mutation rates at a single nucleotide at a single user-defined genomic locus, I set out to measure the diversity generated at the target locus in a single transfection and to characterize EvolvR's mutation window via Illumina sequencing. I again transfected HEK293 BFP cells with plasmids encoding enCas9-PolI5MΔ and a gRNA targeting safe harbor locus AAVS1 or gBFP15. To ensure normalization of substitution frequencies across samples and enrich for substitution variants generated by EvolvR, cells expressing EvolvR were isolated by FACS of mCherry positive cells. After one week of growth, I harvested the genomic DNA of biological duplicates from each condition and quantified the frequency of substitutions at each position using amplicon sequencing (Figure 7). To minimize the frequency of false substitution variants arising from Illumina base calling errors, only perfectly matching paired-end reads were used for variant calling.

Coexpression of EvolvR with gBFP15 significantly elevated the substitution rate throughout the gRNA spacer and beyond compared to the off-target control (Figure 7). EvolvR's elevated substitution rates extended from the nick to the end of the region sequenced, though the frequency of substitutions at each position is markedly higher within the gRNA spacer sequence than outside of it.

Intriguingly, though EvolvR generated mutations asymmetrically in the direction expected during error-prone DNA synthesis, mutations in the opposite direction upstream of the nick were also detectable, albeit sparsely. This bidirectionality in mutagenesis was previously observed in *S. Cerevisiae*^{36,37}, though no model has yet been proposed for explaining this bidirectional mutation window. I postulate that bidirectional mutagenesis occurs due to PolI participation in DNA repair processes initiated during replication fork collisions with EvolvR-generated single-stranded breaks. Specifically, I suggest that PolI initiates error-prone transcription from free 3' ends remaining during MRE-mediated resection of double-stranded breaks, which are known to occur during replication fork collapse arising from single-stranded breaks^{38,39}.

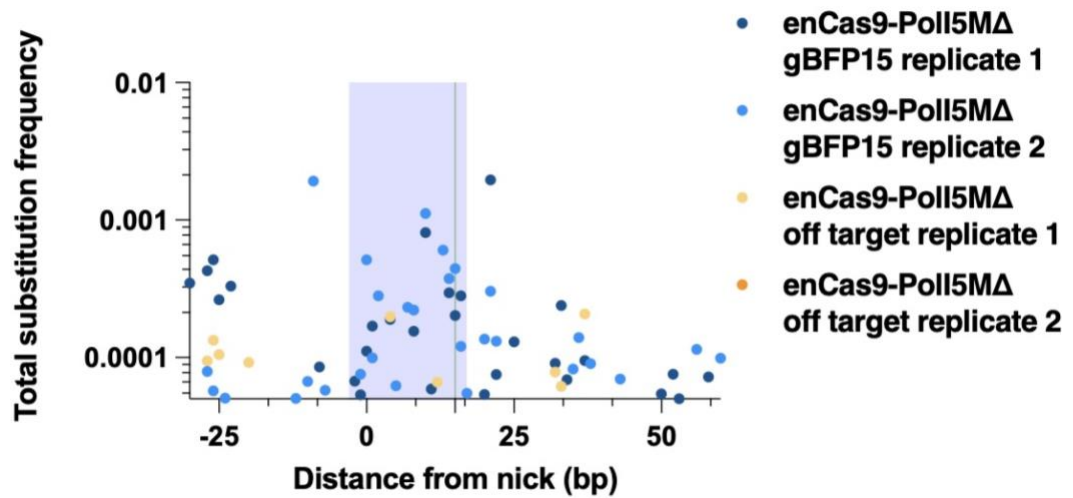


Figure 7. EvolvR elevates substitution rates throughout the gRNA target sequence and further downstream of the nick. The total frequency of substitution variants was quantified at each position within the BFP reference sequence. Positional coordinates are written with reference to the expected direction of the polymerase, where more positive numbers are further in the 3' direction with respect to the gRNA target strand. Position 0 corresponds to the position of the nick generated by enCas9. The blue rectangle marks the footprint of the gRNA target sequence. The green vertical line marks the position of the C to T substitution conferring the H67Y missense mutation.

To investigate EvolvR's substitution bias within its most prominent mutation window, I examined the substitutions that appeared at least twice per 100,000 reads across both biological replicates (Figure 8). Surprisingly, EvolvR's substitution biases were highly inconsistent across the spacer region with respect to the original nucleotide. For example, though G to A and G to C substitutions appeared in both biological replicates 13 bp downstream of the PAM site, the other two G's within the spacer downstream were only detectably mutated to A's, and only in one out of two biological replicates. Additionally, despite the presence of 8 cytosines downstream of the nick and within the spacer, only the C to T substitution conferring the H67Y mutation was detected in two out of two biological replicates.

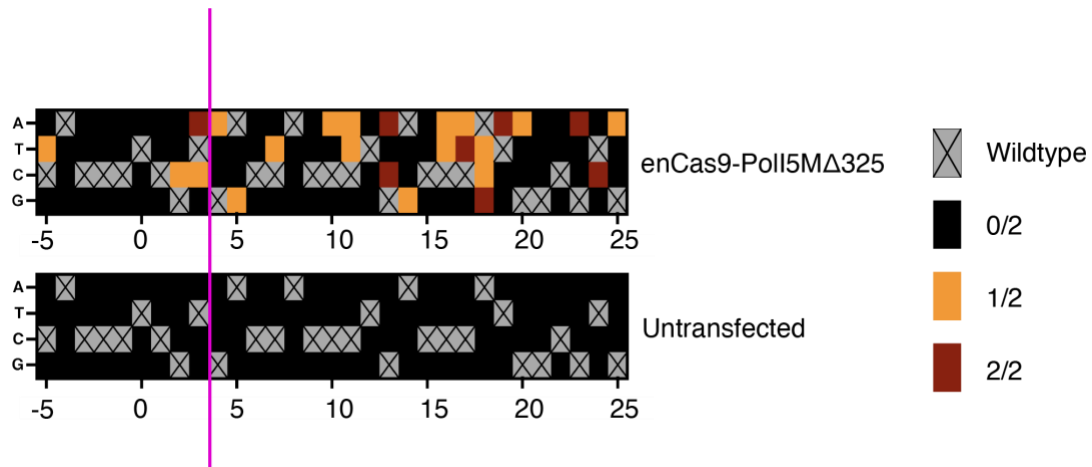


Figure 8. enCas9-PolI5MΔ generates most substitutions inconsistently throughout the gRNA spacer region. Gray boxes represent the BFP reference sequence. Numerical coordinates are set relative to the position of the first nucleotide of the PAM site. The pink vertical line represents the expected cut site of the gRNA used in this experiment. Black, orange, and red boxes represents that a given variant appeared in zero, one, or two out of two biological replicates per 100,000 reads after a background subtraction using the mean frequency of each substitution within an untransfected negative control.

3. Investigating the role of nickase fidelity and gRNA design on EvolvR's editing outcomes.

3.1. Hypothesis for the role of nickase mismatch tolerance on substitution biases and mutation window.

To explain the position bias of EvolvR's substitution frequencies, I first considered that SpCas9 frequently tolerates single mismatches within the 20-bp spacer region in a sequence-specific fashion, both within the seed region and PAM-distal bp⁴⁰. Though the enhanced nicking Cas9 (enCas9) used in EvolvR is derived from an engineered enhanced Cas9 variant (eSpCas91.1)⁴¹ exhibiting lower mismatch tolerance due to its impaired sampling of the docked HNH conformational state necessary for DNA cleavage⁴², its improved fidelity is largely limited to PAM-distal nucleotides and fails to necessarily prevent cleavage of mismatched targets⁴³. These insights led me to envision that substitutions generated by PolI5M in the target strand are frequently tolerated by enCas9, allowing nascent mutation-containing target strands to be re-nicked and consequently resynthesized by PolI5M using the wildtype sequence as a template. Through this process, I hypothesized that substitutions leading to tolerable mismatches allowing continued nicking of the spacer region are frequently overwritten by EvolvR, while those that are not tolerated remain undisturbed to either be repaired by mismatch repair or "locked in" upon cell division. I henceforth refer to this mechanism as the "mismatch tolerance bias" (MTB) model for EvolvR's positional substitution biases (Figure 9).

While the mechanism for enCas9-PolI5M's apparently higher single substitution rates than wild type nCas9-PolI5M has thus far remained mysterious, I note that the MTB model explains their difference in substitution rates both in the fluctuation assays originally described in *E. coli*¹⁷ and in the BFP to GFP assay (Figure 4), especially because both assays measure EvolvR's substitution rates in PAM-distal nucleotides, where eSpCas91.1's nicking fidelity is highest relative to wild type SpCas9⁴³.

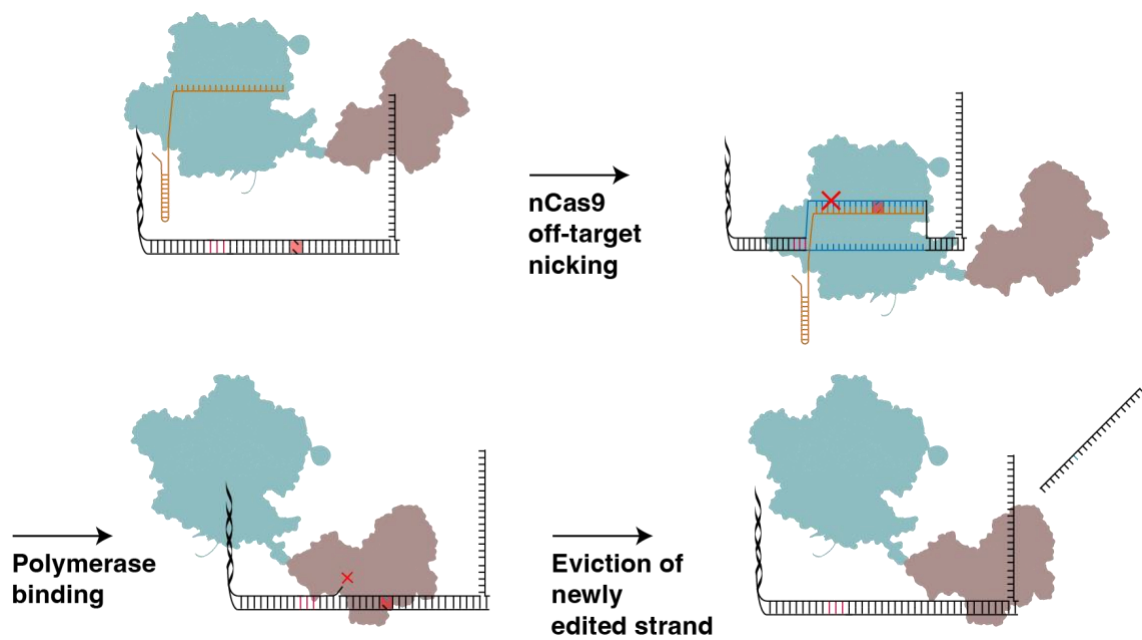


Figure 9. Diagram depicts proposed mechanism for EvolvR substitution biases arising from nCas9's mismatch tolerance. Target strands harboring substitution errors may undergo additional nicking and strand displacement by PolII, resulting in the eviction of substitutions that are tolerated by nCas9.

3.2. Enhancing nCas9-PolI5MΔ substitution rates by truncating gRNAs.

To test the validity of the MTB model, I set out to assess whether improving the fidelity of nCas9 would enhance its capacity to generate GFP positive cells when fused to PolI5MΔ. Cas9's fidelity has previously been improved by using truncated gRNAs to direct cutting, which has been shown to decrease Cas9's off-target activity without decreasing its on-target efficiency for certain gRNAs⁴⁴. Moreover, prior work in *E. coli* has demonstrated an improvement in the efficiency of single-nucleotide genomic edits using Cas9-induced homology-directed repair (HDR) when using 5'-truncated 18 nt-long gRNAs⁴⁵. To test whether the efficiency of single nucleotide edits can also be improved by supplying nCas9 with truncated gRNAs, I targeted nCas9-PolI5M using gRNAs truncated by 2 nt at their 5' end using the BFP to GFP assay. As expected, I measured a drastic elevation in nCas9-PolI5M's mutation rates at the H67 locus when the gRNA was truncated (Figure 10). In contrast, enCas9-PolI5M showed no evidence of mutagenesis when directed by a truncated gRNA, presumably attributable to eSpCas9(1.1)'s sensitivity to loss of PAM-distal gRNA-DNA binding.

As predicted by the MTB model, both BFP to GFP editing rates and the amplicon sequencing of the target locus indicated an increase in the number of unique variants when nCas9-PolI5M targeted with a truncated gRNA compared to when it was targeted by a full-length gRNA (Figures 10, 11, and 12). This finding suggests that as the number nickase-intolerable mismatches decreases, so does the number of variants that can be efficiently generated.

In addition to increasing the total frequency of substitution variants, the use of a truncated gRNA also drastically decreased the frequency of indel variants compared to cells treated with full-length gRNAs (Figure 13). This finding suggests that reducing the number of tolerable substitution variants facilitates installation of substitution variants before the appearance of indels, which are known to be far more intolerable to Cas9 nuclease activity than substitutions^{43,46,47}. More succinctly, reducing the mismatch tolerance of Cas9 appears to redirect mutagenic flux from indels to substitutions.

Additionally, as EvolvR can only be intolerant of substitutions that appear within the spacer region of its gRNA, its mutation rate should be markedly lower outside of its spacer region. Indeed, enCas9-PolI5M's mutation window appears to contain the highest frequency variants within the gRNA spacer window both within the data shown here and in prior work^{17,36}.

Together, the data show that EvolvR's biases are heavily influenced by protospacer sequence-specific and nickase-specific cutting fidelities, and that current measurements of EvolvR's substitution biases are not actually reflective of polymerase biases alone. The MTB model predicts that EvolvR's propensity to generate a given substitution variant should be inversely correlated to its tolerance of that substitution mutation within the spacer region (Figure 14).

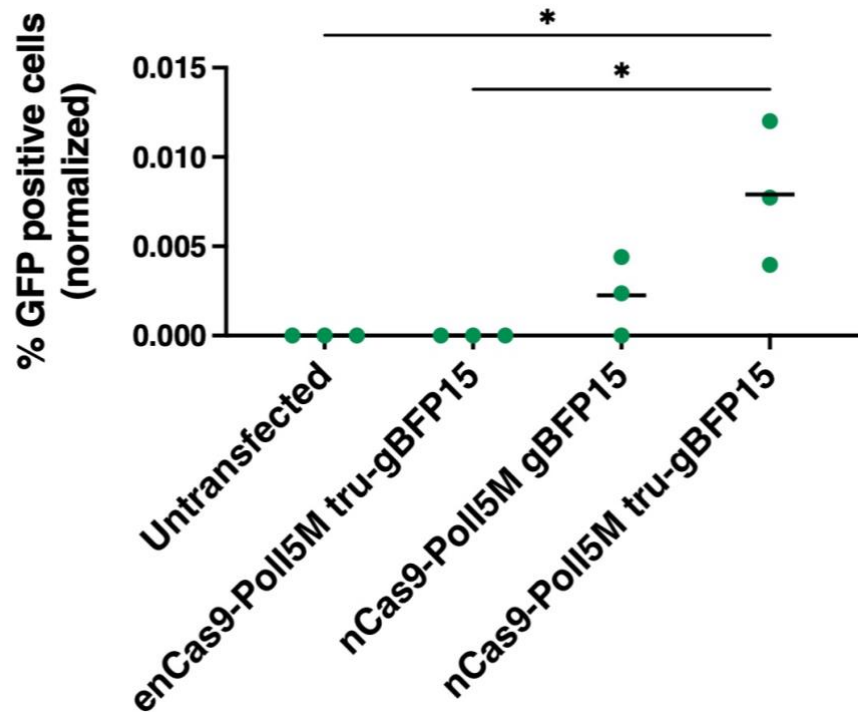


Figure 10. Truncating gBFP15 enhances the frequency of EvolvR-mediated H67Y substitutions when a wildtype nicking Cas9 is fused to Poll but not when a high-fidelity nickase is fused to Poll. The percentage of GFP + cells generated by each construct was measured by flow cytometry and normalized by transfection efficiency. Green dots represent separate biological replicates. Black lines represent mean % GFP positive cells. Asterisks (*) denote statistical significance with p values less than 0.05 as calculated using a one way ANOVA followed by a Tukey's HSD test.

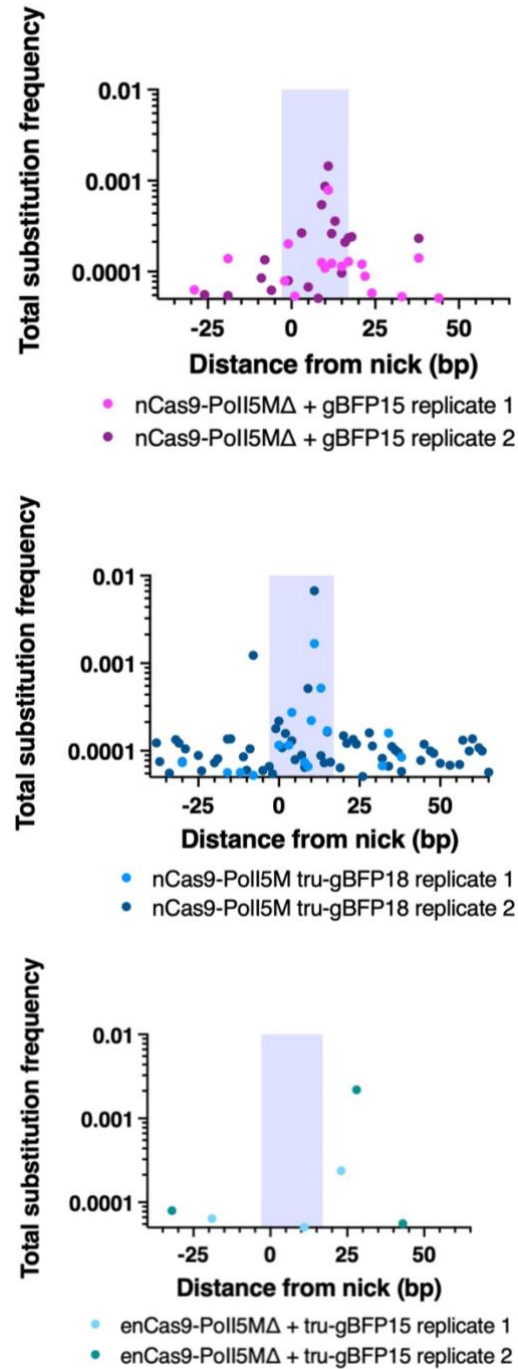


Figure 11. The use of a truncated gRNA enhances the diversity generated by nCas9-PolI5M relative to when a fulllength gRNA is used. The total frequency of substitution variants was quantified at each position within the BFP reference sequence. Positional coordinates are written with reference to the expected direction of the polymerase, where more positive numbers are further in the 3' direction with respect to the gRNA target strand. Position 0 corresponds to the position of the nick generated by nCas9. The blue rectangle marks the footprint of the gRNA target sequence.

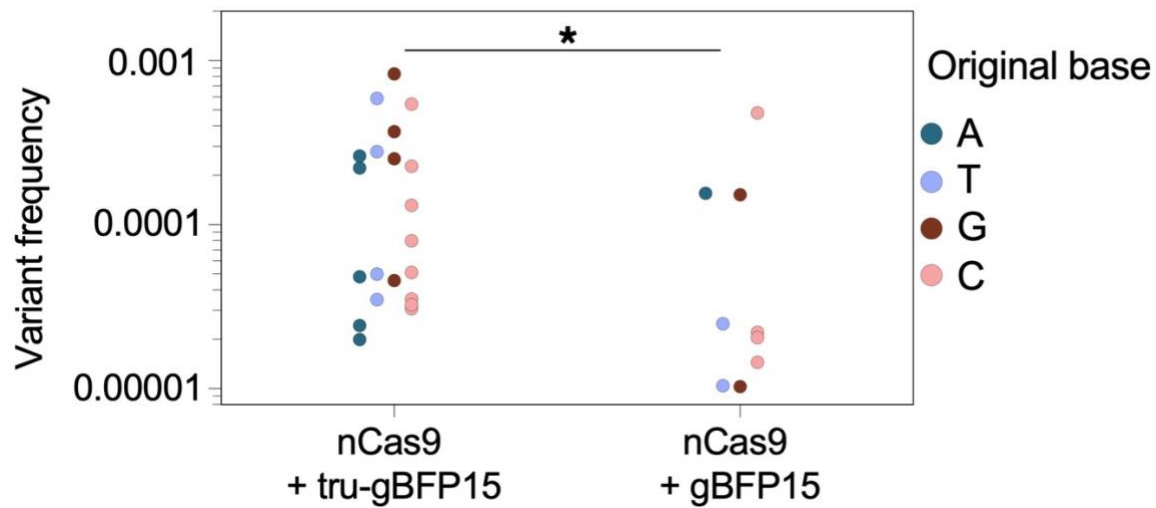


Figure 12. The use of a tru-gRNA increases the number of unique variants detectable by amplicon sequencing within the gRNA target region. Dots represent the mean frequency of individual substitution variants within the gRNA target sequence 18 bp from the PAM site (positions -3 to 15 in Figure 11) across two biological replicates. Asterisk represents a p value of less than 0.05 as calculated using a two-tailed student's t test.

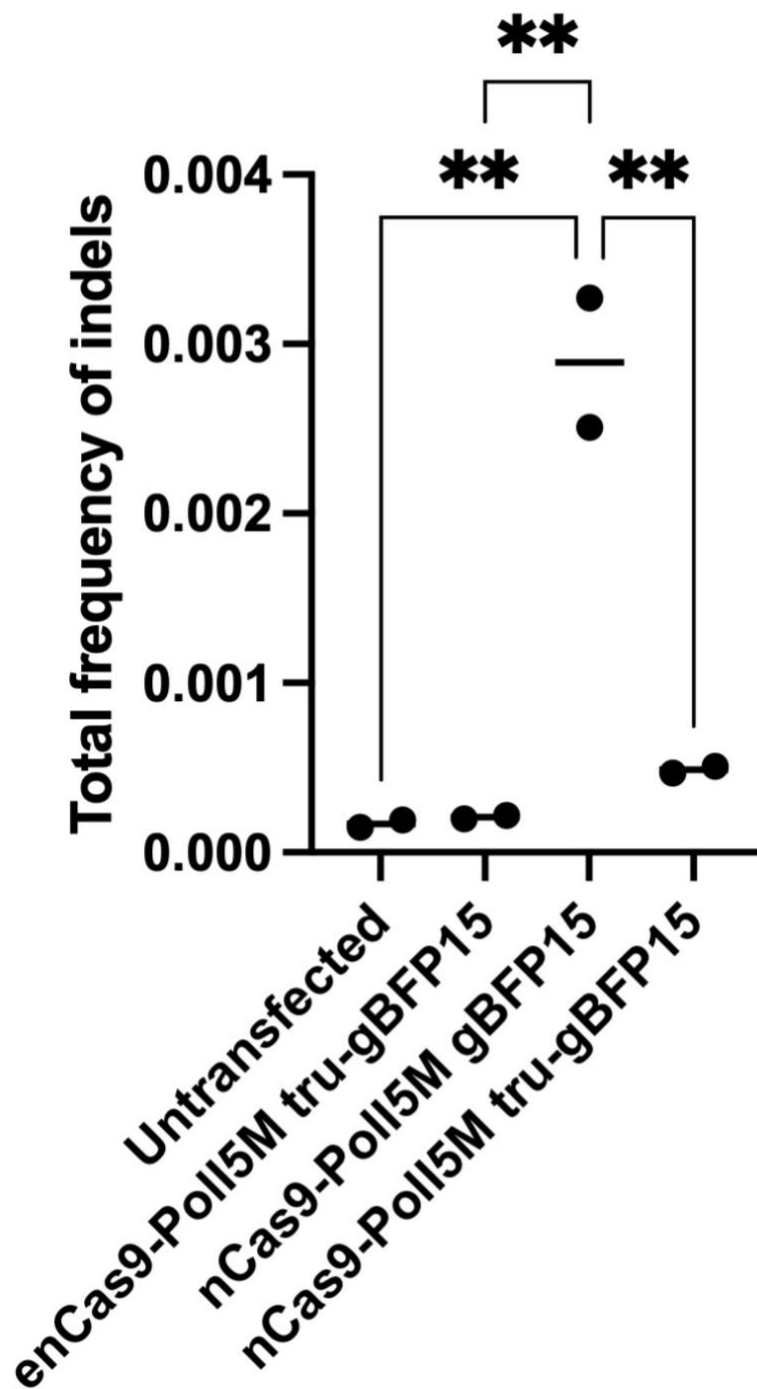


Figure 13. The use of a truncated gRNA decreases the total frequency of indels generated by nCas9-PolI5M relative to when a full-length gRNA is used. ** represents a p value less than 0.01 as calculated using a one-way ANOVA and Tukey's HSD test.

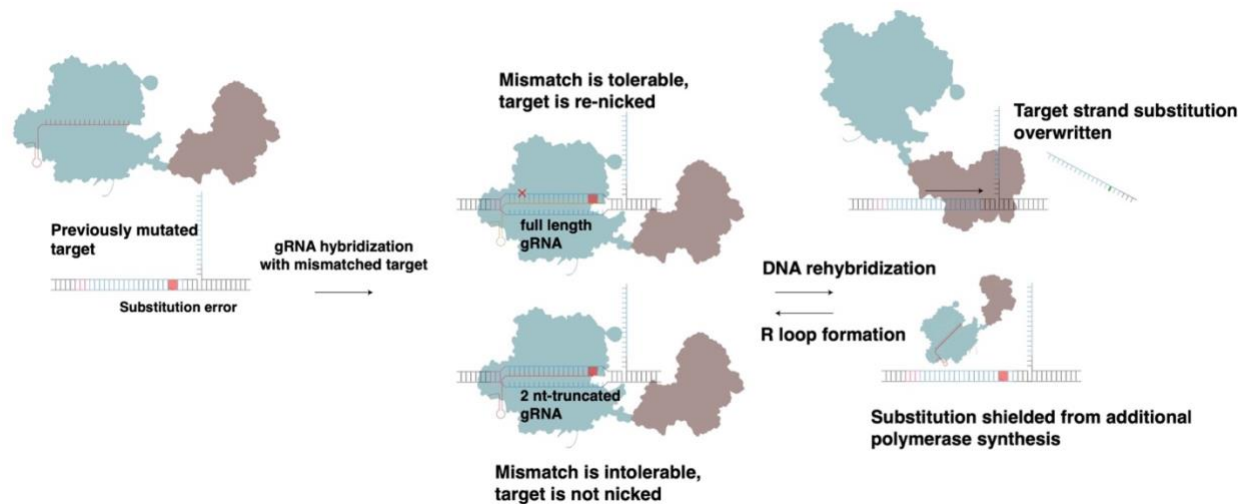


Figure 14. Diagram depicts proposed mechanism underlying increases in substitution rates at certain positions within the spacer region when truncated gRNAs are used instead of full-length gRNAs. Substitutions that are tolerable to nCas9 with full length gRNAs may not be tolerable when complexed with truncated gRNAs, protecting new substitution errors on the target strand from additional cycles of strand displacement.

3.3. Testing engineered high-fidelity nucleases as EvolvR components for improving EvolvR's reliability per gRNA.

The mismatch tolerance model correlates the diversity of EvolvR's edits with its ability to resist cutting mismatched targets. However, mismatch tolerance is highly gRNA-specific and variable, depending on factors like the propensity of a given R-loop to form noncanonical base pairs⁴⁸, base-skipping⁴⁸, and gRNA-DNA free binding energy⁴⁹. Moreover, truncation of gRNAs can impair R-loop formation⁵⁰, which is the rate-limiting step for Cas9 nuclease activity⁵¹. Accordingly, the use of truncated gRNAs alone is undesirable for reliably improving the utility of EvolvR. In contrast, identifying or engineering RNA-guided nickases with reliably low mismatch tolerance and high activity when complexed with all or most gRNAs is an attractive engineering goal.

Prior work has thus far only incorporated wildtype nCas9 (D10A) and enhanced nicking Cas9 (D10A, K848A, K1003A, R1060A) into EvolvR. However, many other Cas9 variants with enhanced fidelities have been engineered, all of which could potentially endow EvolvR with more favorable on and off-target activity profiles. Therefore, I set out to assess a panel of engineered Cas9 variants using the BFP to GFP editing assay described in Chapter 2.

For EvolvR to be useful in interrogating a given substitution variant, it theoretically need only generate the associated substitution at least once within a cell culture under selection. Accordingly, EvolvR's most critical functionality is its ability to generate a given variant consistently across different biological replicates. Therefore, I assessed a panel of different Cas9 nickases derived from various engineered Cas9 variants^{41,42,52,48,53,54,55} for their capacity to generate GFP + variants in 3/3 biological replicates using the BFP to GFP assay with various gRNAs. To emulate variations in R loop formation dynamics, I tested three gRNA variants of each of two distinct spacer regions where one gRNA generates a nick 3 bp away from the codon encoding H67 (gBFP3) and the other generates a nick 15 bp away (gBFP15). For each of the two spacer regions, I tested a 20 nt-long gRNA, an 18 nt-long gRNA, and a 20 nt-long gRNA with a guanine attached to its 5' end, which is conventionally added to enhance gRNA expression from the human U6 promoter.

Expectedly, the performance of all Cas9 variants tested was highly sensitive to both the spacer itself and the design of the gRNA used (Figure 15). While nCas9, enCas9, Sniper-nCas9, LZ3-nCas9, HSC1p2-nCas9, and SpRY-nCas9 all generated GFP positive cells consistently in at least one of the three designs for gBFP15, only SpRY-nCas9 generated GFP positive cells for spacer gBFP3. Furthermore, though SpRY-nCas9 generated GFP positive cells with 20 nt-long gBFP3, it failed to generate any GFP positive cells in all but one replicate with 20 nt-long gBFP15. Though these data demonstrate the potential value of optimizing EvolvR's substitution activity on a per-target-sequence basis by testing various gRNA designs and nickase variants, it does not elucidate clear design principles for easily initiating directed evolution campaigns with EvolvR. As sequencing data presented in Chapter 2 demonstrates that enCas9-PolI5M has the capacity to generate substitution variants throughout the spacer region, I opted to conduct proof of concept directed evolution studies described in Chapter 4 with enCas9.

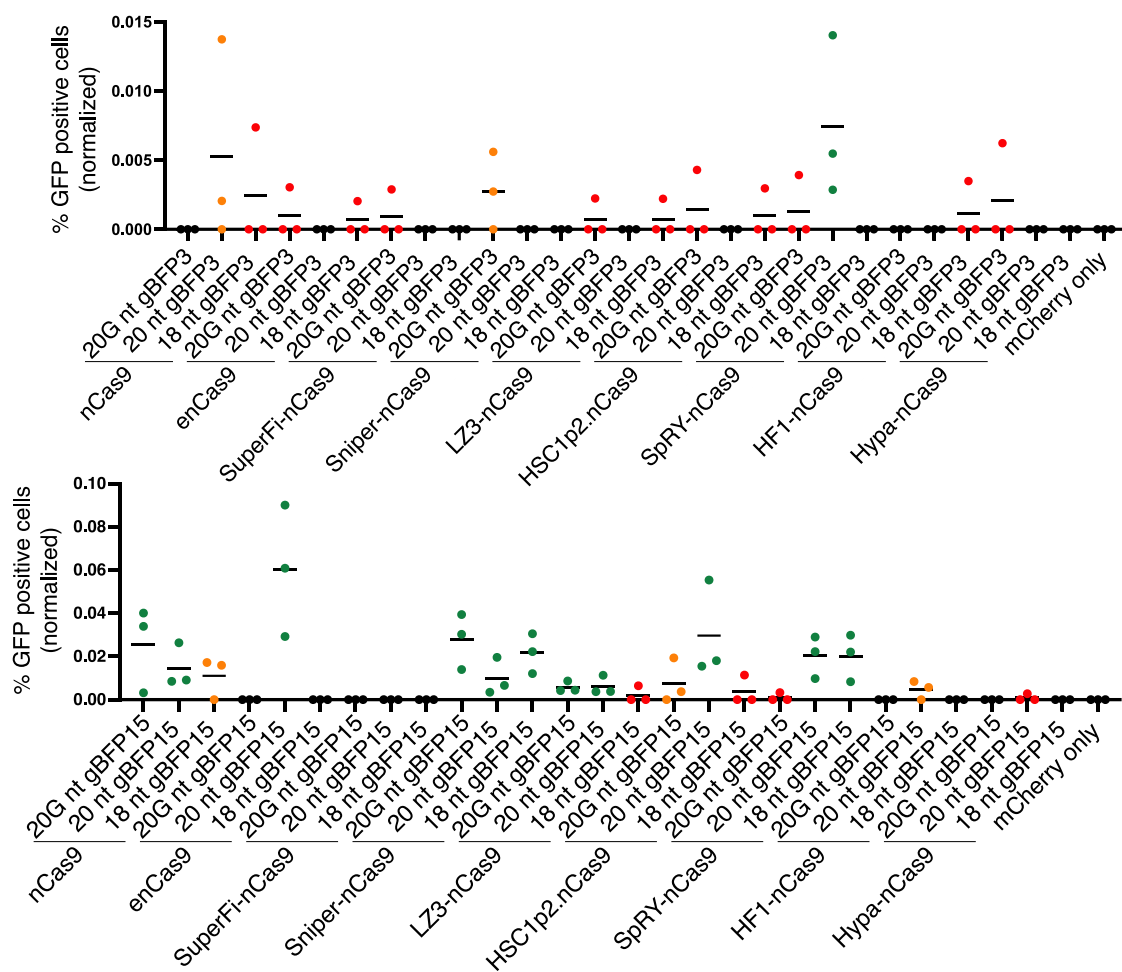


Figure 15. EvolvR performance varies depending on gRNA design and nuclease variant used. The percentage of GFP + cells generated by each construct was measured by flow cytometry and normalized by transfection efficiency. Green, yellow, red, and black dots represent that three, two, one, or zero out of three biological replicates contained GFP + cells, respectively. Black lines represent mean % GFP + cells.

3.4. Postulating a complete model for the role of mismatch tolerance and R loop formation in determining EvolvR's substitution biases.

The variability in EvolvR's capacity to generate GFP positive cells depending on gRNA design, spacer sequence, and Cas9 variant used is consistent with prior work demonstrating the dramatic effects of gRNA design and Cas9 missense mutations on predictors of Cas9 activity such as R loop formation^{50,51} and allosteric intramolecular communication⁵⁶.

Moreover, the failure of all EvolvR nuclease variants to generate GFP positive edits with all six gRNAs tested is consistent with the general tradeoff between activity and fidelity observed previously amongst SpCas9 variants, as it has been measured by tagmentation-based tag integration site sequencing (TTISS) that Cas9 variants that more efficiently cleave their cognate sequences do so at the cost of lower specificity and vice-versa⁵². Analogously, characterization of at least 2540 missense variants of Cas9 revealed that the vast majority of variants with enhanced specificity using a given gRNA exhibit impaired activity or low specificity with a second gRNA. To further complicate predictive models of EvolvR's substitution activity, measurements of Cas9 "activity" often rely on measuring the frequency with which it generates indels at target loci, which is not strictly correlated with nuclease activity. Indeed, it has been demonstrated that indel rates can be positively correlated with Cas9 dissociation induced through R loop disruption by nuclear proteins like FACT⁵⁷ and RNA polymerase II⁵⁸. Though R-loop formation is known to be a limiting factor for DNA cleavage⁵¹, R-loop melting is also known to be a limiting factor for Cas9 dissociation⁵⁹, which is necessary for releasing the free double-stranded breaks (DSBs) that ultimately give rise to indels. Similarly, because nCas9 dissociation is required for the release of the 3' hydroxyl group that PolII initiates DNA synthesis from, R-loop melting is also likely to be a limiting factor for EvolvR-mediated mutagenesis.

Previous work has also demonstrated that efficient cleavage by Cas9 depends on the optimal free energy of binding between both strands of target DNA and RNA-DNA binding in the R loop⁴⁹, such that excessive or insufficient free energy changes during R loop formation result in "low efficiency" gRNAs. This "goldilocks" condition that free energy changes of DNA-DNA hybridization and R-loop formation be within a narrow band for optimal gRNA activity suggests that EvolvR's capacity to generate certain substitutions may be a complex function of (1) efficient R-loop formation and melting with the cognate target strand, and (2) a given substitution sufficiently perturbing both DNA-DNA and gRNA-DNA hybridization to prevent the formation of R-loops with and subsequent nicking of the mutated strand.

For example, a "high efficiency" gRNA within the goldilocks zone of free energy change of R loop formation may be insufficiently perturbed by a single mismatch with its target strand to prevent nuclease activity in the presence of a substitution mutation. However, using an engineered high-fidelity nuclease or truncating that same gRNA may substantially change the energetic cost of the conformational changes underlying cleavage or the free energy change of R-loop formation, respectively, and thereby prevent nuclease activity within a wider range of possible mismatched targets. Applying this logic more broadly, I note that the use of truncated gRNAs and high-fidelity nucleases generally shifts the energetic favorability of R loop formation and allosteric communication within Cas9 such that gRNAs associated with high activity and

low specificity become more specific, while gRNAs exhibiting lower activities and higher specificities at 20 nt in length when complexed with wildtype Cas9 may become inactive upon gRNA truncation or complexing with high-fidelity nickases (Figure 16).

In summary, to explain the dependence of EvolvR's mutagenicity on gRNA design and nuclease fidelity (Figures 10, 11, 12, 13, and 15) I propose that both efficient R-loop formation and nicking of the cognate target and efficient R-loop unwinding are necessary for initial mutagenesis (Figure 17). Thereafter, substitutions generated within the target strand by PolI must sufficiently impair renewed R loop formation with the target strand in order not to be overwritten by PolI in later iterations of nicking and error-prone DNA synthesis. Two potential mechanisms for the stable installation of substitutions on the target strand are simple DNA replication of the substitution error or PolI's participation in homology directed repair upon the replication fork's collision with a nickase-generated single-stranded break.

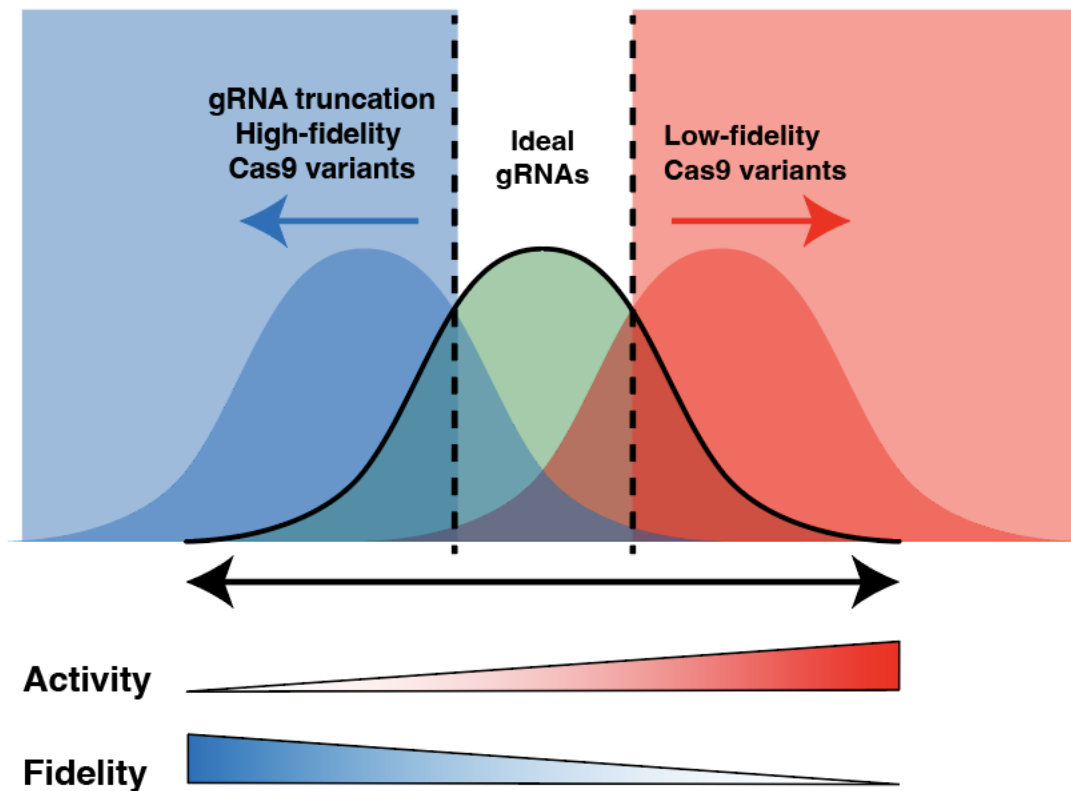


Figure 16. The cartoon above visualizes the tradeoff between activity and fidelity that exists across all possible gRNA sequences as a normal distribution. Truncation of gRNAs or the use of high-fidelity nuclease variants shift the distribution of all possible gRNAs to enhance fidelity but weaken activity such that previously optimal gRNAs will become inactive while low-fidelity gRNAs will become optimal for gene editing. Conversely, the use of low-fidelity Cas9 variants (e.g. SpRY-nCas9) may shift the distribution of all possible gRNAs towards higher activity and lower fidelity such that previously optimal gRNAs will exhibit high mismatch tolerance while previously inactive gRNAs will exhibit low mismatch tolerance. The depiction normally distributed gRNA properties is for ease-of-visualization and is not meant to assert that gRNA properties are normally distributed.

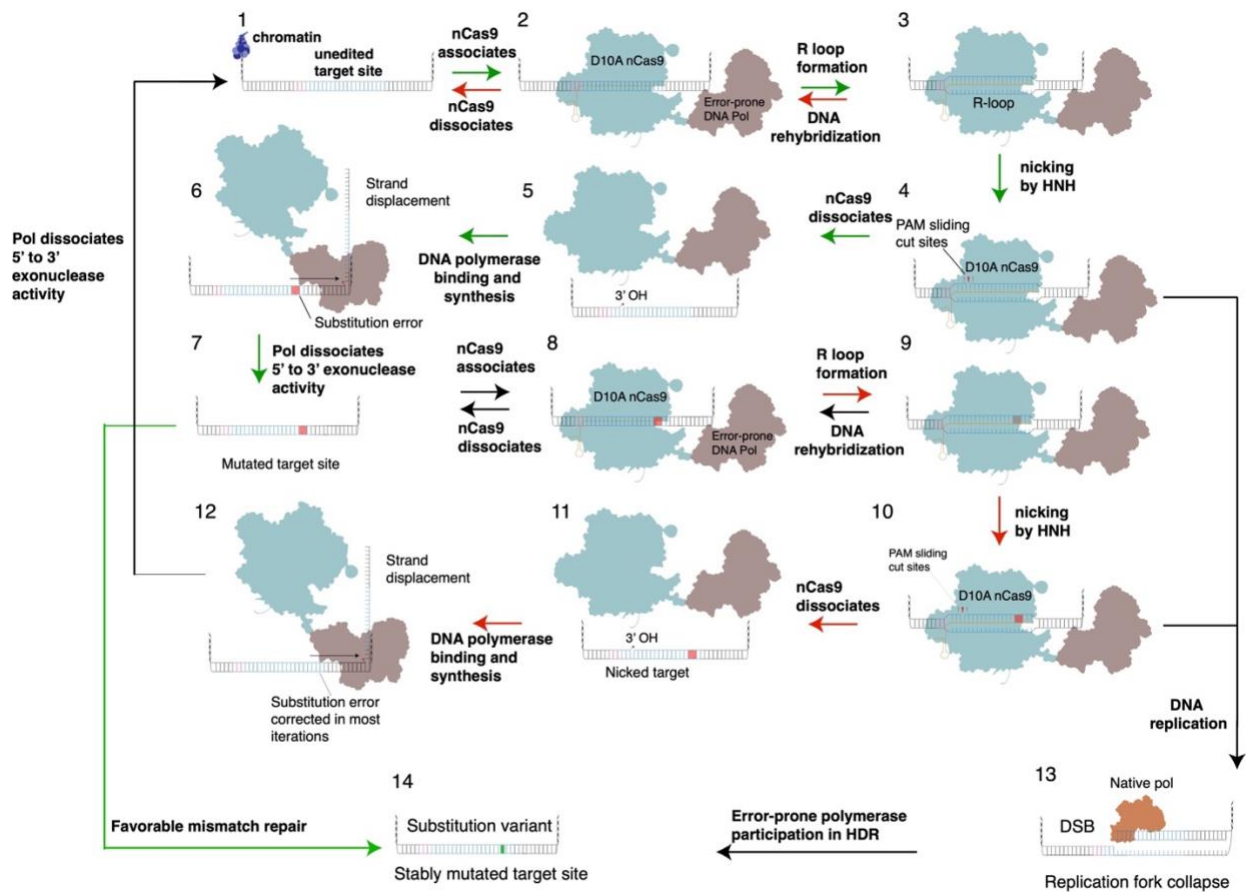


Figure 17. The schematic above depicts a proposed mechanism explaining variability in EvolvR's performance across different gRNA and nickases. Unedited target sites (1) undergo initial recognition by nCas9 (2), enabling the initiation of R loop formation (3). Complete R loop formation enables nCas9 to generate a single-stranded break (4). nCas9 dissociates after DNA-DNA hybridization (5), allowing PolII to initiate strand-displacing DNA synthesis (6). After resection of the 5' flap by native exonucleases (7), nCas9 has the potential to reassociate, nick, dissociate, and perform additional rounds of strand-displacing synthesis (8,9,10,11,12). At steps in which nCas9 is bound to a nicked substrate (4,10), the replication fork can collide with and displace Cas9 resulting in a single-ended double stranded break (13), initiating homology-directed repair which PolII may participate in. Alternatively, when a mismatched but ligated target locus exists at the point of DNA replication (7), mutations may be stably installed as substitution variants in one of the two daughter cells (14). Green arrows represent steps expected to synergize with EvolvR-mediated mutagenesis, while red arrows represent steps expected to impede EvolvR-mediated mutagenesis.

4. Proof of concept of EvolvR's use for directed evolution in mammalian cells.

4.1. Diversifying exon 6 of MAP2K1 in A375 cells to identify drug-resistant variants

Having demonstrated that EvolvR elevates mutation rates at user-defined genomic loci, I tested EvolvR's capacity for generating novel gain of function variants within native genomic loci by targeting EvolvR to exon 6 of MAP2K1, the kinase activity of which A375 cells depend on for growth⁶⁰, and screening the resulting library for variants resistant to MAP2K1 inhibitor selumetinib. To this end, I transiently transfected A375 cells with a plasmid encoding enCas9-PolI5M and one of three gRNAs targeting 3 different loci along MAP2K1 exon 6 (Figure 18), where mutations have been previously identified that confer resistance to selumetinib. After transfection, cells were split into three separate cultures per condition and allowed to recover in culture for six days before being transferred to media containing 1 μ M selumetinib (Figure 19). While non-target control cultures transfected with EvolvR and a gRNA targeting a nonexistent BFP gene (gBFP45) did not noticeably replicate and were not viable after passaging in one of three triplicates, cultures transfected with EvolvR and three gRNAs targeting three nonoverlapping loci in exon 6 contained visible colonies at the end of the selection period (Figure 20).

The cells were harvested from the dish and amplicon sequenced, revealing seven variants that were enriched by at least 10-fold over mean frequencies in a triplicate untreated A375 cell control (Figures 20 and 21). Remarkably, all seven variants are yet to be discovered at the time of preparing this manuscript, revealing EvolvR's potential to access diversity spaces yet unexplored by current *in vivo* diversifiers. Furthermore, one of the seven variants generated by EvolvR was an insertion variant (+S212), demonstrating the potential value of EvolvR's capacity to generate functional indels in addition to substitutions.

Remarkably, though the known resistance-conferring T to A substitution in V211 was enriched by our screen, the substitution was associated entirely with an additional G to C substitution in V211, resulting in a V211H missense mutation rather than the V211D mutation previously reported to confer resistance to selumetinib⁶⁰. This result is consistent with the mismatch tolerance bias model discussed in Chapter 2, suggesting that a T to A substitution alone is tolerated by the gRNA overlapping with it (Figure 18) and that the additional G to C substitution is required to both confer resistance and prevent target recognition. Critically, the identification of a double and triple substitution variant reveals that EvolvR can traverse the diversity space afforded by generating combinations of two and three substitutions within a single spacer region.

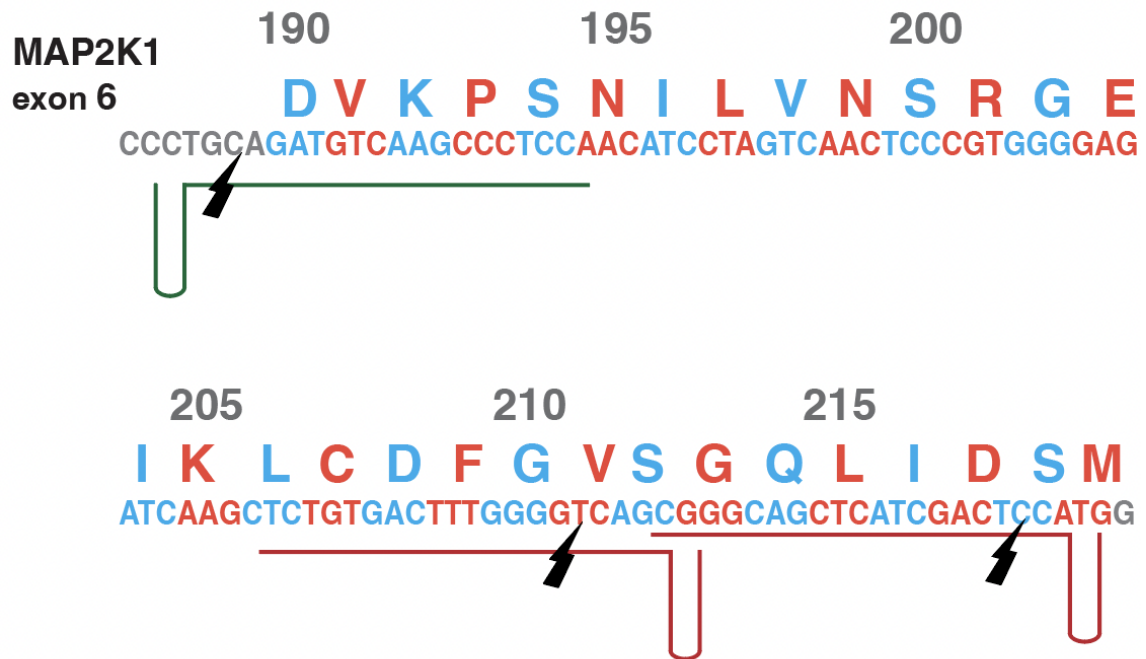


Figure 18. The diagram depicts the use of three gRNAs targeting the sense strand in green and the antisense strand in red of exon 6 of MAP2K1.

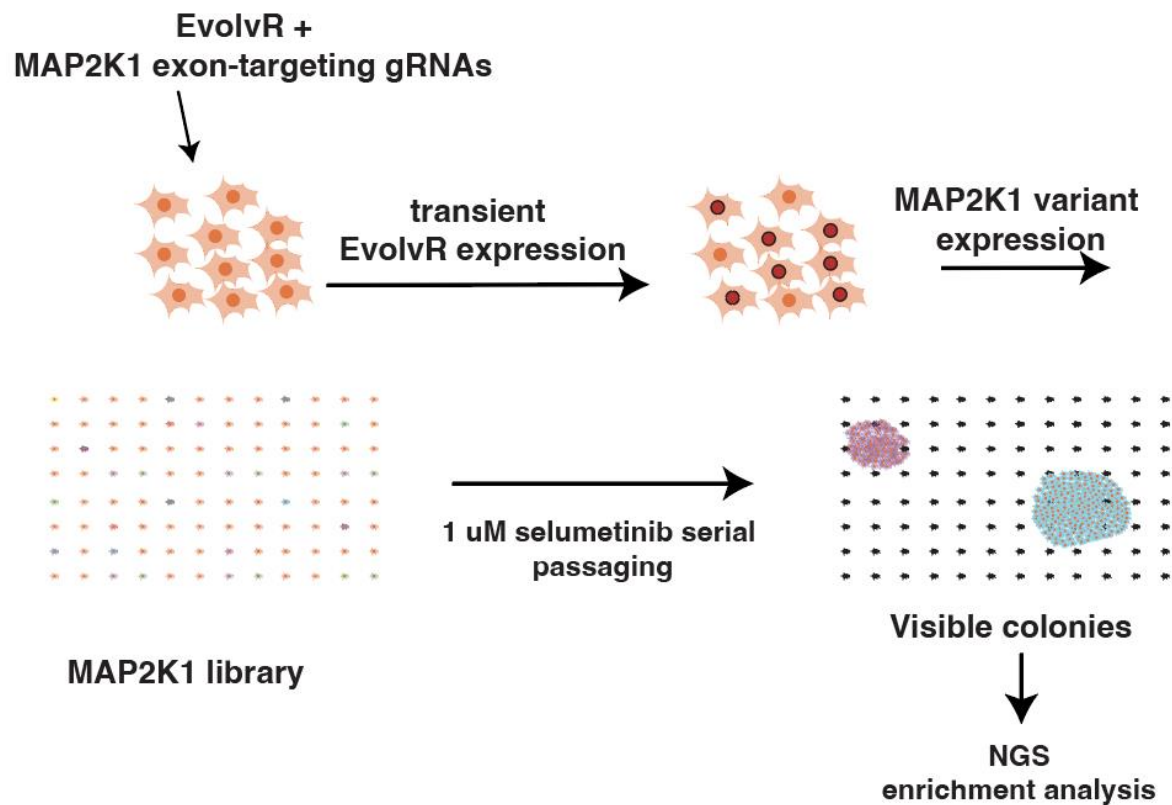


Figure 19. The diagram depicts the workflow used to evolve new selumetinib-resistant MAP2K1 variants using EvolvR. A375 cells were transiently transfected with plasmids encoding expression of EvolvR and gRNAs targeting MAP2K1. Cells were cultured without selection to allow MAP2K1 variants to evolve before initiating selection with media containing 1 μ M selumetinib. Cells were serially passaged until colonies growing rapidly under selective media are visible. gDNA was harvested from the cells and amplicon-sequenced to identify substitution mutations that are enriched in the population.

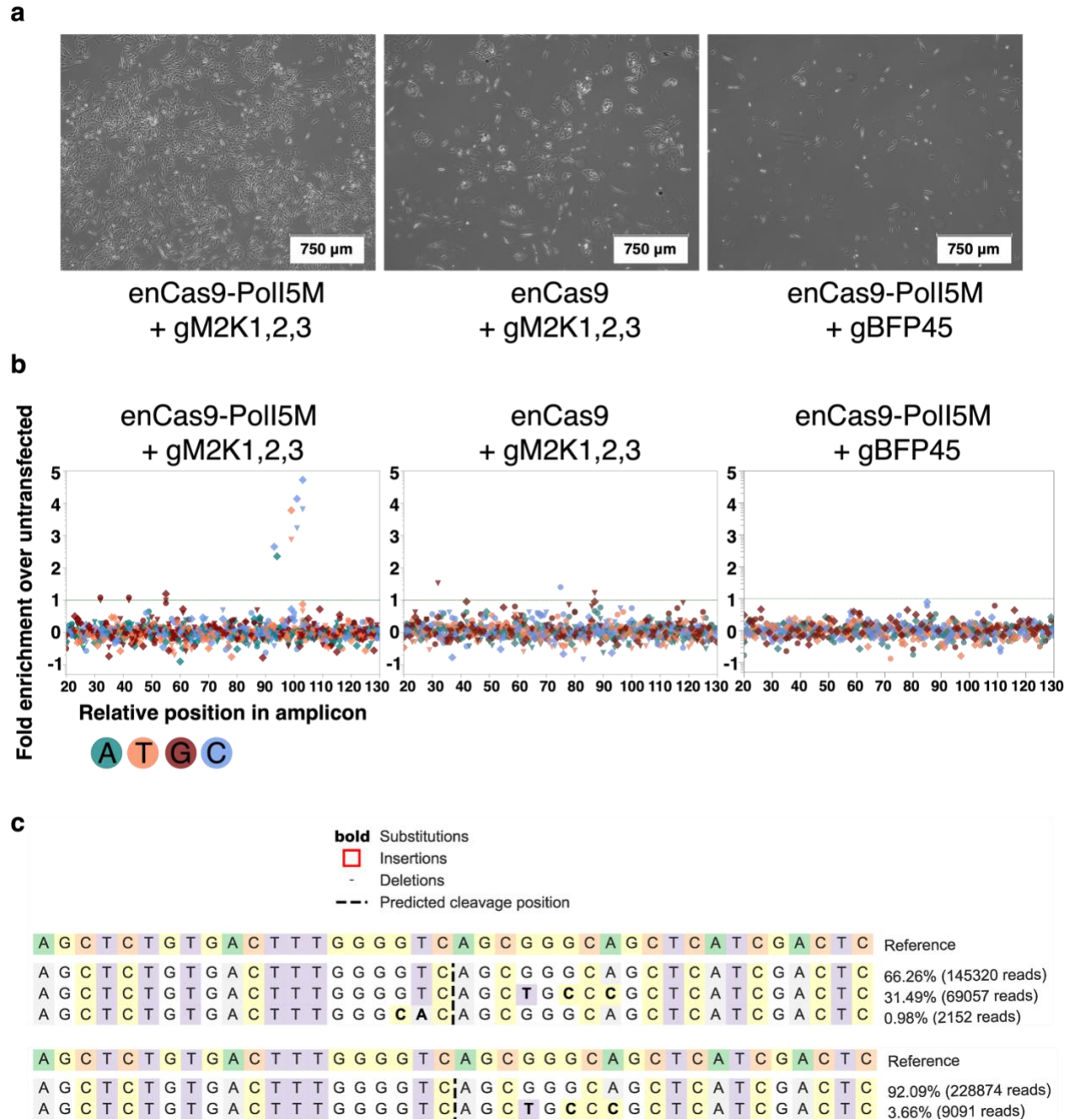


Figure 20. EvolvR generated selumetinib-resistant MAP2K1 substitution variants. **a**, Differences in cell growth under selective media were visible by microscope 36 days after initiation of selection. **b**, EvolvR targeted to exon 6 of MAP2K1 generated substitution variants that were enriched compared to untransfected cells by 10-53,000-fold after 40 days of selection, while enCas9 alone targeted to exon 6 generated fewer substitution variants enriched by at least 10-fold and EvolvR targeted to a nonexistent BFP gene generated no enriched variants. Triangles, diamonds, and circles correspond to different biological replicates. Green, orange, red, and blue points represent A, T, G, and C variants respectively. Green dashed lines mark the 10-fold enrichment threshold. **c**, Representative CRISPRESSO allele tables show enriched alleles

containing multiple substitutions in cell populations generated by EvolvR targeted to exon 6 of MAP2K1.

Mutation	Resulting Missense	Mut. fold enrichment over WT	Allele ID
T>G	D190E	12	Allele 1
T>G	S194A	12	Allele 2
T>G	V198G	16	Allele 3
G>C	V211H	450	Allele 4
T>A	V211H	227	
G>T	G213C	6049, 747	Allele 5
G>C	G213C	13687, 1687	
A>C	Q214P	52708, 6583	
+CAG	+S 212	237	Allele 6

Figure 21. The table shows the substitution types and resulting missense mutations of variants enriched under selection of A375 cells by selumetinib after being transfected by EvolvR. Substitutions not achievable by deaminases are highlighted in red. Fold-enrichment is quantified by dividing the frequency of substitution variants generated by EvolvR by the frequency of those substitution variants in untransfected cells not under selection. Allele labels indicate that mutations were co-represented and appeared together in all reads, resulting in a single selumetinib-resistant variant. Multiple numbers under “Mut. Fold enrichment over WT” correspond to the variant’s enrichment in multiple replicates. Enrichment of variants in different biological replicates are distinguishable by the green, blue, and purple text in the column indicating fold-enrichment.

4.2. Validation and combination of selumetinib-resistant MAP2K1 variants

To validate the three most enriched drug-resistant MAP2K1 variants (V211H, G213C Q214P, +S212), we transiently transfected HEK293T cells with plasmids encoding the cDNA for these variants and measured MAP2K1-ERK activity in the presence of selumetinib using a reporter plasmid for SRE-controlled luciferase expression previously used for measuring MAP2K1 activity in the presence of an inhibitor (Figure 22). As positive and negative controls, I tested the newly discovered variants against known selumetinib-resistant variant V211D and a plasmid encoding wildtype MAP2K1, respectively. V211H, G213C/Q214P and +S212 all showed statistically significantly higher activity at all concentrations of selumetinib tested than wildtype. Additionally, we tested V211H in combination with G213C Q214P and found that its relative MAP2K1-ERK activity was approximately 2.3-fold more than that of V211D and 205.3-fold more active than wildtype MAP2K1 under inhibition by 1 μ M selumetinib. I attribute the absence of V211H G213C Q214P in our selection to insufficient sampling of the $\sim 6.3 \times 10^8$ possible quintuple substitution variants necessary for generating the triple missense variant given my screen size of approximately 10^7 cells.

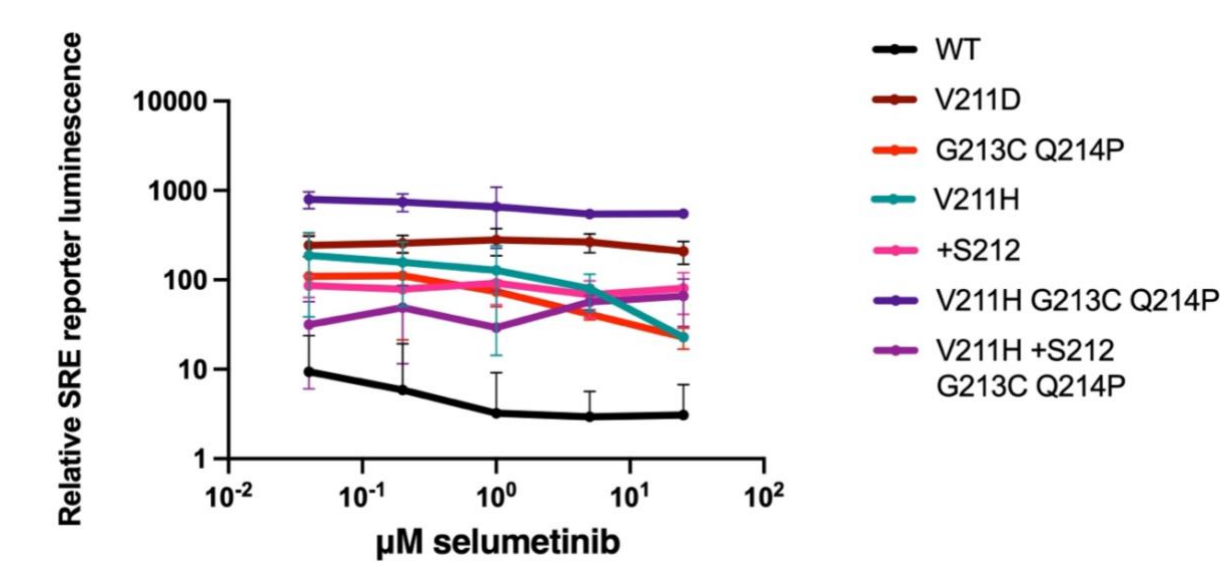


Figure 22. All variants enriched by at least 227-fold exhibited higher MAP2K1-ERK signaling activity than wildtype MAP2K1 at all concentrations tested. MAP2K1-ERK signaling as measured by SRE-driven expression of a luciferase reporter. Error bars represent 95% confidence intervals.

5. Engineering a compact, PAM-flexible EvolvR for superior mutagenic coverage of genetic loci and packaging into lentiviral vectors.

5.1. Approach for increasing the reliability of EvolvR for generating single nucleotide variants

In chapters 2 and 4, I show that EvolvR can diversify all four nucleotides (Chapter 2) and evolve genomic loci to identify previously undescribed gain of function variants that are inaccessible with transition mutations alone (Chapter 4). Nevertheless, the gRNA-specific substitution biases presented by variability in gRNA activity and mismatch tolerance limits the diversity space that EvolvR can access. Though gRNA design and nickase engineering may enable fine-tuning of EvolvR activity at a given target locus, conducting evolution campaigns with multiple gRNA/nickase combinations in parallel is laborious and impractical, partially forfeiting the ease-of-use that motivates the use of *in vivo* continuous directed evolution technologies. Instead, I aimed to increase the probability that a given substitution variant within EvolvR's mutation window is generated by (1) increasing the total length of time over which EvolvR mutagenesis can take place, and (2) increasing the number of gRNAs that overlap with a given target nucleotide to maximize the possibility that at least one of them is favorable for generating a given substitution variant.

To enable long-term mutagenesis and maximize the number of gRNAs that can be applied towards efficient mutagenesis of a target sequence, I sought to engineer a PAM-flexible EvolvR variant that can be efficiently packaged into a lentiviral vector for facile transduction of EvolvR expression cassettes in most human cell lines.

While the packaging efficiency of HIV-1-derived lentiviral vectors decreases geometrically with increasing viral genome length over approximately 5 kb⁶¹, the lengths of enCas9-PolI5M and enCas9-PolI5M Δ alone are 7090 bp and 6118 bp, respectively (Figure 23). Adding additional elements necessary to initiate evolution immediately and ensure stable expression of EvolvR, such as a strong RNA pol II promoter, a fluorescent reporter or selectable marker, a U6 promoter, and a gRNA introduces approximately 1-1.5 kb of additional payload length. Together with the essential components comprising the lentiviral backbone including the LTR's, Rev response element (RRE), and Psi packaging signal, lentiviral transfer vectors for initiating directed evolution with EvolvR in mammalian cells via a single transduction exceed HIV-1's native genome length of roughly 9.2 kb and are inefficiently packageable as lentiviral vectors.

To substantially increase the packaging efficiency of lentiviral vectors for stable expression of EvolvR and gRNAs while widening the set of gRNAs that can target a given nucleotide, I sought to identify compact, PAM-flexible nucleases with favorable activity and specificity profiles to substitute for SpCas9-based nucleases within the EvolvR fusion protein. To this end, I considered two compact RNA-guided nucleases known to exhibit improved specificity compared to Cas9, Nme2⁶² and Slug Cas9⁶³. To improve upon Nme2's native NNNNCC PAM site, I opted to use a variant of Nme2 called eNme2.NR engineered by PACE and shown to recognize NNNNCN PAM sites⁶⁴, while Slug native recognizes NNGR PAM sites⁶⁵. Furthermore, Nme2Cas9 and SlugCas9 are 279 and 313 amino acids shorter in length

than SpCas9, enabling both a shorter genetic payload for lentiviral vectors and improved PAM flexibility compared to Cas9's NGG PAM sites.

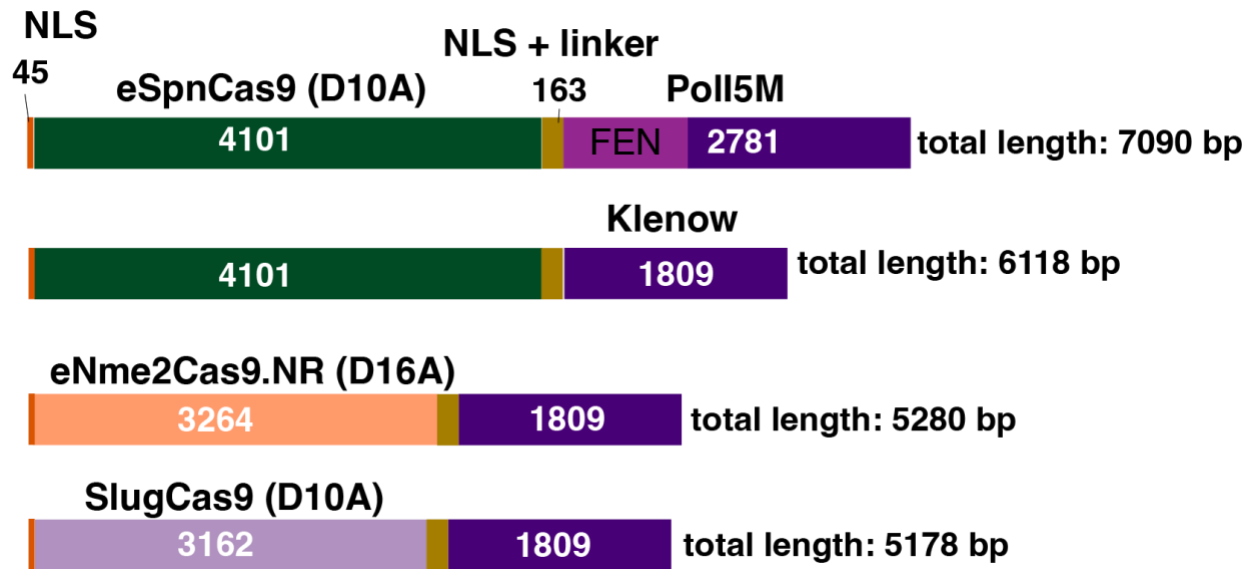


Figure 23. Schematics representing the length of EvolvR variants tested in bp.

5.2. Characterization of eNme2.NR-nCas9 and Slug-nCas9 as nickases for EvolvR using BFP to GFP mutagenesis

To assess the consistency with which eNme2.NR-nCas9-PolI5M Δ and Slug-nCas9 Δ can generate H67Y missense variants with a variety of gRNAs and begin investigating optimal gRNA design, I transfected BFP HEK293 cells with plasmids encoding expression of EvolvR using either eNme2.NR-nCas9 or Slug-nCas9 as nickases and one of a diverse panel of gRNAs (Figures 24 and 25). I tested 20 and 18-nt gRNAs for Slug-nCas9 and 21 and 23-nt gRNAs for eNme2.NR-nCas9, as Nme2Cas9 is conventionally paired with 23 nt gRNAs for optimal editing efficiency.

Out of the 44 gRNAs tested across 22 unique spacer sequences with eNme2.NR-nCas9, only two generated GFP positive cells in all three biological replicates (Figure 24, gRNA 1 and 2T). The relatively poor mutagenicity of eNme2.NR-nCas9-PolI5M Δ may be attributable to Nme2Cas9's relatively weak nuclease activity in mammalian cells⁶⁵, though the degree to which eNme2.NR-Cas9's activity compares to wildtype Nme2Cas9 has not been rigorously quantified.

In contrast, of 24 gRNAs tested across 12 unique spacer sequences with Slug-nCas9, 6 generated GFP positive cells in all three replicates (Figure 24, gRNAs 26, 27, 29 and 31, 26T, and 32T). Of these 6 gRNAs, 4 of them were 20 nt in length (gRNAs 26, 27, 29, and 31) and represented 80% (4/5) of 20 nt gRNAs that both generated nicks upstream of H67 and contained the cytosine that is mutated to generate GFP positive cells within its spacer. Through the lens of the MTB model, these data suggest that Slug-nCas9 is likely to exhibit low mismatch tolerance throughout its spacer region. This finding would be consistent with prior characterization of SlugCas9's activity with thousands of gRNAs revealing that it is the most specific and among the highest activity Cas9 nucleases reported to date⁶⁵.

Given Slug-nCas9's markedly superior reliability for generating BFP to GFP substitutions when incorporated into EvolvR, I abandoned further characterization of eNme2.NR-nCas9 and focused follow-up experiments on optimizing Slug-nCas9-PolI5M Δ .

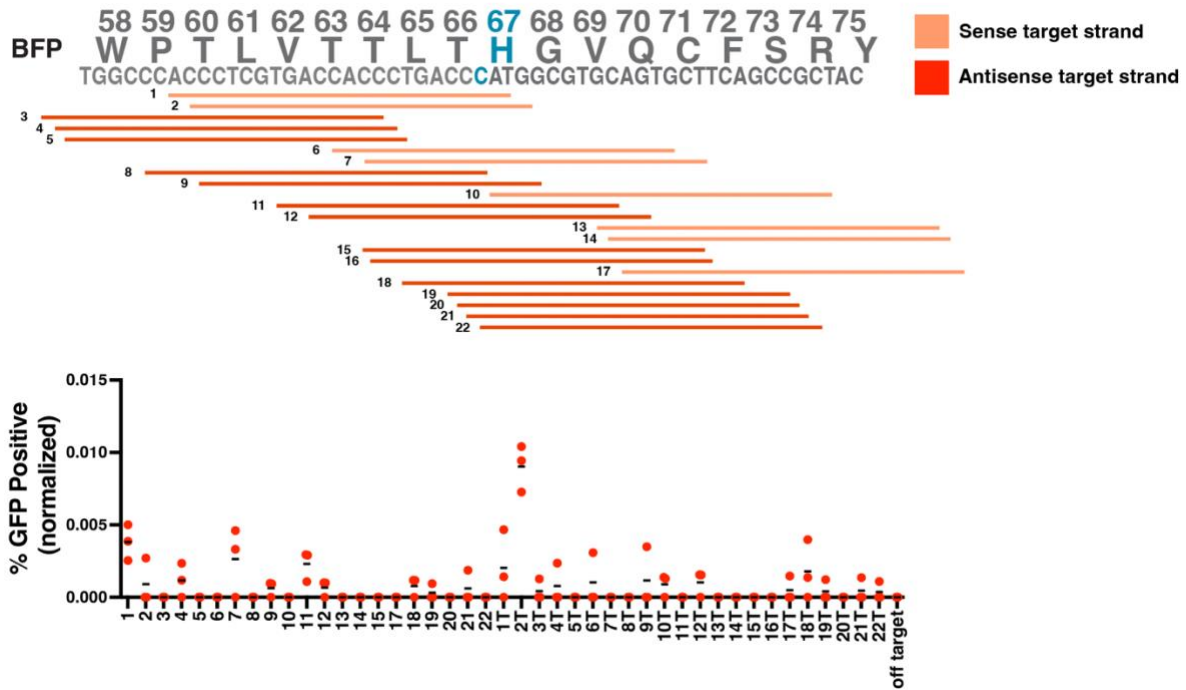


Figure 24. eNme2-PolI5M Δ enables guide-dependent mutagenesis of BFP. The diagram depicts a BFP fluorescent reporter gene for EvolvR mutagenesis and a panel of spacer sequences generating nicks in either strand and either direction from the nucleotide encoding the H67Y missense mutation. Substitution of a T for a C in H67 yields expression of GFP instead of BFP. Dots represent individual biological replicates. The percentage of GFP + cells generated by each construct was measured by flow cytometry and normalized by transfection efficiency. gRNA numbers correspond to the spacers labelled in the diagram. The absence or presence of a “T” after the gRNA label indicates that the gRNA length is 23 nt or truncated to 21 nt, respectively.

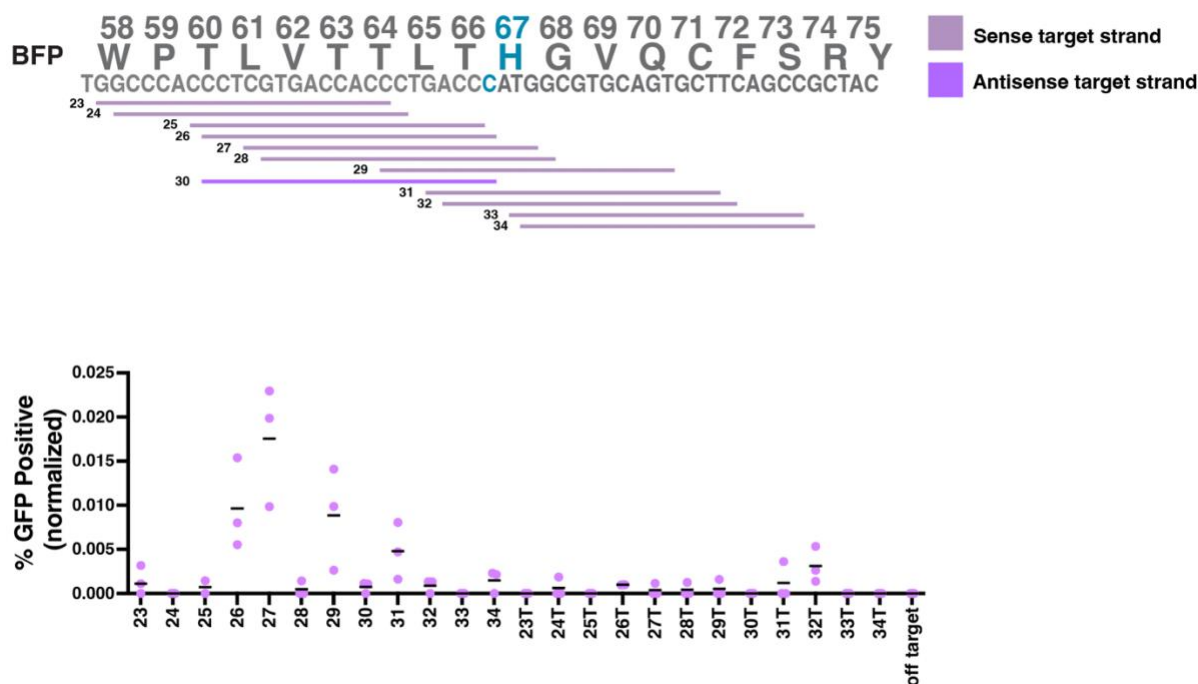


Figure 25. Slug-nCas9-PolI5M Δ enables guide-dependent mutagenesis of BFP. The diagram depicts a BFP fluorescent reporter gene for EvolvR mutagenesis as a panel of spacer sequences generating nicks in either strand and either direction from the nucleotide encoding the H67Y missense mutation. Substitution of a T for a C in H67 yields expression of GFP instead of BFP. Dots represent individual biological replicates. The percentage of GFP + cells generated by each construct was measured by flow cytometry and normalized by transfection efficiency. gRNA numbers correspond to the spacers labelled in the diagram. The absence or presence of a “T” after the gRNA label indicates that the gRNA length is 20 nt or truncated to 18 nt, respectively.

5.3. Establishing design principles for gRNAs with Slug-nCas9-EvolvR

To more thoroughly investigate the effect of gRNA length on Slug-nCas9-PolI5MΔ's reliability in generating substitution variants, I tested an additional panel of gRNAs targeting 8 different spacers near H67 at various lengths. Because 5' terminal purines are optimal for initiating efficient transcription from the U6 promoter, I tested the mutagenicity of each gRNA that does not natively possess a 5' terminal purine (A or G) with and without 5' terminal guanines (Figure 26).

Of the 40 gRNAs tested, 12 gRNAs generated GFP positive cells in all three biological replicates, all of which contained the GFP-conferring C to T substitution within the spacer and downstream of the nick. These 12 gRNAs consist of a single gRNA with 18 bp of complementarity to the target site, 3 gRNAs with 19 bp of complementarity, four with 20 bp of complementarity, and four with 21 bp of complementarity (Figure 27).

Though gRNAs with 20 bp and 21 bp of complementarity to the target strand were similarly reliable in generating GFP positive cells, there was a dramatic increase in the efficiency of BFP to GFP editing for one gRNA (3.4) when a mismatched terminal guanine was appended to its 5' end. To investigate whether adding mismatched 5' terminal purines to gRNAs with 20 bp of complementarity to the target site can be replicated with other gRNAs, I compared EvolvR's mutation rates when gRNAs of length 20, 21, or 21 with the last nucleotide being a mismatched terminal purine were used to target BFP. While the addition of a mismatched terminal purine to gRNAs with 20 bp complementarity markedly enhanced the substitution rates of two gRNAs (Figure 28, gRNA -14 and -13), it appeared to have no effect on the mutation rate of two other gRNAs (-5 and -1) and inactivate an additional gRNA (-2). This variability in outcome depending on individual gRNAs is consistent with our current understanding of gRNA design for classical gene editing as discussed in Chapter 3, by which the nuclease efficiency afforded by gRNAs can be improved by either increasing or decreasing the free energy change associated with R-loop formation⁴⁹ depending on their individual energetic starting points.

The data presented here suggest that the use of gRNAs with at least 20-21 bp of complementarity with the target strand are suitable for mutagenesis with Slug-nCas9 EvolvR. Though the addition of 5' terminal purines appears to have variable effects on the mutagenicity of a given gRNA, the efficient expression of gRNAs and tight control of transcriptional start sites afforded by the addition of 5' terminal guanines may be essential for EvolvR's functionality when expressed from a single lentiviral transgene rather than from the hundreds of copies that enter a given cell during a transient transfection. Therefore, I recommend the use of 20 or 21 nt gRNAs with additional 5' terminal guanines, enabling 20-22 bp complementarity with the target locus.

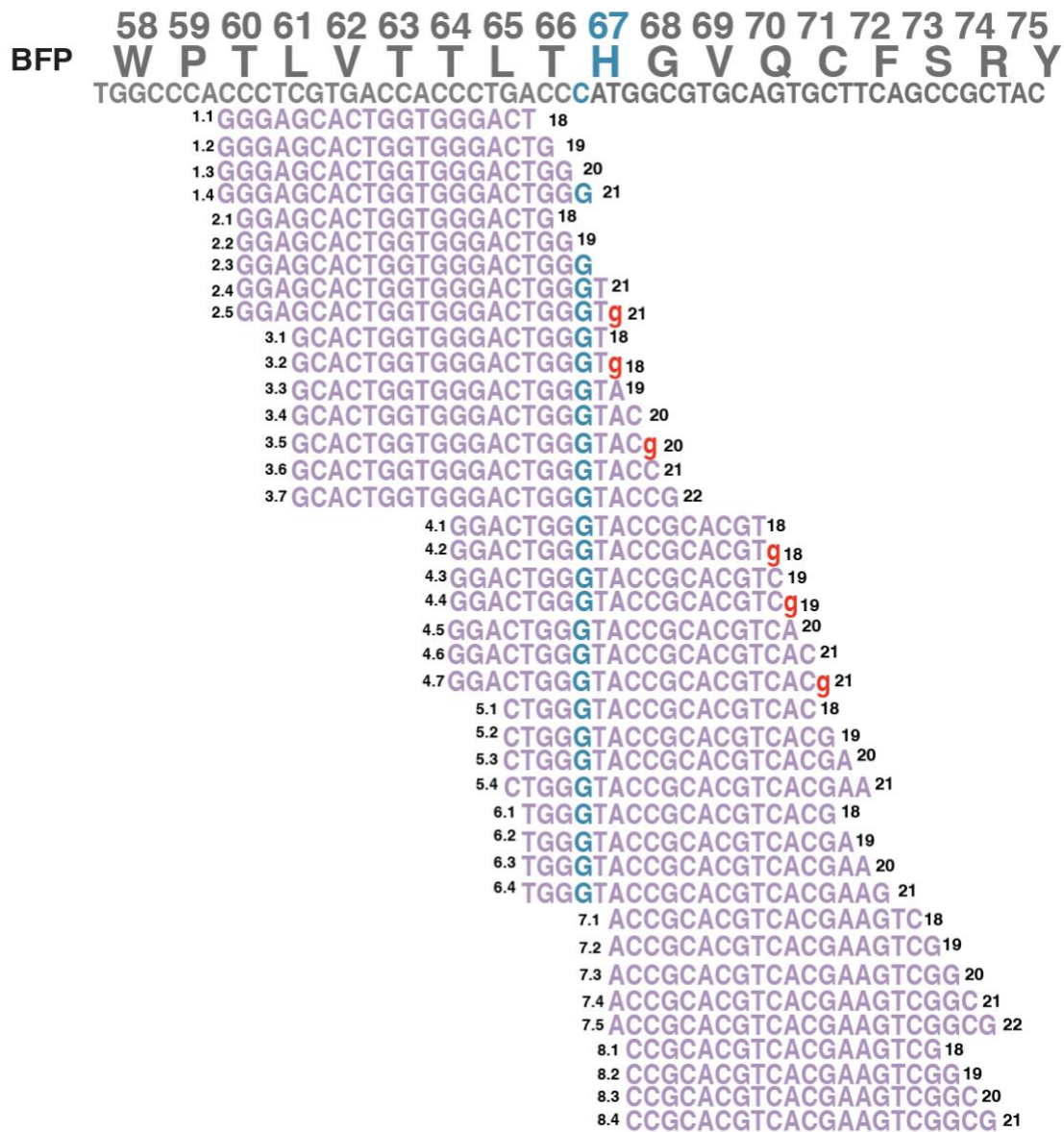


Figure 26. The diagram depicts a fluorescent reporter gene for EvolvR mutagenesis aligned with a panel of gRNAs of various lengths generating nicks in the sense strand near the codon encoding missense mutation H67Y. Substitution of a T for a C in H67 yields expression of GFP instead of BFP. Blue “G” within the gRNA sequences represents the nucleotide pairing with the C residue the mutation of which results in H67Y. Red “g”s at the end of gRNA sequences represent mismatched 5’ terminal guanines. Purple text represents gRNA sequences.

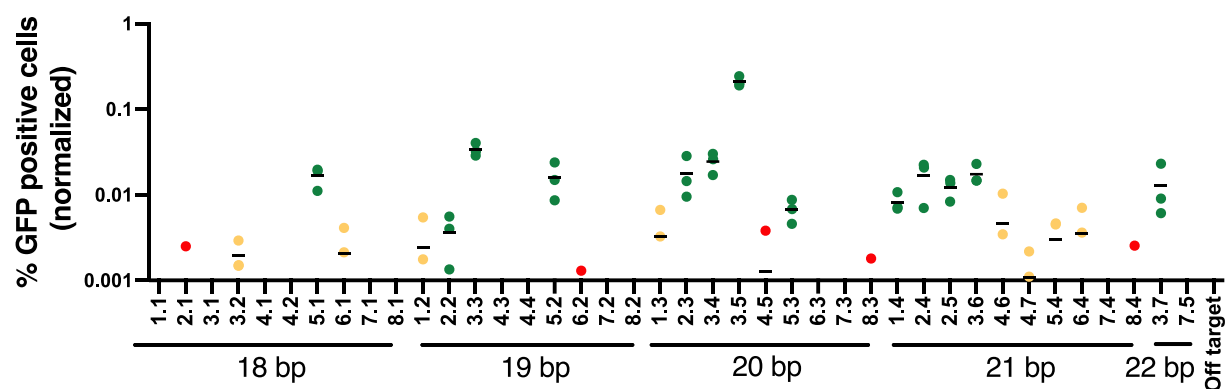


Figure 27. Slug-nCas9-PolI5M Δ enables guide-dependent mutagenesis of BFP most consistently when gRNAs share 20 or 21 bp of complementarity with the target strand. The percentage of GFP positive cells generated by each gRNA when complexed with EvolvR was measured by flow cytometry and normalized by transfection efficiency. Dots represent individual biological replicates. gRNA numbers correspond to the spacers labelled in Figure 26. bp labels under groups of gRNAs indicate the number of nt within a given guide that are complementary to their target strand.

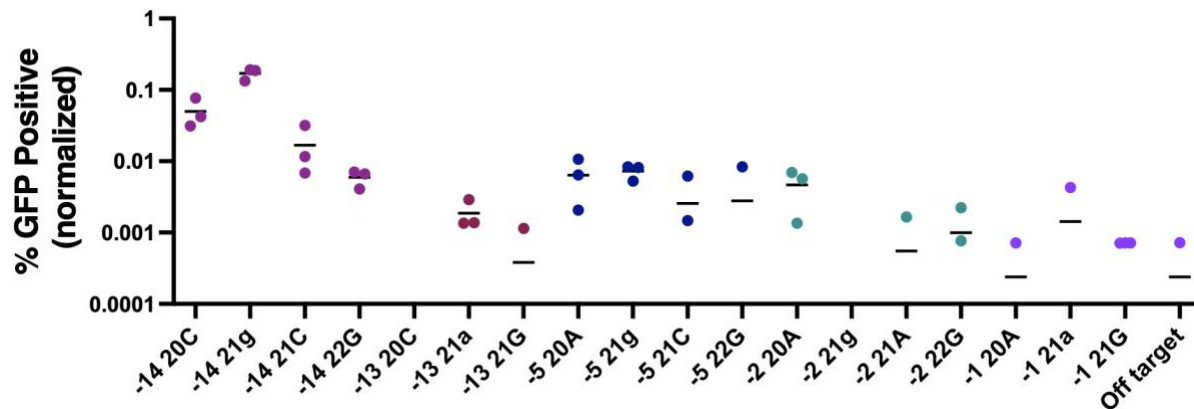


Figure 28. The presence of 5' terminal purines in Slug-nCas9-PolI5MΔ gRNAs affects guide-dependent mutagenesis of BFP. The percentage of GFP positive cells generated by each construct was measured by flow cytometry and normalized by transfection efficiency. Dots represent individual biological replicates. Colors correspond to unique gRNA target sequences. gRNA labels correspond to the position of the cut generated by each gRNA, where negative positions are upstream of the mutation encoding H67Y followed by the total length of the gRNA and the 5' terminal nucleotide of the gRNA. Lowercase nucleotide labels indicate that the 5' terminal nucleotide is mismatched to the target strand.

5.4. Mutagenesis of BFP to GFP using dox-inducible lentiviral vector

Having established that replacement of enCas9 with Slug-nCas9 enables consistent mutagenesis with 20 and 21 nt gRNAs and an approximately 1 kb reduction in cDNA length, Slug-nCas9-PolI5MΔ offers the potential for efficient packaging of the EvolvR system into lentiviral vectors for long term mutagenesis. However, throughout iterations of the BFP to GFP experiment, I consistently observed under a fluorescent microscope that only cells which no longer expressed mCherry-tagged EvolvR were capable of expressing GFP (Figure 32A). I attributed this phenomenon to transcriptional interference by EvolvR's nickase, as would be consistent with previous observations that Cas9 can interfere with transcription at positions downstream of the promoter⁶⁶ and that Cas9 is not dislodged from the target strand by RNA polymerases approaching from the PAM-proximal side⁵⁸. Critically, though SpCas9 is known to be a single-turnover nuclease that resides for hours on its target strand⁶⁷, SlugCas9 is an ortholog of SaCas9⁶³, which has been shown to be a multi-turnover nuclease that dissociates rapidly after cutting its target site⁶⁸. Nevertheless, the mechanisms regulating Cas9 binding are far less stringent than those regulating nuclease activity^{47,43}, such that most mutated target strands are likely to enable binding even if they are intolerable to nuclease activity. As such, because SaCas9's rapid dissociation occurs following nuclease activity, the potential multi-turnover nature of Slug-nCas9 would not negate the possibility of transcriptional interference by binding on the sense strand.

As EvolvR's disruption of the target gene's expression would prevent selection for desired functionality, I transduced BFP 293 cells a lentiviral vector encoding expression of EvolvR driven by a TetOn promoter, a gRNA, and transactivator rtTA3G enabling dox-inducible expression of EvolvR (Figure 29). By supplementing 1 µg/mL doxycycline in 24-hour pulses, I aimed to enable EvolvR mediated mutagenesis of its target site followed by its clearance from the nucleus, enabling unimpeded expression of BFP.

After two 24-pulses of expression followed by a week of incubation following each pulse, I measured the frequency of GFP positive cells amongst mCherry positive cells by flow cytometry (Figure 30). All five gRNAs tested individually in addition to a pool of the same five gRNAs generated GFP positive cells in three triplicates. This suggests that single copies of the EvolvR transgene afford sufficient expression of EvolvR for consistent mutagenesis of the target locus.

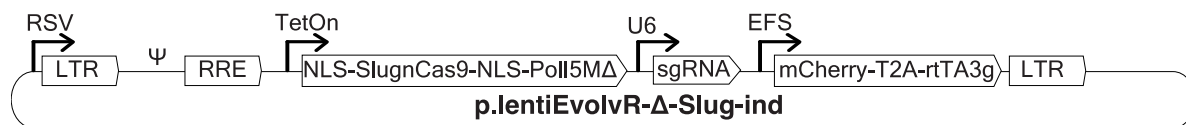


Figure 29. A lentiviral transfer plasmid encoding EvolvR for doxycycline-inducible expression in mammalian cells was constructed as Slug-nCas9 flanked by two nuclear localization sequences and fused to Poll5M's Klenow fragment, labelled here as Poll5MΔ. To enable inducible expression, an rtTA3g transactivator is constitutively expressed by a short EF1α promoter (EFS) along with mCherry T2A as a fluorescent reporter for transduction.

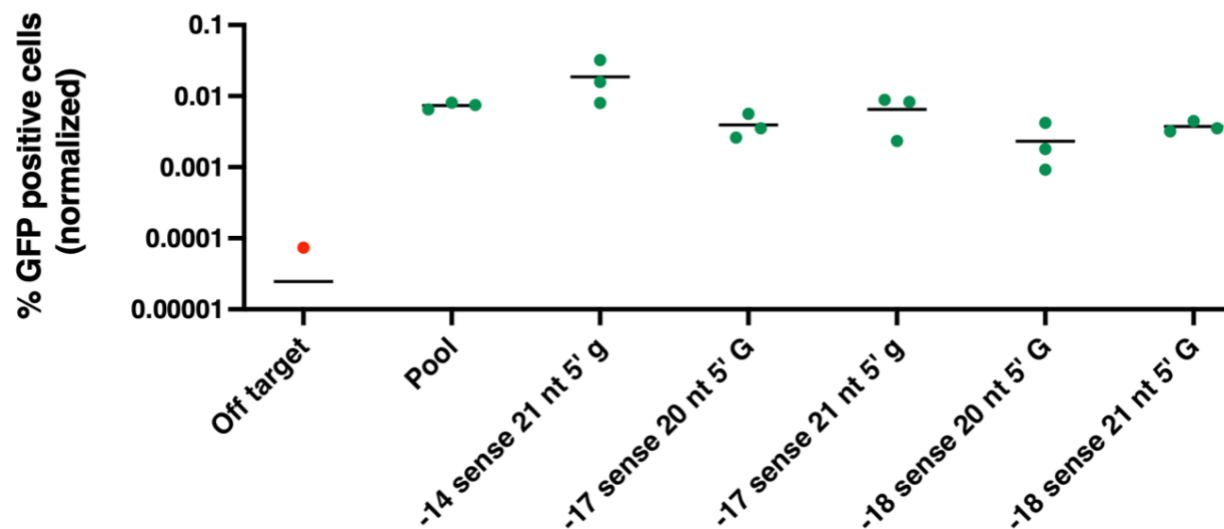


Figure 30. Expression of EvolvR via stable transduction of a lentiviral vector enables consistent mutagenesis of BFP. The percentage of GFP + cells generated by each construct was measured by flow cytometry and normalized by transfection efficiency. Dots represent individual biological replicates. Green dots indicate that GFP positive cells were detected in three out of three biological replicates; red dots indicate that GFP positive cells were detected in one out of three biological replicates. gRNA labels correspond to the position of the cut generated by each gRNA relative to the H67Y missense mutation, where negative positions are upstream of the mutation encoding H67Y followed by the total length of the gRNA and the 5' terminal nucleotide of the gRNA. Lowercase nucleotide labels indicate that the 5' terminal nucleotide is mismatched to the target strand.

5.5. Preparing EvolvR for constitutive expression without transcriptional interference of target genes.

Though iterations of EvolvR expression followed by library expression may enable iterative diversification and readout of a gene of interest's functionality (Chapter 5.4), I sought to engineer EvolvR for continuous diversification and selection. To this end, I sought to modify EvolvR to allow it to efficiently dissociate such that mutagenesis can occur without inhibiting expression of the target gene.

Previous work has shown that recruitment of DNA chaperone protein complex FACT⁵⁷ (facilitates chromatin transcription) can dislodge Cas9 from its target site, while studies in yeast have shown that HMGB domains can recruit FACT⁶⁹. Therefore, I introduced human HMGB1 to the N terminus of Slug-nCas9 (Figure 31) and examined transfected cells under the microscope to search for cells that are both GFP and RFP positive. Remarkably, I found cells expressing both GFP and RFP for the first time exclusively in cells transfected with HMGB1-Slug-nCas9-PolI5MΔ, indicating simultaneous expression of a mutated transgene and EvolvR (Figure 32).

To measure the effect of fusing HMGB1 to Slug-nCas9 on EvolvR's mutation rate, I tested the HMGB1 fusion with three gRNAs: one which generated GFP positive cells with high efficiency, one which generated GFP positive cells with low efficiency, and one which failed to generate any GFP positive cells (-14 21g, -5 21g, and -2 21g, Figure 33). While fusion of HMGB1 with EvolvR slightly reduced the efficiency of the highest performing gRNA, it slightly enhanced the efficiency of the moderately performing gRNA and enabled BFP to GFP edits for the first time in all three replicates for the previously "inactive" gRNA (-2 21g, Figure 28). Notably, fusion of a different chromatin remodeling domain HMGN1 that is not known to recruit FACT but is known to enhance the efficiency of base editors when fused to nCas9⁷⁰ decreased the mutagenicity of all three guides relative to when HMGB1 was fused to EvolvR (Figure 33).

This finding encourages the use HMGB1 for enabling the concurrent expression and hypermutation of target genes, while potentially improving the mutagenicity of previously low-mutagenicity gRNAs.



Figure 31. A plasmid encoding EvolvR for expression in mammalian cells was constructed as an mCherry-tagged Slug-nCas9 flanked by high mobility group box 1 (HMGB1) protein and a nuclear localization sequence and fused to PolI5M's Klenow fragment. PolI5MΔ indicates the deletion of the first 325 amino acids from PolI5M.

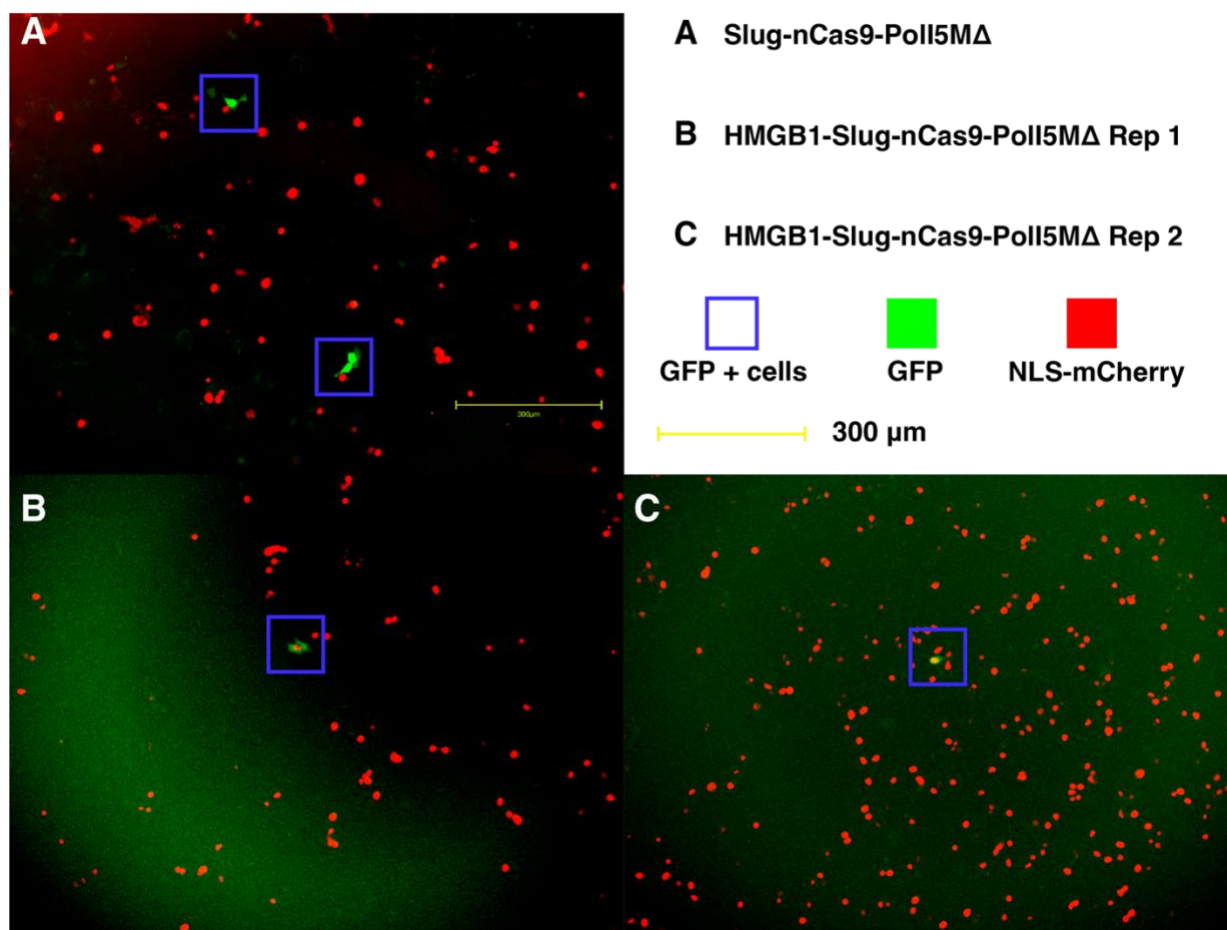


Figure 32. Fusion of HMGB1 to EvolvR enables simultaneous expression and mutagenesis of target DNA. Cells were transfected with EvolvR with or without HMGB1 fused to the N terminus and observed under a fluorescent microscope. Cells expressing EvolvR with HMGB1 fused to its N terminus were observed to express both GFP and mCherry (B,C), while cells expressing GFP that were transfected with EvolvR lacking HMGB1 lacked mCherry fluorescence.

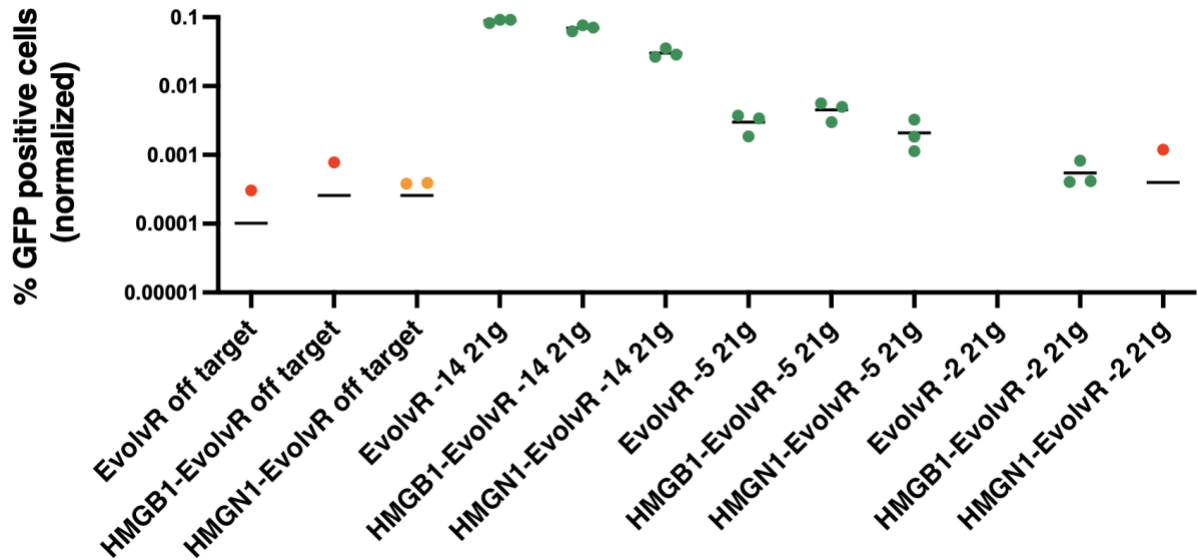


Figure 33. EvolvR's mutation rates are not negatively impacted by fusing HMGB1 to its N terminus. The percentage of GFP + cells generated by each construct was measured by flow cytometry and normalized by transfection efficiency. Dots represent individual biological replicates. Green dots indicate that GFP positive cells were detected in three out of three biological replicates; orange dots indicate the GFP positive cells were detected in two out of three replicates, red dots indicate that GFP positive cells were detected in one out of three biological replicates. gRNA labels correspond to the position of the cut generated by each gRNA, where negative positions are upstream of the mutation encoding H67Y followed by the total length of the gRNA and the 5' terminal nucleotide of the gRNA. Lowercase nucleotide labels indicate that the 5' terminal nucleotide is mismatched to the target strand.

5.6. Further improving EvolvR's PAM flexibility with NNG variant of Slug-nCas9.

While Slug-nCas9 natively recognizes NNGR PAM sites, a variant of Slug-nCas9 recognizing NNG PAM sites was engineered during the preparation of this dissertation⁷¹. Given that the human genome's GC content within 20 kb windows is at least 30%⁷², NNG PAM site recognition would afford the capacity to diversify virtually any region of any gene of interest with EvolvR.

Using 21-22 nt gRNAs depending on whether a nature 5' terminal guanine existed at the end of each gRNA (Figure 34), I tested the reliability of NNG-Slug-nCas9-PolI5MΔ in generating BFP to GFP edits. Among the 24 gRNAs tested, those that efficiently generated GFP positive mutants were definitively represented by those that generated nicks upstream of H64Y, highlighting EvolvR's asymmetrical mutation windows consistent with PolII initiating DNA synthesis from the nick generated by the nickase (Figures 35 and 36), while eight of the 24 gRNAs generated GFP positive cells in all three biological replicates (Figures 35 and 37). Of these eight gRNAs, seven generated nicks upstream of the H67Y substitution mutation (Figure 37), further corroborating the directionality of EvolvR's mutation window. Furthermore, all of these seven gRNAs targeted the sense strand (Figure 37), suggesting a strong dependence of EvolvR's mutagenesis on which strand is targeted. I propose that EvolvR's impaired mutagenicity when targeted to the antisense strand is due to RNA Pol II's collision with single-stranded breaks on the template (antisense) strand, initiating a process known as transcription-coupled repair (TCR) whereby the nicked strand is removed and resynthesized by native polymerases⁷³. Importantly, EvolvR's impaired ability to efficiently mutate the antisense strand further emphasizes the highly enabling PAM flexibility of NNG-Slug-nCas9.

The 7 gRNAs that consistently generated GFP positive cells in the sense strand represent 58% of the sense strand-targeting gRNAs that generate nicks upstream of the H67Y substitution, predicting a favorable probability that at least one gRNA that can efficiently generate a given substitution is available at a given target locus given the relative abundance of NNG PAM sites.

Finally, of the PAM sites differing from Slug-nCas9's native NNGR PAM site recognition within the pool of loci that were possible to test, all three were NNGT PAM sites. Of these three, two generated GFP positive cells consistently, confirming NNG-Slug-nCas9-PolI5MΔ's capacity to recognize PAM sites other than NNGR (Figure 34, 35). The capacity of NNG-Slug-nCas9 to generate productive nicks leading to substitutions as part of the EvolvR fusion protein represents a critical leap in EvolvR's utility as a genomic diversifier, effectively removing PAM availability as a limitation of the EvolvR technology's applicability.



Figure 34. The diagram depicts a fluorescent reporter gene for EvolvR mutagenesis aligned with a panel of gRNAs of various lengths generating nicks in the sense and antisense strands near the codon encoding missense mutation H67Y. Substitution of a T for a C in H67 yields expression of GFP instead of BFP. Blue “G” within the gRNA sequences represents the nucleotide pairing with the C residue the mutation of which results in H67Y. Red “g”s at the end of gRNA sequences represent mismatched 5’ terminal guanines. PAM sequences are highlighted in gold next to gRNA sequences.

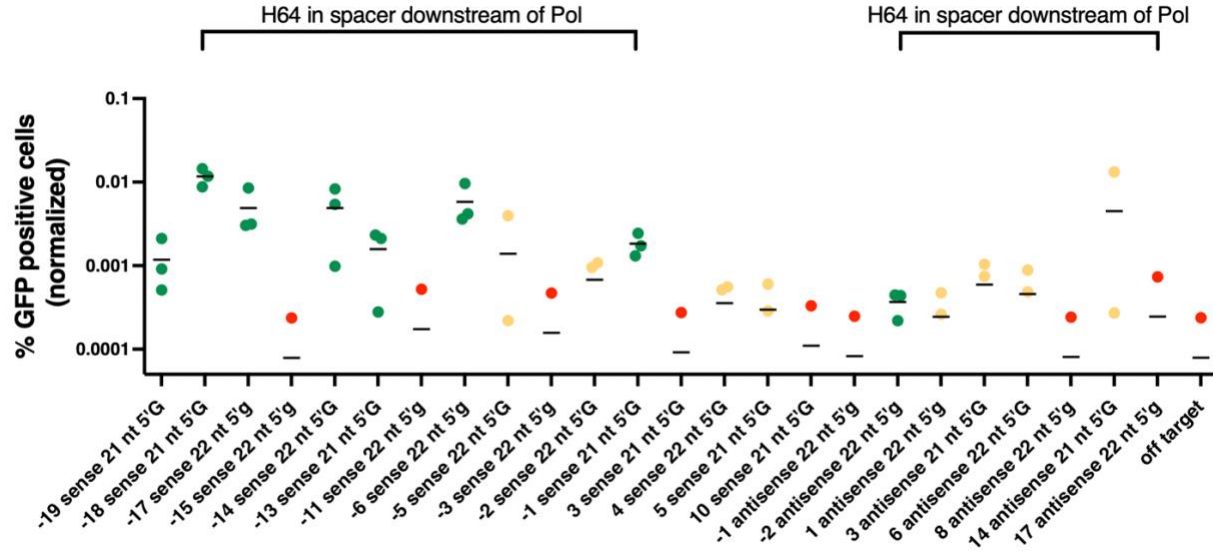


Figure 35. NNG-Slug-nCas9-PolII5M consistently mutates positions within the spacer gRNA spacer region when the sense strand of BFP is targeted. The percentage of GFP + cells generated by each construct was measured by flow cytometry and normalized by transfection efficiency. Dots represent individual biological replicates. Green dots indicate that GFP positive cells were detected in three out of three biological replicates; yellow dots indicate the GFP positive cells were detected in two out of three replicates, red dots indicate that GFP positive cells were detected in one out of three biological replicates. gRNA labels correspond to the position of the cut generated by each gRNA, where negative positions are upstream of the mutation encoding H67Y followed by the total length of the gRNA and the 5' terminal nucleotide of the gRNA. Lowercase nucleotide labels indicate that the 5' terminal nucleotide is mismatched to the target strand.

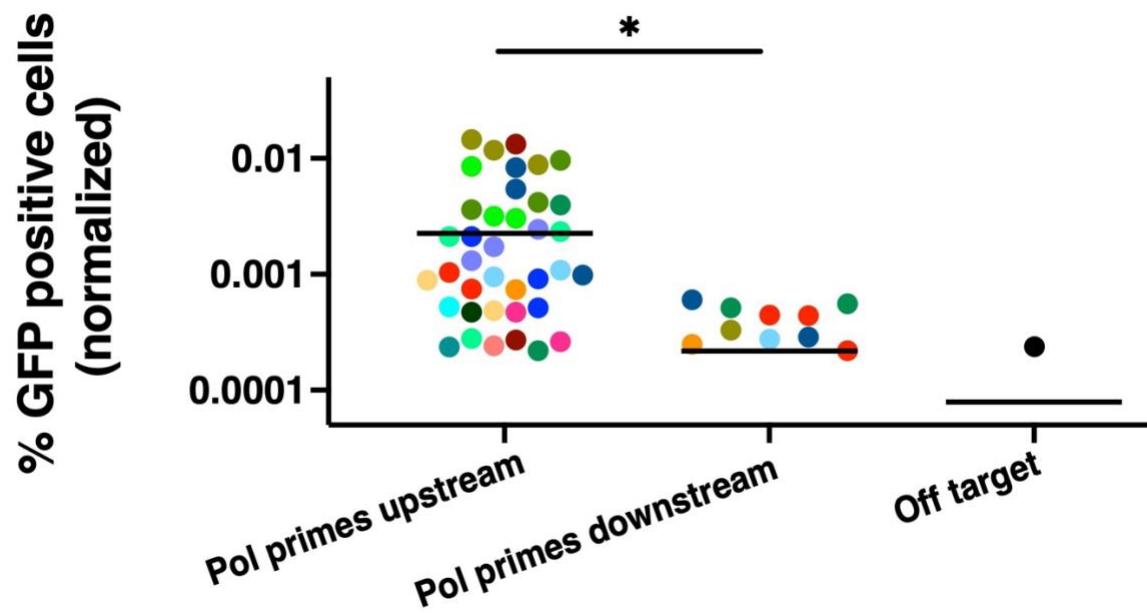


Figure 36. NNG-Slug-nCas9-PolI5M Δ efficiently mutates positions downstream of nCas9-generated nick. The percentage of GFP + cells generated by each construct was measured by flow cytometry and normalized by transfection efficiency. Dots represent individual biological replicates. Identically colored dots indicate biological replicates of the same gRNA condition. Asterisk indicates a p value of less than 0.05, as determined by a two-way ANOVA and Tukey's HSD test.

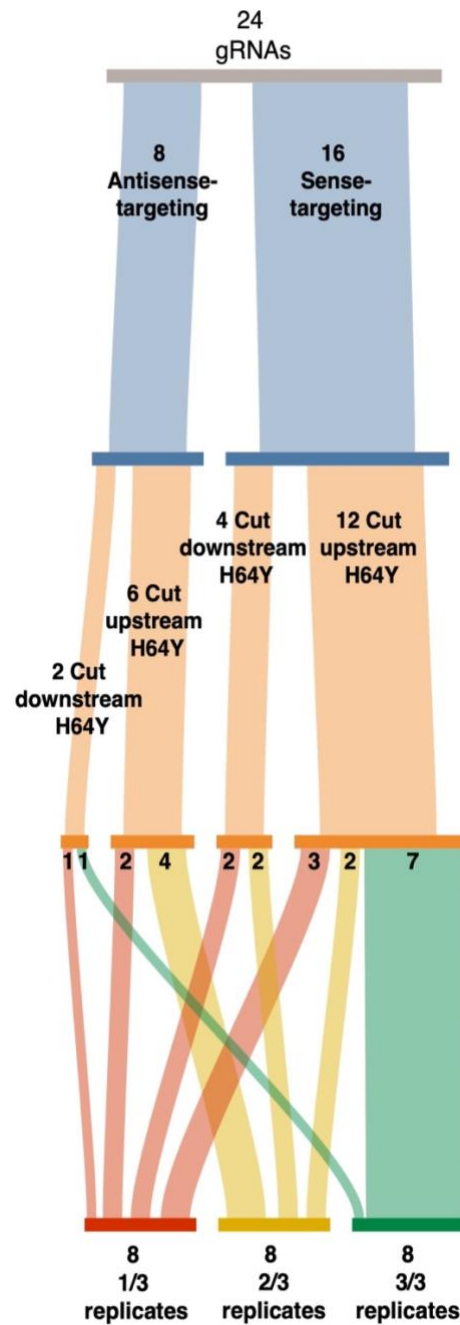


Figure 37. Sankey diagram demonstrates that NNG-Slug-nCas9-PolI5M Δ consistently generates GFP positive cells in over half of gRNAs nicking upstream of H64Y on the sense strand, but not in gRNAs targeting the antisense strand or nicking downstream of H64 do not generally generate H64Y missense mutations consistently.

6. Future work

This dissertation demonstrates EvolvR's capacity to diversify all four nucleotides in mammalian genomic loci for the first time, describes its use for the directed evolution of previously undescribed gain-of-function MAP2K1 variants, and establishes essential groundwork enabling facile genetic engineering of EvolvR-expressing cell lines with flexible PAM-targetability. Critically, the broad tropism of VSVG-pseudotyped lentiviral vectors unlocks the potential to initiate the directed evolution of native genes with a single transduction in most human cell types, including those which have shown promise as cell therapies such as T cells, natural killer cells, and macrophages.

EvolvR's successful diversification native human genes would present the opportunity to engineer complex phenotypes through directed evolution, such as new or enhanced metabolic pathways. Though recent literature has described the use of base editors or synthetic libraries to identify gain of function variants in lymphocytes, these experiments have thus far been limited to single iteration screens and have focused on elucidating the mechanisms underlying T cell antitumor cytotoxicity and differentiation rather than engineering cell therapies^{74,75}. In contrast, pooled gRNA screens using EvolvR would enable the long-term traversal of fitness peaks for as many generations as the host cells can be cultured for or until all target loci are sufficiently mutated to evade recognition by all gRNAs. Therefore, applying EvolvR for the directed evolution of clinically relevant phenotypes is an attractive focus for future work.

For the sake of focusing proposed future work, I will limit my discussion of EvolvR's applications towards engineering T cells below.

6.1. Applying EvolvR to engineer T cells with enhanced fitness within tumor microenvironments.

The immunosuppressive tumor microenvironment (TME) comprising solid tumors hinders immune cells from infiltrating and attacking cancer cells, and is long known to be a key factor underlying the well-documented inefficacy of adoptive cell therapies (ACTs) like CAR-T cells against solid tumors⁷⁶. Immunosuppression in the TME emerges from a complex set of factors, including the presence of ligands that bind to inhibitory receptors (e.g. PD-1, CTLA4)⁷⁷, hypoxia⁷⁸, the physical inaccessibility of the dense stroma comprising tumors^{79,80}, nutrient scarcity⁸⁰, and immunoinhibitory metabolites⁸⁰. Predominantly, approaches for negating immunosuppression in the TME rely on blocking immunoinhibitory T cell signaling pathways, as exemplified by immune checkpoint inhibitors (e.g. anti-PD1 antibodies, anti-CTLA4 antibodies)⁷⁷, knockout of inhibitory receptors⁸¹, and engineering synthetic "switch receptors" that activate T cells upon binding to ligands that are normally inhibitory⁸². In contrast, technologies that enhance T cells' ability to compete for nutrients within the TME are relatively undeveloped, despite evidence showing that nutrient scarcity limits the efficacy of ACTs^{83,84,85,86}.

I hypothesize that the directed evolution of metabolic machinery that resists becoming dysfunctional in environments that emulate immunosuppressive aspects of the TME would elucidate gain-of-function variants that improve adoptive T cell therapies' efficacy against solid tumors. Favorably, dramatic improvements in the efficiency of genetic engineering methods for human T cells have enabled genome-wide CRISPR-based screens in primary cells^{75,87,88,89,90} revealing genetic targets the expression of which can be ablated^{88,89,90}, decreased⁸⁷, increased⁸⁷,

or mutated⁷⁵ to enhance antitumor immunity. Though such screens are capable of identifying genes or gain of function variants that diminish or enhance antitumor efficacy, none of them have thus far leveraged continuous directed evolution of all four nucleotides within genes of interest to enhance antitumor efficacy. Therefore, I propose the use of EvolvR directed evolution campaigns for engineering T cells variants with enhanced metabolic fitness in order to improve T cell potency within the nutrient-poor TME.

It was recently shown that transient glucose restriction (TGR) after CAR-T cell activation “conditions” the cells and improves several predictors of ACT efficacy, including metabolic fitness, cytokine production, tumor clearance, and the persistence of donor cells in an *in vivo* mouse xenograft model⁹¹. Nevertheless, though the set of genes that enhance metabolic fitness after TGR may carry out functions that are amenable to enhancement by directed evolution, they are yet to be systematically identified. To both elucidate the mechanisms underlying the benefits of TGR and identify potential targets for directed evolution, I propose performing genome-wide CRISPR interference and activation screens using stably transduced lentiviral gRNA libraries in primary human T cells as previously described⁸⁷ and expanding the cells under TGR. After TGR and expansion, genetic knockouts with enhanced cytokine production will be enriched by FACS and characterized by NGS as previously described⁸⁷.

Using the findings from the screen described above, I will generate hypotheses for explaining TGR-mediated metabolic changes in T cells, selecting targets for directed evolution according to these hypotheses. In order to maximize the number of generations over which fitness-enhancing mutations can be enriched, and because immortalized cell lines utilize glucose for proliferation⁹², I intend to evolve genetic targets of interest in T cell leukemia-derived Jurkat cells. I will enrich for hits using optimal surrogate biological readouts for CAR-T cell activation as appropriate, potentially including activation markers recapitulated by Jurkat CAR-T cell models (IL-2, CD69)⁹³ or proliferation depending on the genetic target.

6.2. Applying EvolvR to engineer drug-resistant T cells to enhance their competitive fitness against tumors in the presence of glycolysis inhibitors

Competition for glucose between T cells and cancer cells in the TME results in T cell hyporesponsiveness and drives tumor growth⁹⁴. As tumors rely on aerobic glycolysis for proliferation⁹⁴, previous work has investigated the use of glycolysis inhibitors for treating various cancers^{95,96,97}. Unfavorably, T cells also rely on aerobic glycolysis for carrying out effector functions and are likely vulnerable to these glycolysis inhibitors. To endow T cells with a competitive advantage over cancer cells in the presence of glycolysis inhibitors, I propose evolving at least three druggable targets that are essential for glycolysis (GLUT1, HK2, GPI) to identify mutations conferring drug resistance against GLUT1 inhibitor BAY-876⁹⁷ and 2-DG⁹⁵, a glucose analogue that is metabolized by HK2 and consequently inhibits GPI. In combination with clinically-tested glycolysis inhibitors intended for treating cancer, I hypothesize that drug-resistant T cells will more capably outcompete tumors cells for access to glucose and more efficaciously carry out cytotoxic effector functions. Hits will be enriched by culturing in low glucose conditions.

If successful, the work proposed will yield novel biomolecular tools that are generally applicable towards improving the efficacy of all ACTs against solid tumors. Moreover, the T cell diversification and screening methods developed here will set a new standard for engineering ACTs via directed evolution.

7. Conclusion

This dissertation establishes that EvolvR generates diversity within genomic loci in mammalian cells (Chapter 2) and that EvolvR generates sufficient diversity within native genomic loci to identify novel hits not reachable with classical base editors (Chapter 4). Moreover, the data presented here elucidate for the first time that EvolvR's mutation rates are strongly dependent on gRNA design and nickase properties, likely arising from differences in EvolvR's mismatch tolerance and binding kinetics (Chapter 3). With these properties in mind, I successfully make additional engineering improvements to EvolvR, including (1) the identification of Slug-nCas9 as a compact, specific and highly active nickase for improving EvolvR's reliability per gRNA, (2) the removal of the flap endonuclease domain from PolI5M to further miniaturize EvolvR and enable its packaging into lentivirus, (3) the fusion of HMGB1 to Slug-nCas9 to enable simultaneous expression and diversification of the target locus, and (4) the adaptation of a PAM-flexible Slug-nCas9 variant that recognizes NNG PAM sites and vastly increases the number of targetable loci within the human genome. These engineering improvements enable the simultaneous diversification of all four nucleotides and selection for gain of function variants in mammalian genomic loci the first time, facilitating the exploration of previously unexplored, clinically-relevant fitness peaks for human cell therapies.

8. Methods

8.1. Plasmid construction

Plasmids used in transfections were assembled using Golden Gate cloning. Plasmids used for transient transfection of 293 BFP and A375 cells contained a ColE1 origin of replication and AmpR resistance cassette. Prior to transfection, plasmids contained an sfGFP expression cassette in between human U6 promoter and a gRNA scaffold flanked by BsmBI cut sites that would generate overhangs 5'GGTG and 5'TTTG or 5'GTTG depending on the gRNA scaffold sequence. Oligonucleotides containing gRNA sequences and the appropriate overhang sequences 5'CACC and 5'AAAC or 5'CAAC were annealed and knocked in Golden Gate reaction.

Plasmids used for lentiviral packaging of EvolvR transgenes were constructed using Takara Bio's infusion cloning kit, using lentiviral backbone pCW57 (Addgene #41313), and EvolvR transgenes from the plasmids assembled for transient transfections. As described above for the plasmids used in transient transfections, the lentiviral transfer vectors contained an sfGFP expression knockout cassette to be replaced with oligonucleotides encoding gRNAs using a Golden Gate reaction.

8.2. Mammalian cell culture.

HEK293T and A375 cells were purchased from the UC Berkeley Cell Culture Facility. BFP HEK293 cells were generously donated by Jacob Corn's lab.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus L-glutamine (Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco, qualified) and 1x antibiotic-antimycotic. Cells were incubated at a temperature of 37 degrees Celsius and at 5% CO₂ concentration.

8.3. BFP to GFP mutagenesis experiment in BFP HEK293 cells

Unless otherwise noted, cells were transfected using Mirus X2's transfection reagent according to the manufacturer's protocol. Briefly, transfection complexes were prepared by mixing DNA in a 3:1 µg DNA to µL Mirus X2 reagent ratio in 250 µL Opti-MEM serum-free medium. The mixture was incubated at room temperature for 15-30 minutes before dispensing on top of cells. Cells were harvested at 70-90% confluence and reverse transfected by seeding cells at 1 million cells/well in tissue-culture treated 12-well plates immediately before adding transfection mixtures.

16-24 hours after transfection, cells were trypsinized and harvested from 12-well plates and resuspended in 1 mL PBS. 250 µL were transferred to a 96-well plate for flow cytometry analysis on an Attune Nxt flow cytometer, while remaining 750 µL were separated into triplicate 6-well plate wells by dispensing 250 µL into each well. 5-6. 120-144 hours later, cells were once again harvested by trypsinization, resuspended in 300 µL PBS in 96-well plates, and analyzed on the Attune Nxt. Transfection rates and GFP frequencies were determined by analysis on FlowJo of the YL2 and BL1 channels collected on the Attune Nxt.

For high-throughput screens of various high-fidelity nickases shown in Supplementary Figure 5, transfections were scaled down to 48-well plates by seeding 30,000 cells in 48-well plates per condition one day prior to transfection. When the cells were 70-90% confluent, the cells were transfected with Mirus X2 reagent according to the manufacturer's protocol by mixing 390 ng DNA in a 1:3 ng DNA to μ L transfection reagent ratio with 1.17 μ L transfection reagent in 39 μ L Opti-MEM reduced serum medium. The cells were transfected and incubated as described previously. 16-24 hours later, the cells were resuspended in 400 μ L enzyme-free dissociation buffer and split into four 100 μ L volumes, where three were passaged into 2 mL growth media in 6-well plates and the remaining 100 μ L volume was analyzed for mCherry fluorescence on an Attune NxT flow cytometer. The cells were incubated at 5% CO₂ and 37 degrees Celsius for approximately 7 days to enable mutagenesis and were then harvested and analyzed by flow cytometry as previously described.

8.4. Next-generation sequencing experiments with HEK293 BFP cells

HEK293 BFP cells were seeded at approximately 5,000,000 cells per plate in 10 cm tissue culture plates. When cells were at approximately 70% confluence, transfection complexes were prepared by mixing 15 μ g plasmid with 45 μ L Mirus LT1 reagent ratio in 250 1.5 mL Opti-MEM serum-free medium. The mixture was incubated at room temperature for 15-30 minutes before dispensing on top of cells. Approximately 36 hours later, cells were trypsinized and resuspended in 350 μ L PBS and passed through a cell strainer into a FACS tube. 100,000 mCherry positive cells from each transfection were sorted on a Sony SH800Z sorter into 2 mL growth media in 15 mL Falcon tubes for each biological replicate and seeded onto 10 cm plates and incubated for two weeks.

The cells were resuspended in 200 μ L PBS and gDNA was harvested using a Qiagen DNEasy kit according to the manufacturer's protocol.

8.5. Library preparation for next generation sequencing of BFP

The BFP locus was amplified from 500 ng of gDNA for 20 cycles of PCR using oligonucleotides BFP-F and BFP-R. The PCR product was purified using PCR Reaction Cleanup beads from the UC Berkeley Sequencing Facility. The beads were warmed to room temperature and mixed thoroughly in 9:5 volumetric ratio with the PCR product and incubated at room temperature for 5 minutes. Beads were separated from the solution by placing them on magnetic racks and washed with 70% ethanol twice. Ethanol was removed and beads were allowed to dry at room temperature for 30 minutes. The beads were removed from the magnetic rack and thoroughly resuspended in water to elute PCR 1 product. The concentration of PCR 1 product was quantified with a Qubit fluorometer using the high-sensitivity DNA kit. 10 ng of PCR 1 product was applied to a second PCR, in which Illumina adapter sequences and unique dual index pairs were attached. The samples were delivered to the University of California Berkeley Vincent J. Coates Genomics Sequencing Laboratory, where the samples were purified via bead cleanup and size selected by Pippin Prep. The molar concentration of each library prep was quantified by qPCR prior to pooling. PhiX was spiked in at 2% molar ratio relative to the library and diluted to a final concentration of 14 pM. The library was sequenced

using an Illumina MiSeq V2 150 paired-end kit on an Illumina MiSeq sequencer. Samples were demultiplexed using bcl2fastq (v2.20).

8.6. Sequencing data analysis and variant calling

The last 5 nt of each read were trimmed to eliminate low quality reads using seqtk. Trimmed fastq.gz files were merged using NGmerge and were filtered for any read pairs that were not perfectly matching with “-p 0”. The merged fastq files were aligned with the BFP reference sequence with bowtie2. The resulting BAM files were indexed and converted to SAM files using samtools. Mpileup files were generated from the resulting SAM files using samtools mpileup filtering for bases calls with a quality score of at least 30 using -Q 30 and no mapping quality filter. Variant calling was performed by running mpileup files through SiNple⁵⁷ using a theta value (prior probability of a substitution variant) of 0.9.

To reduce the influence of base calling errors and prior mutations on variant calling, the mean variant frequency at each position for two untransfected negative control replicates was subtracted from the frequency of that same variant in all other conditions for all possible variants. Then, substitutions appearing at less than 0.00001 frequency were set to 0 to filter out variants that do not appear in at least 1/100,000 reads.

8.7 MAP2K1 evolution

A375 cells were transfected using Mirus X2's transfection reagent according to the manufacturer's protocol. Briefly, cells were seeded in 6-well plates one day prior to transfection. Once they reached 70% confluence, transfection complexes were prepared by mixing 1 µg plasmid in 3 µL Mirus X2 reagent ratio in 250 µL Opti-MEM serum-free medium. The mixture was incubated at room temperature for 15-30 minutes before dispensing on top of cells.

Approximately 60 hours later, the cells were trypsinized and resuspended into four 250 µL aliquot. Each of three aliquots was seeded into separate 10 cm tissue culture plates, while the fourth was analyzed on a Sony SH800Z sorter to verify consistent transfection efficiencies between samples. After one week of growth, the cells were split in 1:10 fashion into new 10 cm dishes with selective growth media containing 1 µM selumetinib. Selective media was replaced routinely every 48-72 hours until large colonies were visible by the naked eye (approximately 41 days later).

The cells remaining on each plate were harvested and gDNA was harvested with a Qiagen DNEasy kit according to the manufacturer's protocol.

8.8. Library preparation for MAP2K1 evolution experiment

A section of the MAP2K1 exon 6 locus was amplified from 360 ng of gDNA (approximately 100,000 genomes) for 20 cycles of PCR using oligonucleotides

GCTCTTCCGATCTNNNNNCCCTCCTTTTCTATTTTCTCTTCCCTGCAG and GCTCTTCCGATCTNNNNNCCGACATGTAGGACCTTGTGCCC. The PCR product was purified using PCR Reaction Cleanup beads from the UC Berkeley Sequencing Facility. The beads were warmed to room temperature and mixed thoroughly in 9:5 volumetric ratio with the PCR product and incubated at room temperature for 5 minutes. Beads were separated from the solution by placing them on magnetic racks and washed with 70% ethanol twice. Ethanol was removed and beads were allowed to dry at room temperature for 30 minutes. The beads were removed from the magnetic rack and thoroughly resuspended in water to elute PCR 1 product. The concentration of PCR 1 product was quantified with a Qubit fluorometer using the high-sensitivity DNA kit. 10 ng of PCR 1 product was applied to a second PCR, in which Illumina adapter sequences and unique dual index pairs were attached. The samples were delivered to the Innovative Genomic Institute Sequencing Core, where the samples the molar concentration of each library prep was quantified by qPCR prior to equimolar pooling. The library was sequenced using an Illumina MiSeq V2 150 paired-end kit on an Illumina MiSeq sequencer. Samples were demultiplexed using bcl2fastq (v2.20).

8.9. Sequencing analysis for MAP2K1 evolution experiment.

Raw fastq.gz files were processed into SiNPLe variant calling files as described previously. To calculate fold-enrichment of each substitution, the frequency of each variant in EvolvR on target conditions were divided by the frequency of those variants in untransfected cells not having undergone selection.

8.10. SRE reporter assay

cDNA encoding wildtype MAP2K1 from which the enriched MAP2K1 was cloned by PCR was ordered as a gBlock and cloned into a simple mammalian expression cassette under the control of a CMV promoter (pMAP2K1). HEK293T cells were transfected. HEK293 cells were seeded at 30,000 cells per well 96 well white assay plates. After overnight incubation, the cells were transfected with Lipofectamine 2000 according to the manufacturer's protocol with 50 ng pMAP2K1. Approximately 7 hours later, the cells were washed twice with PBS and incubated with media containing selumetinib for 12 hours. After 12 hours of incubation at 37 degrees Celsius and 5% CO₂, the cells were washed with twice PBS and their media was replaced with media containing 10 ng/mL human epidermal growth factor (hEGF) and 0.5% FBS. Approximately 6 hours later, MAP2K1 activity was measured using the SRE Reporter Kit (BPS Bioscience) according to the manufacturer's protocol using a Tecan Spark plate reader. To quantify MAP2K1 activity, background luminescence was determined by measuring the luminescence per the manufacturer's protocol and subtracting the background luminescence from all other reads. To determine relative MAP2K1 activity between wells, background-subtracted Firefly luciferase luminescence was divided by luminescence generated by luminescence generated by Renilla luciferase, a luminescent reporter of transfection efficiency.

8.11. Lentivirus production and generation of EvolvR cell lines

HEK293T cells were reverse transfected by seeding 25 million cells per 10 cm dish in 10 mL growth media lacking antibiotics. To generate transfection complexes, 15 µg DNA comprising 8.4 µg EvolvR transfer vector, 4.2 µg psPax2 (Addgene #12260) and 2.4 µg pCMV-VSV-G (Addgene #8454) was added to 1.5 mL Opti-MEM. 45 µL of 1 mg/mL polyethylenimine was added to the DNA in OptiMEM and incubated for 15 minutes at room temperature. The transfection mixture was dispensed on top of the cells and the cells were incubated at 37 degrees Celsius, 5% CO₂. After 12 hours, cell media was replaced. Approximately 36 hours later, viral supernatant was passed through a 0.45 µm filter and concentrated using the Lenti-X concentrator according to the manufacturer's protocol. Concentrated viral particles were resuspended in 2 mL growth media. To generate EvolvR cell lines, BFP HEK293 cells were seeded directly in 1 mL growth media in 6-well plates.

8.12. Fluorescence microscopy

Microscopy images were captured using an EVOS 5000 fluorescent microscope. To optimally visualize GFP and RFP fluorescent cells and account for differences in background noise between samples and region of the culture plates, the exposure and gain was adjusted between photographs. The contrasts were further adjusted in ImageJ to visualize the absence or presence of GFP and RFP fluorescence and emphasize regions where the signals overlap.

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10. Appendix.

10.1. Code used for generating inputs to alignment pipeline

```
import sys, argparse
import os
import csv
import numpy as np
from scipy import stats
from os import listdir
from os.path import isfile, join

def main(args):

    list_files = (listdir(args.files))

    list_headers = ["Forward Read Filename", "Reverse Read Filename", "Paired end", "Name of reference", "Number
of cores to use for alignment", "Number of threads for merging", "Alignment speed",
                    "mpileup maxdepth", "mpileup min base Q", "mpileup min map q", "output folder", "output base name"]
    starting_tab_delimited_output = ('\t'.join(list_headers) + '\n')

    for x in range(0, len(list_files)):
        if x % 2 == 0:
            condition_base_name = list_files[x][0:int(args.basenamecharacters)]

            list_inputs = [(args.files + "/" + list_files[x]), (args.files + "/" + list_files[x+1]), "Y", args.reference,
args.threadstouse, args.threadstouse, args.alignmentspeed, args.maxdepth, args.minbaseq, args.minmapq,
condition_base_name, condition_base_name, args.percentmismatchidentityfilter]

            starting_tab_delimited_output += ('\t'.join(list_inputs) + '\n')

    with open(args.outputinputname, 'w') as f:
        f.write(starting_tab_delimited_output)
```

```

if __name__ == "__main__":
    parser = argparse.ArgumentParser()
    parser.add_argument("-f", "--files", type=str, help="Path to folder containing raw fastq gzips")
    parser.add_argument("-q", "--minmapq", type=str, help="min map q for mpileup")
    parser.add_argument("-Q", "--minbaseq", type=str, help="min base Q for mpileup")
    parser.add_argument("-r", "--reference", type=str, help="reference FASTA file")
    parser.add_argument("-c", "--threadstouse", type=str, help="threads to use for ngmerge and bowtie")
    parser.add_argument("-bc", "--basenamecharacters", type=str, help="first n letters to use as output name")
    parser.add_argument("-as", "--alignmentspeed", type=str, help="alignment speed to set Bowtie2 to")
    parser.add_argument("-md", "--maxdepth", type=str, help="maximum depth to allow on mpileup")
    parser.add_argument("-o", "--outputinputname", type=str, help="name of final tab delimited file")
    parser.add_argument("-p", "--percentmismatchidentityfilter", type=str, help="The maximum percent (from 0 to 100)
of two paired end reads that can be mismached and still included in analysis.")

args = parser.parse_args(sys.argv[1:])

main(args)

```

10.2. Code comprising alignment and mpileup generation pipeline

```

import sys, argparse
import os
import csv
import pandas as pd
import numpy as np
from scipy import stats
from os import listdir
from os.path import isfile, join

```

```

def main(args):

    #The purpose of the following for loop is to process the input manifest into inputs for the functions comprising the
    pipeline.

    # After the first line is skipped, the excel file is read as a tab delimited file. The new lines are removed and replaced
    # with empty characters. The line elements (line_elems) are are a list of the inputs. If

    with open(args.manifest) as manifest:

        line_count = 0
        for line in manifest:
            if line_count == 0:
                line_count += 1
                continue
            line = line.replace("\n","")
            line_elems = line.split("\t")
            raw_data_path_fwd = line_elems[0]
            raw_data_path_rev = line_elems[1]
            paired_end_or_not = line_elems[2]
            ref = line_elems[3]
            num_cores_merge = line_elems[4]
            num_cores_align = line_elems[5]
            bt2_setting = line_elems[6]
            base_qual = line_elems[8]
            mpileup_depth = line_elems[7]
            mapping_qual = line_elems[9]
            output_folder= line_elems[10]
            output_basename = line_elems[11]
            percent_match_filter = line_elems[12]

            if paired_end_or_not == "Y":
                os.mkdir(output_folder)
                pforngmerge = float(percent_match_filter)/100

                base_output_directory = output_folder + "/" + output_basename

                print('Merging reads for sample: ' + raw_data_path_fwd + ' with NGMerge. Allowing reads with up to ' +
str(percent_match_filter) + ' percent mismatching.')

```

```

        os.system("NGmerge-master/NGmerge -p " + str(pforngmerge) + " -u 41 -w NGmerge-master/jh_qp.txt -n "
+ num_cores_merge + " -1 " + raw_data_path_fwd + " " +
        "-2 " + raw_data_path_rev + " " + "-o" + base_output_directory + "merged.fastq.gz -j " + output_basename
+ "_NGmerge.log" )

    if line_count == 1:

        print('Building bowtie2 database using ' + ref + ".")
        os.system("bowtie2-build " + ref + ".fasta " + ref)
        print('Creating samtools indeces for reference with faidx')
        os.system("samtools faidx " + ref + ".fasta")

    else:

        print("Already set up indeces, moving on.")

    print('Aligning to reference with Bowtie2 on ' + bt2_setting + " with " + num_cores_align + " cores.")
    os.system("bowtie2 -p " + num_cores_align + " --" + bt2_setting + "--no-unal -x " + ref + "-U " +
base_output_directory + "merged.fastq.gz "
        + "-S " + base_output_directory + ".sam")
    print('Creating BAM file out of SAM file.')
    os.system('samtools view -b ' + base_output_directory + ".sam" + "> " + base_output_directory + ".bam")
    print('Sorting BAM file.')
    os.system("samtools sort " + base_output_directory + ".bam > " + base_output_directory + "_sorted.bam")
    print('Indexing sorted BAM file.')
    os.system("samtools index " + base_output_directory + "_sorted.bam")
    print('Creating mpileup from a maximum of ' + mpileup_depth + " with min mapping quality " +
mapping_qual + " and min base quality " + base_qual + ".")
    mpileup_output_filename = (base_output_directory + "_minbaseQ_" + base_qual + "_minmapq_" +
mapping_qual + "_mpileup.mpileup")
    os.system("samtools mpileup " + "-q " + mapping_qual + "-Q " + base_qual + "-f " + ref + ".fasta"
        + "-d " + mpileup_depth + " " + base_output_directory + "_sorted.bam " + "-o " +
mpileup_output_filename)

    print('mpileup done. Here is a command you can run if you are using SiNPlE: /SiNPlE -t 0.95 -i '
        + mpileup_output_filename + "-o " + base_output_directory + "_variants.txt")
    print("Saving parameter information.")

```

```

text_file = open(base_output_directory + "_parameters.txt", "w")
list_labels = ["Forward File Directory: ", " Reverse File Directory: ",
               "Paired end? (Y for Yes or N for No): ",
               "Name of reference FASTA: ",
               "Number of cores used for merging: ",
               "Number of cores used for alignment: ",
               "Bowtie2 alignment aetting: ",
               "mpileup starting coordinate: ",
               "mpileup min base Q: ",
               "mpileup max depth: ",
               "mpileup min quality score: ",
               "Output file folder: ",
               "Output file base name: "]

for x in range(0, len(line_elems)-1):
    print("Adding " + str(list_labels[x]) + " information: " + line_elems[x])
    text_file.write(list_labels[x] + line_elems[x] + "\n")

    print("DONE with " + base_output_directory + ". The following file contains the parameters used in the
manifest submitted for this analysis run: "
        + base_output_directory + "_parameters.txt")

    if paired_end_or_not == "N":
        print("Nonpaired end functionality not written yet.")

    line_count = line_count + 1

if __name__ == "__main__":
    parser = argparse.ArgumentParser()
    parser.add_argument("-M", "--manifest", type=str, help="Path to text file containing arguments")
    args = parser.parse_args(sys.argv[1:])

    main(args)

```

10.3. Code for variant calling from mpileups in parallel using SiNPlE

```
import sys, argparse
import os
import csv
import numpy as np
from scipy import stats
from os import listdir
from os.path import isfile, join
import multiprocessing as mp
import os
from joblib import Parallel, delayed
os.environ["OPENBLAS_MAIN_FREE"] = "1"

def simpler(i, t, o, n) :
    n = int(n)
    output_filename_raw = i.split("/")
    output_filename_raw = output_filename_raw[-1]
    output_filename = str(output_filename_raw[0:n])
    os.system("simple_master/simple/SiNPlE -i " + i +
              " -t " + t + " -o " + o + "/" + output_filename + ".txt")

def main(args):
    os.mkdir(args.outputfolder)
    directory = args.directory_mpileups
    processors = int(args.numthreads)
    priorprob = args.priorsubprob
    outputfolder = args.outputfolder
    n = args.numchars
    list_files = (listdir(directory))

    list_file_with_directories = []

    for x in list_files:
        branch1 = directory + "/" + x
        sublist_files = listdir(branch1)
        for y in sublist_files:
```

```

        if y.endswith(".mpileup"):
            list_file_with_directories.append(branch1 + "/" + y)

    Parallel(n_jobs=processors)(delayed(simpler)(mpileup, priorprob, outputfolder, n) for mpileup in
list_file_with_directories)

if __name__ == "__main__":
    parser = argparse.ArgumentParser()
    parser.add_argument("-d", "--directory_mpileups", type=str, help="list of subdirectories containing the mpileups to
be analyzed.")
    parser.add_argument("-t", "--priorsubprob", type=str, help="Prior probability of a mutant to feed into SiNPlE.
Between 0 and 1, a higher value results in higher sensitivity and higher false positives.")
    parser.add_argument("-o", "--outputfolder", type=str, help="Where to dump the output files.")
    parser.add_argument("-p", "--numthreads", type=str, help="How many threads to use")
    parser.add_argument("-n", "--numchars", type=str, help="first number of mpileup filename characters to use in
output name")

args = parser.parse_args(sys.argv[1:])

main(args)

```

10.4. Code for analysis of variant frequencies at each position and identifying unique variants within windows of interest from SiNPlE output files.

```
import matplotlib.pyplot as plt
```

```

from pylab import *
import pandas as pd
import csv
from scipy.stats import gamma
from scipy.stats import norm
from scipy.stats import entropy
import numpy as np
import seaborn as sns
from os import path
from cmcrameri import cm
import matplotlib.font_manager as fm

### Set a few things
save_plots = False
#out_dir = '/Users/libbykoolik/Documents/Research/Miscellaneous/juan_rolling_code'

sns.set_context('notebook')
sns.set_style('white')

file_directory = '/Users/juanhurtado/Desktop/Manuscript_2024'

### Define a function for the output file
def import_df(fn, poi, file_directory=file_directory):

#Takes a SiNPlE output text file as fn and some minimum and maximum positions and outputs a pandas dataframe
containing the data.

    filedir = path.join(file_directory, fn) # directory provided of the raw text file
    final_output = open(filedir) # open the text file

    reader = csv.reader(final_output, delimiter="\t") #create a csv reader for the raw file

    data = list(reader) #The data is converted into a list where each entry is a different line of the csv file.

    proto_df = [] # list of lists to become a dataframe at the end

```

```

for position_data in data: #iterate through each line of the csv file in the list form known as "data".

    position = int(position_data[1])

    if poi[0] <= position <= poi[1]: # Before doing anything, check to make sure that the position is within the range
that we provided as max_poi and min_poi.

        possible_snps = ['A','T','C','G'] # List of possible substitution variants before we know the reference nucleotide

        position_proto_df = []
        num_unique_variants = int((len(position_data)-2)/4) #Total number of variants for each list is equal to the
length of the data -2 divided by 4. This is because each variant has 4 parameters, but the list contains an empty spot
followed by the position of the variants along the reference, which only show up once.

        total_reads_at_position = 0 # Start a variable for the total number of reads in the position that we will add to to
generate a total read count for each position (i.e. coverage).

        for b in range(0,num_unique_variants): # Iterating over the number of different variants.

            varr_index = 2+4*b # The variant sequence appears every 4 times on the list, so the first read count
appears in index 2 and after that 6, etc.

            read_count_index = 3 + 4*b # The read count appears every 4 times on the list, so the first read count
appears in index 3 and after that 7, etc.

            quality_index = 4 + 4*b # The average quality score appears every 4 times on the list, so the first read
count appears in index 4 and after that 8, etc.

            postprob_index = 5 + 4*b # The posterior probability of the variant being real appears every 4 times on the
list, so the first read count appears in index 5 and after that 9, etc.

```

```

varr = position_data[varr_index] #The variant sequence is identified.
read_count = int(position_data[read_count_index]) # The variant read count is identified.
total_reads_at_position += read_count #Adds the variant's read counts to the total read counts at the
position.

quality = float(position_data[quality_index]) # The variant's mean quality score is identified.
postprob = float(position_data[postprob_index]) #The variant's probability of being real is identified.


if len(varr) > 1: #If the string of the variant is longer than one, it is an indel.
    variant_type = "indel"
else:
    variant_type = "snp" #Otherwise it's a snp.
    possible_snps.remove(varr) # get rid of the varr from possible SNPs remaining to be found


row_proto_df = [position,0, varr, read_count, quality, postprob, variant_type, 0, 0]

position_proto_df.append(row_proto_df)


reference_nuc = str(position_proto_df[0][2])

for x in range(0,len(position_proto_df)): # Calculates the frequency of each variant at the current position.
    position_proto_df[x][-2] = position_proto_df[x][3]/total_reads_at_position
    position_proto_df[x][1] = position_proto_df[0][2]
    position_proto_df[x][-1] = str(position_proto_df[x][0]) + '-' + str(position_proto_df[x][2])


if len(possible_snps) > 0:
    for variant in possible_snps:
        print("Missing var " + str(variant))
        print("Creating the following entry.")
        zero_row = [position, reference_nuc, variant, 0, 0 ,0, 'snp', 0, str(str(position) + '-' + variant)]
        print(zero_row)
        position_proto_df.append(zero_row)

```

```

proto_df.extend(position_proto_df)

output_dataframe= pd.DataFrame(proto_df, columns =
['Position','Reference','Variant','Counts','MeanQ','BProbability','Type', 'Freq', 'VarCode'])

return (output_dataframe)

def AddGammapVals(df):

def fit_gamma(frequencies):
    """
    Iteratively fits a gamma distribution to the provided group.
    Outliers are identified and removed after fitting by the three-sigma-rule, and then refit
    """

    from scipy import stats
    frequencies = frequencies.tolist()
    converged = False
    if len(frequencies) < 2:
        return [10000,10000,10000]
    tofit = frequencies
    while not converged:
        param = stats.gamma.fit(tofit, floc=0)
        std = stats.gamma.std(param[0], param[1], param[2])
        mean = stats.gamma.mean(param[0], param[1], param[2])
        if len(tofit) > 5 and (mean + 3 * std) < max(tofit): # three-sigma-rule for identifying outliers
            tofit.remove(max(tofit)) # exclude extreme outlier and reiterate
        else:
            return param[0], param[1], param[2]

def calc_pvalue(frequency, a_param, c_param): # calculates a p value based on a gamma distribution generated
by fit_gamma

```

```

pval = 1 - gamma.cdf(frequency, a_param, 0, c_param)

return(pval)

pvalled_dfs = [] #list of sub dfs now containing gamma p values

substitution_types = [['A','T'], ['A','C'], ['A','G'],
                      ['T','A'], ['T','C'], ['T','G'],
                      ['C','A'], ['C','T'], ['C','G'],
                      ['G','A'], ['G','C'], ['G','T']]

for snp in substitution_types:

    filtered_df = df[(df.Reference == snp[0]) & (df.Variant == snp[1])].copy()

    frequencies_of_this_type = filtered_df['Freq'].values

    a,b,c = fit_gamma(frequencies_of_this_type)

    filtered_df['gamma_p'] = filtered_df.apply(lambda x: calc_pvalue(x['Freq'], a, c), axis=1)

    pvalled_dfs.append(filtered_df)

# indel_df = df[df.Type=="indel"]
# indel_df['gamma_p'] = 1.0
# pvalled_dfs.append(indel_df)

return(pd.concat(pvalled_dfs))

def filterbyGamma(df, max_p): # returns a dataframe with only rows that pass the gamma p value filter.

```

```

    return (df.loc[df['gamma_p'] <= max_p,:])

def filterbyBayes(df, min_p): # returns a dataframe with only rows that pass the gamma p value filter.
    return (df[df.BProbability >= min_p])

def filterbyType(df, subtype): # returns a dataframe with only variants that are indels or snps

    return(df[df.Type==subtype])

def filterbyQ(df, minQ): # returns a dataframe with only variants meeting minimum average quality scores

    return(df[df.MeanQ>=minQ])

def filterbyN(df, N): # returns a dataframe with only SNP variants of a user defined nucleotide (A,T,C,G)
    return(df[df.Variant==N])

def filterbyFreq(df, f, subFreq = False): # returns a dataframe with only variants of at least a user defined frequency.

    if subFreq == False:

        return(df[df.Freq>=f])

    else:
        df['SubFreq'] = df['SubFreq'].apply(lambda x: 0 if x < f else x)
        return(df)

def countuniqueVars(list_df, subtype): #count the total number of unique variants that show up between all samples

    reference_free_df_list = []

    for df in list_df:
        reference_free_df_list.append(df[(df.Reference != df.Variant) & (df.Type == subtype)])

    merged_df = pd.concat(reference_free_df_list)

    # print(merged_df)

```

```

count_uniques = len(pd.unique(merged_df["VarCode"]))

return(count_uniques)

def removePriors(e_df, c_df):

    list_control_codes = c_df["VarCode"].unique().tolist()

    # print(list_control_codes)

    subtracted_df = e_df.loc[~e_df["VarCode"].isin(list_control_codes)]

    return(subtracted_df)

def meanfreqVars(df): ### function inputs a df and outputs another df where it only has a single frequency for a single
variant representing the average

    output_df = df.groupby(["VarCode", "Reference", "Type", "Variant"]).mean().reset_index()

    return(output_df)

def subtractPriors(varcode, e_df, c_df):

    e_row = e_df[e_df.VarCode==varcode].reset_index()

    if varcode in c_df["VarCode"].values:

        c_row = c_df[c_df.VarCode==varcode].reset_index()

        subtracted_freq = e_row.at[0, 'Freq'] - c_row.at[0, 'Freq']

        if subtracted_freq < 0:

```

```

        subtracted_freq = 0

    else:
        subtracted_freq = e_row.at[0,'Freq']

    return(subtracted_freq)

def calculatePercentileFreq(df, percentile): ### calculates the frequency value of a user defined percentile within the
data supplied by the user

## not debugged

list_freqs = df['Freq'].to_list()

arr_freqs = np.array(list_freqs)
# print(arr_freqs)

percentile_freq = np.percentile(arr_freqs, percentile)

return(percentile_freq)

def preparebiasesHeatmap(df): # takes a df and pulls out the total frequencies of each type of substitution variant.
Outputs a heatmappable df

list_total_freqs = []

df = df[df.Type!='snp'] #Get rid of indel values

sum_snps = df['Freq'].sum()

substitution_types = ['A','T','C','G']

for snp1 in substitution_types:

```

```

ref_list = []

for snp2 in substitution_types:

    if snp1 == snp2:
        ref_list.append(0)
        continue

    filtered_df = df[(df.Reference == snp1) & (df.Variant == snp2)]

    frequencies_of_this_type_sum = filtered_df['Freq'].values.sum()

    ref_list.append(frequencies_of_this_type_sum)

ref_list = ref_list/sum_snps

list_total_freqs.append(ref_list)

returnable_df = pd.DataFrame(list_total_freqs, substitution_types, substitution_types)

return(returnable_df)

def preparediversityHeatmap(df, lowerpos, higherpos, relative, subFreq = False, binary=False, mincount = 1): # takes
a df and pulls out the total frequencies of each type of substitution variant per position. Outputs a heatmappable df

df = df[df.Type=='snp'] #Get rid of indel values

df = df.sort_values (by=['Position'], ascending = True) # sort df by position

df = df[(df.Position >= lowerpos) & (df.Position <= higherpos)] # filter df by positions of interest

if subFreq:
    sum_snps = df['SubFreq'].sum()

```

```

else:
    sum_snps = df['Freq'].sum()

    positions = range(lowerpos, higherpos + 1)

    status_by_position = [[],[],[],[]]

    substitution_types = ['A','T','C','G']

    for position in positions:

        position_data = df[(df.Position == position)]

        # print(position_data)

        for nucindex in range(0,len(substitution_types)):

            relevant_var = substitution_types[nucindex]

            reference_nuc_column = position_data['Reference'].values.tolist()

            vars_at_position = position_data['Variant'].values.tolist()

            if relevant_var in reference_nuc_column:
                status_by_position[nucindex].append(None)

            else:

                if binary:

                    if relevant_var in vars_at_position:

```

```

countedtimes = vars_at_position.count(relevant_var)

status_by_position[nucindex].append(countedtimes)

else:
    status_by_position[nucindex].append(0)

else:

    if relevant_var in vars_at_position:

        varposition_df = position_data[position_data.Variant == relevant_var]

        if subFreq:

            sumfreqs = varposition_df['SubFreq'].values.sum()

        else:
            sumfreqs = varposition_df['Freq'].values.sum()

        if relative:

            relative_freq = sumfreqs/sum_snps

            status_by_position[nucindex].append(relative_freq)

        else:

            status_by_position[nucindex].append(sumfreqs)
    else:
        status_by_position[nucindex].append(0)

# print(status_by_position)

```

```

returnable_df = pd.DataFrame(status_by_position)

return(returnable_df)

def plotResults(df, yscale, xmin, xmax, ymin, ymax, title, DifferentiateSNPs, otherFreq = None): # Plots a df's
positions vs frequency of variants using SeaBorn. If DifferentiateSNPs is true, it plots the dots by color.

    if otherFreq == None:
        freq = "Freq"
    else:
        freq = otherFreq

    if DifferentiateSNPs:
        df = df[df.columns].sort_values("Variant").copy()
        sns.set_theme(style = "whitegrid")
        f, ax = plt.subplots(figsize=(6.5, 6.5))

        sns.scatterplot(x="Position", y=freq, hue="Variant", #style="Variant",
                        # palette="ch:r=-.2,d=.3_r",
                        palette='pastel',
                        sizes=(1, 8), linewidth=0,
                        data=df, ax=ax)
        sns.set(font_scale=2)
        plt.title(title)
        plt.yscale("log")
        plt.xlim(xmin,xmax)
        plt.ylim(ymin,ymax)
        plt.ylabel("Variant Frequency")
        plt.xlabel("Position")

    else:
        print("Not differentiating SNPs")

    return f, ax

```

```

def get_entropy(df):
    """
    # Calculate the entropy for each position and base pair
    df['entropy'] = np.abs(df['Freq'])*np.log2(df['Freq'])

    # Get the entropy for each position
    entropied_df = df.groupby('Position')[['entropy']].sum().reset_index()

    return entropied_df

def sumentropy_within_window(df, lowerposition, upperposition):

    filtered_df = df[(df['Position'] >= lowerposition) & (df['Position'] <= upperposition)]

    return filtered_df['entropy'].sum()

def filterdfbywindow(df, lowerposition, upperposition):

    filtered_df = df[(df['Position'] >= lowerposition) & (df['Position'] <= upperposition)]

    return filtered_df

#%%

#####
#####

##Script starts below##

#####
#####

condition_groups = [['01','02'],
                    ['03','04'],
                    ['05','06'],
                    ['07','08'],
                    ['09','10'],

```

```

        ['11','12'],
        ['13','14'],
        ['15','16']]

list_condition_names = [['nCas9-Poll5M + 18 full length R1', 'nCas9-Poll5M + 18 full length R2'],
                        ['nCas9-Poll5M + 5 full length R2', 'nCas9-Poll5M + 5 full length R2'],
                        ['nCas9-Poll5M + 18 truncated R1', 'nCas9-Poll5M + 18 truncated R2'],
                        ['enCas9-Poll5M off target R1', 'enCas9-Poll5M off target R2'],
                        ['enCas9-Poll5M + 18 full length R1', 'enCas9-Poll5M + 18 full length R2'],
                        ['enCas9-Poll5M + 5 full length R1', 'enCas9-Poll5M + 5 full length R2'],
                        ['enCas9-Poll5M + 18 truncated R1', 'enCas9-Poll5M + 18 truncated R2'],
                        ['untransfected R1', 'Untransfected R2']]

list_condition_sets = [ 'wtEvolvR + PAM distal gRNA', 'wtEvolvR + PAM proximal gRNA', 'wtEvolvR + truncated PAM
                        distal gRNA',
                        'enhEvolvR + off target gRNA', 'enhEvolvR + PAM distal gRNA', 'enhEvolvR + PAM proximal gRNA',
                        'enhEvolvR + truncated PAM distal gRNA', 'Untransfected'
                        ]

negative_control_index= -1 #####USER DEFINED#####

roi = [140,250] #####USER DEFINED#####

prefix = 'tDJH_TG/tDJH_TG' #####USER DEFINED#####
suffix = '.txt' #####USER DEFINED#####

maxgamma_pval = 1.0 #####USER DEFINED#####
min_single_p = 0.0#####USER DEFINED#####

minfreq = 0.00008 #####USER DEFINED#####
minaveq = 0 #####USER DEFINED#####

freq_ymin = 0.000001
freq_ymax = 0.1

norm_ymin = 1

```

```

norm_ymax = 100000

ymin = 0.000005
ymax = 0.01

xmin = 140
xmax = 250

yscale = "log"

snp_colors = ['b', 'r', 'g', 'm']

#

#%%###Import raw dataframes###/
list_of_raw_snp_dataframes = []
list_of_raw_indel_dataframes = []

for condition in condition_groups:
    condition_snp_df_list = []
    condition_indel_df_list = []
    for replicate in condition:
        filename = prefix + replicate + suffix
        sample_df = import_df(filename, roi)
        #gammapad_df = AddGammapVals(sample_df)

        indel_df = filterbyType(sample_df, "indel")
        snp_df = filterbyType(sample_df, "snp")

        condition_snp_df_list.append(snp_df)
        condition_indel_df_list.append(indel_df)

    list_of_raw_snp_dataframes.append(condition_snp_df_list)
    list_of_raw_indel_dataframes.append(condition_indel_df_list)

```

```

#%%Analyze snps based on prior variant subtraction only, then pass a frequency filter on the subtracted frequencies
save_plots = False

control_combined_df = pd.concat(list_of_raw_snp_dataframes[negative_control_index])
means_control_combined_df = meanfreqVars(control_combined_df)
minfreq = 0.00001

list_priorsubtractedfrequencyfiltered_dfs = []

for i in range (0, len(list_of_raw_snp_dataframes)):

    list_priorsubtractedfrequencyfiltered_dfs.append([])

    condition_name = list_condition_sets[i]

    first_df = True

    for j in range(0,len(list_of_raw_snp_dataframes[i])):

        replicate_df = list_of_raw_snp_dataframes[i][j]

        copy_df = replicate_df

        replicate_df['SubFreq'] = replicate_df.apply(lambda x: subtractPriors(x['VarCode'],
copy_df,means_control_combined_df), axis=1)

        priors_subtracted_replicate_df = replicate_df

        priors_subtracted_freq_filtered_df = filterbyFreq(priors_subtracted_replicate_df, minfreq, subFreq=True)
        list_priorsubtractedfrequencyfiltered_dfs[i].append(priors_subtracted_freq_filtered_df)

```

```

if first_df:
    concat_df = priors_subtracted_freq_filtered_df
    first_df = False

else:
    concat_df = pd.concat([concat_df, priors_subtracted_freq_filtered_df])

plottable_df = concat_df

title = str(condition_name) + '_' + "prior_subtracted_and_freq_filtered_" + str(minfreq) + ".png"

f, ax = plotResults(plottable_df, yscale, xmin, xmax, ymin, ymax, condition_name, True, otherFreq='SubFreq')

if save_plots:
    f.savefig(path.join(out_dir, title), transparent = True, dpi = 300)

for i, df_list in enumerate(list_priorsubtractedfrequencyfiltered_dfs):
    for j, df in enumerate(df_list):
        df = df.sort_values(by='Position') # Filter out rows where Reference is equal to Variant

        varsums_df = df[df['Reference'] != df['Variant']]

        # Group by 'Position' and sum 'SubFreq', then reset the index to turn 'Position' back into a column
        varsums_df = varsums_df.groupby('Position')['SubFreq'].sum().reset_index()

        # Rename columns for clarity
        varsums_df.columns = ['Position', 'Total SubFreq']

        # Generate a unique filename
        filename = f'dataframe_{i}_{j}.csv'
        filename_sums = f'dataframe_{i}_{j}_sums.csv'

        # Export the DataFrame to a CSV file

```

```

df.to_csv(filename, index=False)

#varsums_df.to_csv(filename_sums, index = False)

print(f'Exported {filename}')
print(f'Exported {filename_sums}')

### Generate violin plots of the mean frequencies of the substitution variants between two duplicates within a given
window
woi = [182,197]

list_means = []

for i in list_priorsubtractedfrequencyfiltered_dfs:
    firstdf = True
    for j in i:
        if firstdf:
            concat_df = j
        else:
            concat_df = pd.concat(concat_df, j)

    appendable_df = filterdfbywindow(concat_df, woi[0], woi[1])

    list_means.append(meanfreqVars(appendable_df))

###
# Formatting
sns.set_style('ticks')
cmap = cm.berlin
ref_pal = [cmap(0), cmap(0.99), cmap(0.75), cmap(0.25)]

# Grab the relevant ones
dfs = [list_means[0], list_means[1]] # [list_means[6]]

```

```

# Add labels
dfs[0]['CATEGORY'] = 'nCas9 + tru-gRNA'
dfs[1]['CATEGORY'] = 'nCas9 + gRNA'
#dfs[2]['CATEGORY'] = 'enCas9 + tru-gRNA'

# Update to spread by reference
shift_x_dict = {'A':-0.1, 'T':-0.05, 'G':0.0, 'C':0.05}

for df in dfs:
    df['SHIFT'] = df['Reference'].map(shift_x_dict)

# Assign X-value for later
dfs[0]['X'] = 0 + dfs[0]['SHIFT']
dfs[1]['X'] = 1 + dfs[1]['SHIFT']
#dfs[2]['X'] = 2 + dfs[2]['SHIFT']

# Concatenate
df_to_plot = pd.concat(dfs)

# Get rid of everything that is >1%
df_to_plot = df_to_plot[df_to_plot['SubFreq']<=0.01].copy()

fig, ax = plt.subplots(figsize=(6,4))

# Add the violin plot
sns.boxplot(data=df_to_plot, x='CATEGORY', y='SubFreq', color='lightgray', width=0.4,
            showfliers=False, linewidth=0.5, ax=ax )
for patch in ax.artists:
    patch.set_facecolor('none')

# Add the dots for each condition
sns.scatterplot(data=df_to_plot, x='X', y='SubFreq', hue='Reference', palette=ref_pal,
               legend=False, ax=ax)

# Final formatting
ax.set_yscale('log')
ax.set_ylim([0.000008,0.002])

```

```

ax.set_xticks(ax.get_xticks(),
              labels=['nCas9 + tru-gRNA', 'nCas9 + full gRNA'], #enCas9 + tru-gRNA'],
              fontsize=14, rotation=15)
ax.set_xlabel("")
ax.set_ylabel('Variant Frequency', fontsize=16)
plt.yticks(fontsize=14)
fig.tight_layout()
#plt.savefig('truncDistributionwithzeros.svg', format = 'svg')

#%%%

filenames = ['nCas9trugRNA', 'nCas9gRNA', 'enCas9trugRNA']
index = 0
for df in dfs:
    df = df.sort_values(by='Position')
    # Generate a unique filename
    filename = filenames[index] + '.csv'
    index = index + 1

    # Export the DataFrame to a CSV file
    #df.to_csv(filename, index=False)
    print(f'Exported {filename}')

```

10.5 Code for quantification of fold-enrichment of MAP2K1 variants over untransfected cells

```

import matplotlib.pyplot as plt
from pylab import *
import matplotlib.ticker as ticker
import seaborn as sns
sns.set_style('ticks')

```

```

def simplyplotmuts(fn, minaveq, minbp, min_freq, rarity_threshold,min_poi, max_poi):

    from scipy.stats import gamma
    import matplotlib.pyplot as plt
    import csv
    from statistics import median
    import numpy as np
    from scipy.stats import norm

    def fit_gamma_jh(frequencies):
        """
        Iteratively fits a gamma distribution to the provided group.
        Outliers are identified and removed after fitting by the three-sigma-rule, and then refit
        """
        from scipy import stats
        converged = False
        if len(frequencies) < 2:
            return [10000,10000,10000]
        tofit = frequencies
        while not converged:
            param = stats.gamma.fit(tofit, floc=0)
            std = stats.gamma.std(param[0], param[1], param[2])
            mean = stats.gamma.mean(param[0], param[1], param[2])
            if len(tofit) > 5 and (mean + 3 * std) < max(tofit): # three-sigma-rule for identifying outliers
                tofit.remove(max(tofit)) # exclude extreme outlier and reiterate
            else:
                return param[0], param[1], param[2]

    min_snp_freq = min_freq # minimum frequency for a variant to be considered real.
    min_qual = minaveq # minimum quality for a base to be considered. The min qual is the min average base qualities
    for entire reads that contain a given variant.

    min_p = minbp # minimum bayesian probability of true variant given by SINPLE for a given variant/
    master_snp_freqs = [] # creating a list for the frequencies of each variant/
    master_snp_subs = [] # creating a list for the specific substitutions made for each base
    master_snp_bayes_pval_outcome = [] # Stores a tuple for whether or not a variant at a given position passes the
    Bayesian prior probability threshold. Each nested list represents a position.

```

```

master_snp_positions = [] #Will store the position of each substitution in the reference as we find it.

filedir = fn # directory provided of the raw text file
final_output = open(filedir) # open the text file

reader = csv.reader(final_output, delimiter="\t") #create a csv reader for the raw file

data = list(reader) #The data is converted into a list where each entry is a different line of the csv file.

#print(data)

for x in data: #iterate through each line of the csv file in the list form known as "data".
    position = int(x[1])

    if min_poi <= position <= max_poi: # Before doing anything, check to make sure that the position is within the
range that we provided as max_poi and min_poi.

        # print(position)

        relevant_pos_data = x # Rename the entire line to the position's data for intuitive human readability.
        total_reads_at_position = 0 # Start a variable for the total number of reads in the position that we will add to to
generate a total read count for each position (i.e. coverage).

        num_vars = int((len(relevant_pos_data)-2)/4) #Total number of variants for each list is equal to the length of
the data -2 divided by 4. This is because each variant has 4 parameters, but the list contains an empty spot followed
by the position of the variants along the reference, which only show up once.

        snp_variants = [] # Create a list to contain specific variants.
        snp_frequencies = [] # Create a list to contain each variant's frequencies.
        snp_b_pval_outcomes = [] # Stores a tuple for whether or not a variant at a given position passes the
Bayesian prior probability threshold

        snp_positions = [] #Stores the position of each line along the reference sequence provided.
        for b in range(0,num_vars): # Iterating over the number of different variants.
            read_count_index = 3 + 4*b # The read count appears every 4 times on the list, so the first read count
appears in index 3 and after that 7, etc.

            total_reads_at_position += int(relevant_pos_data[read_count_index]) #The total number of reads for a given
variant is added to the total number of reads at the position/

```

```

    for b in range (0,num_vars): # Iterating over the number of different variants again. Not the most efficient but it
was more readable for me.

        varr_index = 2+4*b # The read count appears every 4 times on the list, so the first read count appears in
index 2 and after that 6, etc.

        varr = relevant_pos_data[varr_index] #The variant sequence is identified.

        if len(varr) > 1: #If the string of the variant is longer than one, it is an indel and is ignored.

            continue

        if float(relevant_pos_data[4+4*b]) > min_qual and
((int(relevant_pos_data[3+4*b])/total_reads_at_position))>= min_snp_freq: # Otherwise, if the variant has an average
quality score higher than the min quality and higher than the min frequency.

            snp_b_pval_outcomes.append(float(relevant_pos_data[5+4*b]) >= min_p) # Note whether it meets the
minimum Bayesian prior probability generated by SiNPLE.

            snp_variants.append(varr) #Add the varr sequence to the variants list.

            snp_frequencies.append(((int(relevant_pos_data[3+4*b])/total_reads_at_position))) #Then add the
corresponding variant frequency by dividing the number of reads with this variant by the total number of reads at the
position

            snp_positions.append(position)


        master_snp_freqs.append(snp_frequencies) #For each variant, add the final snp_frequencies list to the
master list of lists containing snp frequencies at each line.

        master_snp_subs.append(snp_variants) #For each variant, add the final subs list to the master list of lists
containing snp subs at each line.

        master_snp_bayes_pval_outcome.append(snp_b_pval_outcomes) #Store the list of pval outcomes (1 or 0 ) for
the SNPs in a given position.

        master_snp_positions.append(snp_positions) #Add the positions the master list we created earlier to be able
to call the position of each variant later on.


    #Next, we fit distributions to the background rate of each type of substitution mutation to assign a frequency-based
p value to each variant based on how unlikely it is that each variant would be background noise given the distribution
of background noise for its substitution type.


    #First create lists to contain frequencies of each of 12 types of substitutions.

    list_A_T = []
    list_A_G = []
    list_A_C = []

```

```

list_T_A = []
list_T_G = []
list_T_C = []

list_C_T = []
list_C_G = []
list_C_A = []

list_G_T = []
list_G_A = []
list_G_C = []

#Create variables to store the total amount of As, Ts, Cs, and Gs.

total_A = 0

total_T = 0

total_C = 0

total_G = 0

for x in range (0,len(master_snp_freqs)): #We iterate over the master list of lists of substitution frequencies.
    if len(master_snp_freqs[x]) > 0: #If there is at least one substitution in a given position...
        consensus_base = master_snp_subs[x][0] #The consensus base is listed as the first one thanks to SiNPlE.
        previous_base = 'A' #Set a dummy previous base so that we can use the first position

```

```
for y in range(1, len(master_snp_freqs[x])): #For each line, we iterate over the entire line starting at position 1
since we are not including the reference base.
```

```
submade = master_snp_subs[x][y] #The substitution made is equal to the entry in the master_snp_subs
```

```
if submade == 'A': #Here things get repetitive: If the substitution made is A, look at the reference base. If
the reference base is T, add the substitution frequency to the T to A list.
```

```
if consensus_base == 'T':
```

```
list_T_A.append(float(master_snp_freqs[x][y]))
```

```
if consensus_base == 'G':
```

```
list_G_A.append(float(master_snp_freqs[x][y]))
```

```
if consensus_base == 'C':
```

```
list_C_A.append(float(master_snp_freqs[x][y]))
```

```
if submade == 'T':
```

```
if consensus_base == 'A':
```

```
list_A_T.append(float(master_snp_freqs[x][y]))
```

```
if consensus_base == 'G':
```

```
list_G_T.append(float(master_snp_freqs[x][y]))
```

```
if consensus_base == 'C':
```

```
list_C_T.append(float(master_snp_freqs[x][y]))
```

```
if submade == 'G':
```

```
if consensus_base == 'T':
```

```
list_T_G.append(float(master_snp_freqs[x][y]))
```

```
if consensus_base == 'C':
```

```
list_C_G.append(float(master_snp_freqs[x][y]))
```

```
if consensus_base == 'A':
```

```
list_A_G.append(float(master_snp_freqs[x][y]))
```

```
if submade == 'C':
```

```
if consensus_base == 'A':
```

```
list_A_C.append(float(master_snp_freqs[x][y]))
```

```

        if consensus_base == 'T':
            list_T_C.append(float(master_snp_freqs[x][y]))

        if consensus_base == 'G':
            list_G_C.append(float(master_snp_freqs[x][y]))

#Fit a gamma distribution to the frequencies for each type of substitution.

a_A_T, b_A_T, c_A_T = fit_gamma_jh(list_A_T)
a_A_C, b_A_C, c_A_C = fit_gamma_jh(list_A_C)
a_A_G, b_A_G, c_A_G = fit_gamma_jh(list_A_G)

a_T_A, b_T_A, c_T_A = fit_gamma_jh(list_T_A)
a_T_C, b_T_C, c_T_C = fit_gamma_jh(list_T_C)
a_T_G, b_T_G, c_T_G = fit_gamma_jh(list_T_G)

a_C_A, b_C_A, c_C_A = fit_gamma_jh(list_C_A)
a_C_T, b_C_T, c_C_T = fit_gamma_jh(list_C_T)
a_C_G, b_C_G, c_C_G = fit_gamma_jh(list_C_G)

a_G_A, b_G_A, c_G_A = fit_gamma_jh(list_G_A)
a_G_T, b_G_T, c_G_T = fit_gamma_jh(list_G_T)
a_G_C, b_G_C, c_G_C = fit_gamma_jh(list_G_C)

# Calculate the sum frequency of each type of substitution variant in the unprocessed data (that has only been
optionally filtered by average quality and frequency potentially).

raw_total_AT = sum(list_A_T)
raw_total_AC = sum(list_A_C)
raw_total_AG = sum(list_A_G)

raw_total_TA = sum(list_T_A)
raw_total_TC = sum(list_T_C)
raw_total_TG = sum(list_T_G)

```

```

raw_total_CA = sum(list_C_A)
raw_total_CT = sum(list_C_T)
raw_total_CG = sum(list_C_G)

raw_total_GA = sum(list_G_A)
raw_total_GT = sum(list_G_T)
raw_total_GC = sum(list_G_C)

raw_master_totals = [raw_total_AT, raw_total_AG, raw_total_AC, raw_total_TA, raw_total_TG, raw_total_TC,
raw_total_CT, raw_total_CG, raw_total_CA, raw_total_GT, raw_total_GA, raw_total_GC]

# Calculate the total frequency of all types of mutations.

raw_total_total = sum(raw_master_totals)

#Normalize the frequency of each type of mutation to the total frequency of all mutations. We might need these
later to compare processed vs unprocessed biases.

raw_norm_AT = raw_total_AT/raw_total_total
raw_norm_AC = raw_total_AC / raw_total_total
raw_norm_AG = raw_total_AG / raw_total_total

raw_norm_TA = raw_total_TA / raw_total_total
raw_norm_TG = raw_total_TG / raw_total_total
raw_norm_TC = raw_total_TC / raw_total_total

raw_norm_CG = raw_total_CG / raw_total_total
raw_norm_CA = raw_total_CA / raw_total_total
raw_norm_CT = raw_total_CT / raw_total_total

raw_norm_GT = raw_total_GT / raw_total_total
raw_norm_GA = raw_total_GA / raw_total_total
raw_norm_GC = raw_total_GC / raw_total_total

#Next create lists to store their positions. The positions and frequencies should be matchable by their indices in
each list.

```

```

df_list_positions_A = []
df_list_positions_T = []
df_list_positions_C = []
df_list_positions_G = []

df_list_varfreqs_A = []
df_list_varfreqs_T = []
df_list_varfreqs_C = []
df_list_varfreqs_G = []

df_list_varfreqs_AT = []
df_list_varfreqs_AC = []
df_list_varfreqs_AG = []

df_list_varfreqs_TA = []
df_list_varfreqs_TC = []
df_list_varfreqs_TG = []

df_list_varfreqs_CA = []
df_list_varfreqs_CT = []
df_list_varfreqs_CG = []

df_list_varfreqs_GA = []
df_list_varfreqs_GT = []
df_list_varfreqs_GC = []

gamma_pvals_list = []

for x in range (0,len(master_snp_freqs)):

    if len(master_snp_freqs[x]) > 0:

        for y in range (1, len(master_snp_freqs[x])): #starting at 1 instead of 0 because 1 is the consensus sequence

```

```

submade = master_snp_subs[x][y]
if submade == 'A':
    if master_snp_subs[x][0] == 'T':

        relpval = 1 - gamma.cdf(master_snp_freqs[x][y], a_T_A, 0, c_T_A)
        gamma_pvals_list.append(relpval)

        if 1 - gamma.cdf(master_snp_freqs[x][y], a_T_A, 0, c_T_A) <= maxgamma_pval and
master_snp_bayes_pval_outcome[x][y] == True:
            df_list_varfreqs_A.append(master_snp_freqs[x][y])
            df_list_positions_A.append(master_snp_positions[x][y])
            df_list_varfreqs_TA.append(master_snp_freqs[x][y])

    if master_snp_subs[x][0] == 'C':

        relpval = 1 - gamma.cdf(master_snp_freqs[x][y], a_C_A, 0, c_C_A)
        gamma_pvals_list.append(relpval)

        if 1 - gamma.cdf(master_snp_freqs[x][y], a_C_A, 0, c_C_A) <= maxgamma_pval and
master_snp_bayes_pval_outcome[x][y] == True:
            df_list_varfreqs_A.append(master_snp_freqs[x][y])
            df_list_positions_A.append(master_snp_positions[x][y])
            df_list_varfreqs_CA.append(master_snp_freqs[x][y])

    if master_snp_subs[x][0] == 'G':

        relpval = 1 - gamma.cdf(master_snp_freqs[x][y], a_G_A, 0, c_G_A)
        gamma_pvals_list.append(relpval)

        if 1 - gamma.cdf(master_snp_freqs[x][y], a_G_A, 0, c_G_A) <= maxgamma_pval and
master_snp_bayes_pval_outcome[x][y] == True:
            df_list_varfreqs_A.append(master_snp_freqs[x][y])
            df_list_positions_A.append(master_snp_positions[x][y])
            df_list_varfreqs_GA.append(master_snp_freqs[x][y])

if submade == 'T':

```

```

if master_snp_subs[x][0] == 'A':

    relpval = 1 - gamma.cdf(master_snp_freqs[x][y], a_A_T, 0, c_A_T)
    gamma_pvals_list.append(relpval)

    if 1 - gamma.cdf(master_snp_freqs[x][y], a_A_T, 0, c_A_T) <= maxgamma_pval and
master_snp_bayes_pval_outcome[x][y] == True:
        df_list_varfreqs_T.append(master_snp_freqs[x][y])
        df_list_positions_T.append(master_snp_positions[x][y])
        df_list_varfreqs_AT.append(master_snp_freqs[x][y])

if master_snp_subs[x][0] == 'C':

    relpval = 1 - gamma.cdf(master_snp_freqs[x][y], a_C_T, 0, c_C_T)
    gamma_pvals_list.append(relpval)

    if 1 - gamma.cdf(master_snp_freqs[x][y], a_C_T, 0, c_C_T) <= maxgamma_pval and
master_snp_bayes_pval_outcome[x][y] == True:
        df_list_varfreqs_T.append(master_snp_freqs[x][y])
        df_list_positions_T.append(master_snp_positions[x][y])
        df_list_varfreqs_CT.append(master_snp_freqs[x][y])

if master_snp_subs[x][0] == 'G':

    relpval = 1 - gamma.cdf(master_snp_freqs[x][y], a_G_T, 0, c_G_T)
    gamma_pvals_list.append(relpval)

    if 1 - gamma.cdf(master_snp_freqs[x][y], a_G_T, 0, c_G_T) <= maxgamma_pval and
master_snp_bayes_pval_outcome[x][y] == True:
        df_list_varfreqs_T.append(master_snp_freqs[x][y])
        df_list_positions_T.append(master_snp_positions[x][y])
        df_list_varfreqs_GT.append(master_snp_freqs[x][y])

if submade == 'G':

    if master_snp_subs[x][0] == 'T':

```

```

relpval = 1 - gamma.cdf(master_snp_freqs[x][y], a_T_G, 0, c_T_G)
gamma_pvals_list.append(relpval)

if 1 - gamma.cdf(master_snp_freqs[x][y], a_T_G, 0, c_T_G) <= maxgamma_pval and
master_snp_bayes_pval_outcome[x][y] == True:
    df_list_varfreqs_G.append(master_snp_freqs[x][y])
    df_list_positions_G.append(master_snp_positions[x][y])
    df_list_varfreqs_TG.append(master_snp_freqs[x][y])

if master_snp_subs[x][0] == 'A':

    relpval = 1 - gamma.cdf(master_snp_freqs[x][y], a_A_G, 0, c_A_G)
    gamma_pvals_list.append(relpval)

    if 1 - gamma.cdf(master_snp_freqs[x][y], a_A_G, 0, c_A_G) <= maxgamma_pval and
master_snp_bayes_pval_outcome[x][y] == True:
        df_list_varfreqs_G.append(master_snp_freqs[x][y])
        df_list_positions_G.append(master_snp_positions[x][y])
        df_list_varfreqs_AG.append(master_snp_freqs[x][y])

if master_snp_subs[x][0] == 'C':

    relpval = 1 - gamma.cdf(master_snp_freqs[x][y], a_C_G, 0, c_C_G)
    gamma_pvals_list.append(relpval)

    if 1 - gamma.cdf(master_snp_freqs[x][y], a_C_G, 0, c_C_G) <= maxgamma_pval and
master_snp_bayes_pval_outcome[x][y] == True:
        df_list_varfreqs_G.append(master_snp_freqs[x][y])
        df_list_positions_G.append(master_snp_positions[x][y])
        df_list_varfreqs_CG.append(master_snp_freqs[x][y])

if submade == 'C':

    if master_snp_subs[x][0] == 'T':

        relpval = 1 - gamma.cdf(master_snp_freqs[x][y], a_T_C, 0, c_T_C)
        gamma_pvals_list.append(relpval)

```

```

        if 1 - gamma.cdf(master_snp_freqs[x][y], a_T_C, 0, c_T_C) <= maxgamma_pval and
master_snp_bayes_pval_outcome[x][y] == True:
            df_list_varfreqs_C.append(master_snp_freqs[x][y])
            df_list_positions_C.append(master_snp_positions[x][y])
            df_list_varfreqs_TC.append(master_snp_freqs[x][y])

    if master_snp_subs[x][0] == 'A':

        relpval = 1 - gamma.cdf(master_snp_freqs[x][y], a_A_C, 0, c_A_C)
        gamma_pvals_list.append(relpval)

        if 1 - gamma.cdf(master_snp_freqs[x][y], a_A_C, 0, c_A_C) <= maxgamma_pval and
master_snp_bayes_pval_outcome[x][y] == True:
            df_list_varfreqs_C.append(master_snp_freqs[x][y])
            df_list_positions_C.append(master_snp_positions[x][y])
            df_list_varfreqs_AC.append(master_snp_freqs[x][y])

    if master_snp_subs[x][0] == 'G':

        relpval = 1 - gamma.cdf(master_snp_freqs[x][y], a_G_C, 0, c_G_C)
        gamma_pvals_list.append(relpval)

        if 1 - gamma.cdf(master_snp_freqs[x][y], a_G_C, 0, c_G_C) <= maxgamma_pval and
master_snp_bayes_pval_outcome[x][y] == True:
            df_list_varfreqs_C.append(master_snp_freqs[x][y])
            df_list_positions_C.append(master_snp_positions[x][y])
            df_list_varfreqs_GC.append(master_snp_freqs[x][y])

    # Calculate the sum frequency of each type of substitution variant in the unprocessed data (that has only been
    optionally filtered by average quality and frequency potentially).

    df_total_AT = sum(df_list_varfreqs_AT)
    df_total_AC = sum(df_list_varfreqs_AC)
    df_total_AG = sum(df_list_varfreqs_AG)

    df_total_TA = sum(df_list_varfreqs_TA)

```

```

df_total_TC = sum(df_list_varfreqs_TC)
df_total_TG = sum(df_list_varfreqs_TG)

df_total_CA = sum(df_list_varfreqs_CA)
df_total_CT = sum(df_list_varfreqs_CT)
df_total_CG = sum(df_list_varfreqs_CG)

df_total_GA = sum(df_list_varfreqs_GA)
df_total_GT = sum(df_list_varfreqs_GT)
df_total_GC = sum(df_list_varfreqs_GC)

filtered_master_totals = [df_total_AT, df_total_AG, df_total_AC, df_total_TA, df_total_TG, df_total_TC, df_total_CT,
                           df_total_CG, df_total_CA, df_total_GT, df_total_GA, df_total_GC]

# Calculate the total frequency of all types of mutations.

df_total_total = sum(filtered_master_totals)

#Normalize the frequency of each type of mutation to the total frequency of all mutations. We might need these
later to compare processed vs unprocessed biases.

df_norm_AT = df_total_AT/df_total_total
df_norm_AC = df_total_AC / df_total_total
df_norm_AG = df_total_AG / df_total_total

df_norm_TA = df_total_TA / df_total_total
df_norm_TG = df_total_TG / df_total_total
df_norm_TC = df_total_TC / df_total_total

df_norm_CG = df_total_CG / df_total_total
df_norm_CA = df_total_CA / df_total_total
df_norm_CT = df_total_CT / df_total_total

df_norm_GT = df_total_GT / df_total_total
df_norm_GA = df_total_GA / df_total_total
df_norm_GC = df_total_GC / df_total_total

```

```

    return [[df_list_varfreqs_A, df_list_varfreqs_T, df_list_varfreqs_C, df_list_varfreqs_G], [df_list_positions_A,
df_list_positions_T, df_list_positions_C, df_list_positions_G],
            [[raw_norm_AC, raw_norm_AG, raw_norm_AT], [raw_norm_CA, raw_norm_CG, raw_norm_CT],
[raw_norm_GA, raw_norm_GC, raw_norm_GT], [raw_norm_TA, raw_norm_TC, raw_norm_TG]],
            [[df_norm_AC, df_norm_AG, df_norm_AT], [df_norm_CA, df_norm_CG, df_norm_CT], [df_norm_GA,
df_norm_GC, df_norm_GT], [df_norm_TA, df_norm_TC, df_norm_TG]]]

```

```

def merge_reads(list_of_simply_plot_muts_outputs):

```

```

    master_A_freqs = []

```

```

    master_T_freqs = []

```

```

    master_C_freqs = []

```

```

    master_G_freqs = []

```

```

    master_A_positions = []

```

```

    master_T_positions = []

```

```

    master_C_positions = []

```

```

    master_G_positions = []

```

```

    for x in list_of_simply_plot_muts_outputs:

```

```

        master_A_freqs.extend(x[0][0])

```

```

        master_T_freqs.extend(x[0][1])

```

```

        master_C_freqs.extend(x[0][2])

```

```

        master_G_freqs.extend(x[0][3])

```

```

        master_A_positions.extend(x[1][0])

```

```

        master_T_positions.extend(x[1][1])

```

```

        master_C_positions.extend(x[1][2])

```

```

        master_G_positions.extend(x[1][3])

```

```

    return[[master_A_freqs, master_T_freqs, master_C_freqs, master_G_freqs], [master_A_positions,
master_T_positions, master_C_positions, master_G_positions]]

```

```

def take_mean_of_merge (merge_reads_output, num_reps):

```

```

    mean_freqs_and_pos = [[[],[],[],[]],[[],[],[],[]]]

```

```

    for subtype in merge_reads_output[1]:

```

```

subtype_index = merge_reads_output[1].index(subtype)

for pos in subtype:

    pos_index = subtype.index(pos)

    if pos in mean_freqs_and_pos[1][subtype_index]:
        continue

    tot_subvar_freq_at_current_position = merge_reads_output[0][subtype_index][pos_index]

    for pos_c in range(0, len(subtype)):

        if pos_c == pos_index:
            continue

        if subtype[pos_c] == pos:
            tot_subvar_freq_at_current_position += merge_reads_output[0][subtype_index][pos_c]

    mean_subvar_freq_at_current_position = tot_subvar_freq_at_current_position/num_reps
    mean_freqs_and_pos[0][subtype_index].append(mean_subvar_freq_at_current_position)
    mean_freqs_and_pos[1][subtype_index].append(pos)

print(mean_freqs_and_pos[0][1][24])
print(mean_freqs_and_pos[1][1][24])
return mean_freqs_and_pos

def convertstrandedness(list_of_sub_freqs_per_nucleotide):
    #A>C is T>G
    #A>T is T>A
    #A>G is T>C

```

```
#T>A is A>T
```

```
#T>C is A>G
```

```
#T>G is A>C
```

```
#C>A is G>T
```

```
#C>G is G>C
```

```
#C>T is G>A
```

```
#G>A is C>T
```

```
#
```

```
corrected_list = [[0,0,0],[0,0,0],[0,0,0],[0,0,0]]
```

```
corrected_list[0][0] = list_of_sub_freqs_per_nucleotide[3][2]
```

```
corrected_list[0][1] = list_of_sub_freqs_per_nucleotide[3][1]
```

```
corrected_list[0][2] = list_of_sub_freqs_per_nucleotide[3][0]
```

```
corrected_list[1][0] = list_of_sub_freqs_per_nucleotide[2][2]
```

```
corrected_list[1][1] = list_of_sub_freqs_per_nucleotide[2][1]
```

```
corrected_list[1][2] = list_of_sub_freqs_per_nucleotide[2][0]
```

```
corrected_list[2][0] = list_of_sub_freqs_per_nucleotide[1][2]
```

```
corrected_list[2][1] = list_of_sub_freqs_per_nucleotide[1][1]
```

```
corrected_list[2][2] = list_of_sub_freqs_per_nucleotide[1][0]
```

```
corrected_list[3][0] = list_of_sub_freqs_per_nucleotide[0][2]
```

```
corrected_list[3][1] = list_of_sub_freqs_per_nucleotide[0][1]
```

```
corrected_list[3][2] = list_of_sub_freqs_per_nucleotide[0][0]
```

```
return(corrected_list)
```

```
def id_uniques(control_merged_reads, experimental_merged_reads):
```

```
    unique_positions = [[],[],[],[]]
```

```
    unique_freqs = [[],[],[],[]]
```

```

for x in range(0, len(experimental_merged_reads[1])):
    for y in range(0, len(experimental_merged_reads[1][x])):
        # print('experimental')
        # print(experimental_merged_reads[1][x])
        # print("control")
        # print(control_merged_reads[1][x])
        if experimental_merged_reads[1][x][y] in control_merged_reads[1][x]:
            print("NOT UNIQUE")
            continue
        else:
            unique_positions[x].append(experimental_merged_reads[1][x][y])
            unique_freqs[x].append(experimental_merged_reads[0][x][y])

return [unique_freqs, unique_positions]

def sum_variants(merge_reads_output):
    for x in range(0, len(merge_reads_output[1])):
        for y in range(0, len(merge_reads_output[1][x])):
            position = merge_reads_output[1][x][y]
            frequency = merge_reads_output[0][x][y]
            if merge_reads_output[1][x][y] in total_var_freqs_by_position.keys():
                total_var_freqs_by_position[position] += frequency
            else:
                total_var_freqs_by_position[position] = frequency
    return [list(total_var_freqs_by_position.keys()), list(total_var_freqs_by_position.values())]

def normalize_by_control(control_merged_reads, experimental_merged_reads, norm_threshold):
    norm_positions = [[], [], [], []]
    norm_freqs = [[], [], [], []]

    for x in range(0, len(experimental_merged_reads[1])):
        for y in range(0, len(experimental_merged_reads[1][x])):
            experimental_pos = experimental_merged_reads[1][x][y]
            control_pos_list = control_merged_reads[1][x]
            if experimental_pos in control_pos_list:

```

```

corresponding_control_freq_index = control_pos_list.index(experimental_pos)

# if experimental_merged_reads[1][x][y] == 51 and x == 1:
    # print("FIFTY ONE!")
    # print(experimental_merged_reads[0][x][y])
    # print(corresponding_control_freq_index)
    # print(control_merged_reads[0][x][corresponding_control_freq_index])
    # print(control_pos_list[corresponding_control_freq_index])

norm_value =
(experimental_merged_reads[0][x][y]/control_merged_reads[0][x][corresponding_control_freq_index])
if norm_value >= norm_threshold:
    norm_positions[x].append(experimental_merged_reads[1][x][y])
    norm_freqs[x].append(norm_value)

else:
    # print("ONLY PRESENT IN CONTROL")
    continue

return[norm_freqs, norm_positions]

def remove_out_of_range(unique_plottable, relevant_coords):
    filtered_unique_plottable = [[[],[],[],[]],[[],[],[],[]]] #filtered_unique_plottables is just an empty form of unique_plottable
    that we fill with filtered data points
    for xy in unique_plottable[1]: # xy will be a list of lists of coordinates of variants of each substitution type.
        for coord in xy: # coord will be a number that will be tested for whether it's in our relevant coordinates.
            for range in relevant_coords: #range will be a number range in the form of a list (e.g. [100,200]) for the
positions we are considering.
                # print("Coord is ")
                # print(coord)
                if coord >= range[0] and coord <= range[1]:
                    substitution_index = unique_plottable[1].index(xy)
                    coordinate_index = xy.index(coord)
                    corresponding_variant_frequency = unique_plottable[0][substitution_index][coordinate_index]

```

```

        # print("SFSDFSDF")
        # print(corresponding_variant_frequency)
        filtered_unique_plottable[1][substitution_index].append(coord)
        filtered_unique_plottable[0][substitution_index].append(corresponding_variant_frequency)
    print(filtered_unique_plottable)
    return filtered_unique_plottable

#####
#####
##Script starts below##
#####
#####

identifiers_for_each_plot = [['01','02','03'],
                              ['10','11'],
                              ['04','05','06'],
                              ['07','08','09']]

list_condition_names = ['Untransfected R1','Untransfected R2','Untransfected R3',
                        'EvolvR Off target R1','EvolvR Off target R2',
                        'enCas9 On target R1','enCas9 On target R2', 'enCas9 On target R3',
                        'EvolvR On target R1','EvolvR On target R2', 'EvolvR On target R3,']
list_condition_sets = ['Untransfected','EvolvR + gBFP45 ', 'enCas9 + gM2K1.1,2,3', 'EvolvR + gM2K1.1,2,3']
rc1 = [613, 732] #####USER DEFINED#####
rc2 = [48300,48429]#####USER DEFINED#####
rc3 = [48421, 48575] #####USER DEFINED#####
rc4 = [50014,50166]#####USER DEFINED#####
rc5 = [20,130] #####USER DEFINED#####

list_relevant_coords = [ #####USER DEFINED#####
                        [rc5,rc5,rc5],
                        [rc5,rc5],
                        [rc5,rc5,rc5],
                        [rc5,rc5,rc5]
                        ]

```

```

prefix = 'tA375map2k1cmv/tA375map2k1cmv' #####USER
DEFINED#####
suffix = '.txt' #####USER DEFINED#####

maxgamma_pval = 1.00 #####USER DEFINED#####
min_single_p = 0.00 #####USER DEFINED#####

minfreq = 0.000 #####USER DEFINED#####
minaveq = 0 #####USER DEFINED#####

freq_ymin = 0.00001
freq_ymax = 0.1

norm_ymin = 0.05
norm_ymax = 100000

normalized_by_control = True #####USER DEFINED#####
differentiate_reps = True #####USER DEFINED#####
norm_thresh = 0.01 #####USER DEFINED#####
negative_control_index = 0 #####USER DEFINED#####

snp_colors = ['teal', 'coral', 'cornflowerblue', 'maroon']

for id_set in identifiers_for_each_plot: #conditions is the list of lists of prefixes corresponding to different PCRs for the
same condition.
    normed_merge_reads_input = []
    merge_reads_input = []
    control_merge_reads_input = []

    identifier_index = identifiers_for_each_plot.index(id_set) #This is an integer that represents the index of the lists of
indices within the larger conditions list.

    if identifier_index == negative_control_index:

```

```

    control_set = True
else:
    control_set = False

set_of_windows_of_interest = list_relevant_coords[identifiers_for_each_plot.index(id_set)]

for id in id_set: #id will represent a single amplicon within a list of PCRs.

    fn1 = prefix + id + suffix #putting together the filename to open the file.


    position_range_of_interest = set_of_windows_of_interest[id_set.index(id)]

    results = simplyplotmuts(fn1, minaveq, min_single_p, minfreq, maxgamma_pval, position_range_of_interest[0],
position_range_of_interest[1])


    if control_set:
        control_merge_reads_input.append(results)
    else:
        merge_reads_input.append(results)
        normed_results = normalize_by_control(mean_whole_control_condition_results, results, norm_thresh)
        normed_merge_reads_input.append(normed_results)

if control_set:
    whole_control_condition_results = merge_reads(control_merge_reads_input)
    mean_whole_control_condition_results = take_mean_of_merge(whole_control_condition_results,3)
else:
    whole_exp_condition_results = merge_reads(merge_reads_input)

if normalized_by_control and control_set==False:
    final_condition_results = normalize_by_control(mean_whole_control_condition_results,
whole_exp_condition_results,norm_thresh)
else:

```

```

if control_set:
    final_condition_results = whole_control_condition_results
else:
    final_condition_results = whole_exp_condition_results

if normalized_by_control:
    ymin = norm_ymin
    ymax = norm_ymax
else:
    ymin_ = freq_ymin
    ymax = freq_ymax

list_markers = ['o', 'D', 'v']

fig, ax = plt.subplots()
for rep in normed_merge_reads_input:

    for sub_type in rep[0]:

        xmin = position_range_of_interest[0]
        xmax = position_range_of_interest[1]

        subtype_index = rep[0].index(sub_type)
        rep_index = normed_merge_reads_input.index(rep)
        rep_marker = list_markers[rep_index]

        variants = rep[0][subtype_index]
        positions = rep[1][subtype_index]

        # Add scatterpoints
        ax.scatter(positions, variants, c = snp_colors[subtype_index], marker = rep_marker,
                    alpha=0.75)

```

```

ax.axhline(y=10, color='g', linestyle='--', linewidth=1)

# Formatting steps
ax.set_ylabel("Variant fold enrichment over untransfected")
ax.set_xlabel("Position")
ax.set_yscale("log")
ax.set_xticks(ticks=[20,30,40,50,60,70,80,90,100,110,120,130])

# plt.xticks(np.arange(xmin, xmax + 1, 10))
ax.set_ylim(ymin, ymax)
ax.set_xlim(xmin, xmax)
ax.set_title(list_condition_sets[identifiers_for_each_plot.index(id_set)])

pltname = list_condition_sets[identifiers_for_each_plot.index(id_set)] + "_filtered_10.svg"
#print(pltname)
#plt.savefig(pltname)
plt.show()

```

10.6. Plasmids used in this study

p.m-mCherry	CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA TCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGA AAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTC CCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACG CTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTG GGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGT TTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTT TGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGC AACTCGGTGCGCCGATACACTATTCTCAGAATGACTTGTTGAGTA CTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAG AGAATTATGCAGTGCTGCCATAACCATGAGTGATAAACTGCGGC CAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGC TTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGG GAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACC ACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATTAAC GGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGA TGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCC GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGT TCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCC GTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATG AACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGC ATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGA TTTAAAACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTT TTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTTCGTTCC ACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAG ATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACCC ACCGCTACCAGCGGTGGTTTGTGTTGCCGGATCAAGAGCTACCAACT CTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAAT ACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT CTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGT GGCTGCTGCCAGTGCGGATAAGTCGTGTCTTACCGGGTTGGACTCA AGACGATAGTTACCGGATAAAGCGCAGCGGTGCGGGCTGAACGGGG GGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAA CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCC GAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTTCGG AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGT ATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGAAAAACGC CAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTG CTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGT ATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGACCCGAACG ACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCC AATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAATTAATGC AGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGC AACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT
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ATAACAATTTACACAGGAAACAGCTATGACCATGATTACGCCAA
GCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCA
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TCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGA
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CGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACG
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CTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTTGGCAG
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CGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAA
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GGGCGAGGGCGAGGGCCGCCCTACGAGGGCACCCAGACCGCCA
AGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACA
TCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCA
CCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGC
TTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGTTG
ACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTAC
AAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTA
ATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATG
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AAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACC
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GTGGAACAGTACGAACGCGCCGAGGGCCGCGCACTCCACCGGCGGC
ATGGACGAGCTGTACAAG GGATCCGGTATGTAATGGCTGAAAAC
GGGACCAGGCTCACTAACTCAGATCCTACTAGGTTTAATAAAC ATC
TTTATTTTCATTACATCTGTGTGTTGGTTTTTTGTGTG GTACCCAAT

	TCGCCCTATAGTGAGTCGTATTACTCACTGGCCGTCGTTTTACAAC GTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGC AGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCG CACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATG GGACGCGCCCTGTAGCGGCGCATTAAAGCGCGGCGGGTGTGGTGGT TACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCT CCTTTCGCTTCTTCCCTTCCTTCTCGCCACGTTGCGCGGCTTTCCC CGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTG CTTTACGGCACCTCGACCCCAAAAACTTGATTAGGGTGATGGTTC ACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACG TTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAACTGGAA CAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATT TTGCCGATTTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAA AATTTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTAGGT GGCACTTTTCGGGGAAATGTG Ampicillin resistance cassette-ColE1 origin of replication-hU6 promoter- gRNA sequence-SpCas9 gRNA scaffold-CMV promoter-NLS-mCherry- GSGM-stop-SV40 polyA
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p.m- enCas9	CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA TCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGA AAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTC CCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACG CTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTG GGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGT TTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTC TGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGC AACTCGGTGCGCCGATACACTATTCTCAGAATGACTTGGTGAGTA CTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAG AGAATTATGCAGTGCTGCCATAACCATGAGTGATAAACTGCGGC CAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACC GC TTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGG GAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACC ACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATTAAC GGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGA TGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCC GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGT TCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCC GTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATG AACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGC ATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGA TTTAAAACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTT TTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCC ACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAG ATCCTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACC ACCGCTACCAGCGGTGGTTTGTGTTGCCGGATCAAGAGCTACCAACT CTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAAT ACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT CTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGT GGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCA AGACGATAGTTACCGGATAAAGGCGCAGCGGTGCGGGCTGAACGGGG GGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAA CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCC GAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTTCGG AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGT ATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGAAAAACGC CAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTG CTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGT ATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGACCCGAACG ACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCC AATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAATTAATGC AGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGC AACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT
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p.m- PolI5M	CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA TCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGA AAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTC CCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACG CTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTG GGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGT TTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTC TGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGC AACTCGGTGCGCCGCATACACTATTCTCAGAATGACTTGGTGAGTA CTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAG AGAATTATGCAGTGCTGCCATAACCATGAGTGATAAACTGCGGC CAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGC TTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGG GAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACC ACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATTAAC GGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGA TGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCC GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGT TCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCC GTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATG AACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGC ATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGA TTTAAAACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTT TTTGATAATCTCATGACCAAATCCCTTAACGTGAGTTTTCGTTCC ACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAG ATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACCC ACCGCTACCAGCGGTGGTTTGTGTTGCCGGATCAAGAGCTACCAACT CTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAAT ACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT CTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGT GGCTGCTGCCAGTGCGGATAAGTCGTGTCTTACCGGGTTGGACTCA AGACGATAGTTACCGGATAAAGCGCAGCGGTGCGGGCTGAACGGGG GGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAA CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCC GAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGG AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGT ATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGAAAAACGC CAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTG CTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGT ATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGACCCGAACG ACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCC AATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAATTAATGC AGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGC AACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT
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	TAAGGGATTTTGCCGATTTTCGGCCTATTGGTTAAAAAATGAGCTGA TTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGCTTAC AATTTAGGTGGCACTTTTCGGGGAAATGTG Ampicillin resistance cassette-ColE1 origin of replication-hU6 promoter- gRNA sequence-SpCas9 gRNA scaffold-CMV promoter-NLS-mCherry- GSGM-NLS-linker-PolI5M-stop-SV40 polyA
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p.mEvolvR -nCas9	CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA TCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGA AAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTC CCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACG CTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTG GGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGT TTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTC TGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGC AACTCGGTGCGCCGCATACACTATTCTCAGAATGACTTGGTGAGTA CTCACAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAG AGAATTATGCAGTGCTGCCATAACCATGAGTGATAAACTGCGGC CAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGC TTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGG GAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACC ACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATTAAC GGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGA TGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCC GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGT TCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCC GTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATG AACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGC ATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGA TTTAAAACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTT TTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTTCGTTCC ACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAG ATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACC ACCGCTACCAGCGGTGGTTTGTGTTGCCGGATCAAGAGCTACCAACT CTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAAT ACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT CTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGT GGCTGCTGCCAGTGCGGATAAGTCGTGTCTTACCGGGTTGGACTCA AGACGATAGTTACCGGATAAAGCGCAGCGGTGCGGGCTGAACGGGG GGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAA CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCC GAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGG AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGT ATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGAAAAACGC CAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTG CTCACATGTTCTTTCTGCGTTATCCCCTGATTCTGTGGATAACCGT ATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGACCCGAACG ACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCC AATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAATTAATGC AGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGC AACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT
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 gRNA sequence-SpCas9 gRNA scaffold-CMV promoter-NLS-mCherry-
 GSGM-nCas9(D10A)-NLS-linker-Poll5M-stop-SV40 polyA

p.mEvolvR
-H840A

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Ampicillin resistance cassette-Cole1 origin of replication-hU6 promoter-
 gRNA sequence-SpCas9 gRNA scaffold-CMV promoter-NLS-mCherry-
 GSGM-nCas9(H840A)-NLS-linker-Poll5M-stop-SV40 polyA

p.mEvolvR -dCas9	CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA TCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGA AAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTC CCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACG CTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTG GGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGT TTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTT TGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGC AACTCGGTGCGCCGATACACTATTCTCAGAATGACTTGGTGAGTA CTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAG AGAATTATGCAGTGCTGCCATAACCATGAGTGATAAACTGCGGC CAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGC TTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGG GAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACC ACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATTAAC GGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGA TGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCC GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGT TCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCC GTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATG AACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGC ATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGA TTTAAAACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTT TTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCC ACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAG ATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAC ACCGCTACCAGCGGTGGTTTGTGTTGCCGGATCAAGAGCTACCAACT CTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAAT ACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT CTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGT GGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCA AGACGATAGTTACCGGATAAAGCGCAGCGGTGCGGGCTGAACGGGG GGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAA CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCC GAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGG AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGT ATCTTTATAGTCCTGTGCGGGTTTCGCCACCTCTGACTTGAGCGTCG ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGAAAAACGC CAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTG CTCACATGTTCTTTCTGCGTTATCCCCTGATTCTGTGGATAACCGT ATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGACCCGAACG ACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCC AATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAATTAATGC AGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGC AACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT
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Ampicillin resistance cassette-Cole1 origin of replication-hU6 promoter-
 gRNA sequence-SpCas9 gRNA scaffold-CMV promoter-NLS-mCherry-
 GSGM-dCas9(D10A, H840A)-NLS-linker-PolII5M-stop-SV40 polyA

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 gRNA sequence-SpCas9 gRNA scaffold-CMV promoter-NLS-mCherry-
 GSGM-enCas9(D10A)-NLS-linker-PolI3M-stop-SV40 polyA

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Ampicillin resistance cassette-Cole1 origin of replication-hU6 promoter-
 gRNA sequence-SpCas9 gRNA scaffold-CMV promoter-NLS-mCherry-
 GSGM-enCas9(D10A)-NLS-linker-PolI5M-stop-SV40 polyA

p.mEvolvR -Δ-enh	CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA TCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGA AAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTC CCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACG CTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTG GGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGT TTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTC TGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGC AACTCGGTGCGCCGATACACTATTCTCAGAATGACTTGGTGAGTA CTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAG AGAATTATGCAGTGCTGCCATAACCATGAGTGATAAACTGCGGC CAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGC TTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGG GAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACC ACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATTAAC GGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGA TGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCC GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGT TCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCC GTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATG AACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGC ATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGA TTTAAAACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTT TTTGATAATCTCATGACCAAATCCCTTAACGTGAGTTTTTCGTTCC ACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAG ATCCTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACCC ACCGCTACCAGCGGTGGTTTGTGTTGCCGGATCAAGAGCTACCAACT CTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAAT ACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT CTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGT GGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCA AGACGATAGTTACCGGATAAAGCGCAGCGGTGCGGGCTGAACGGGG GGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAA CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCC GAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGG AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGT ATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGAAAAACGC CAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTG CTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGT ATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGACCCGAACG ACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCC AATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAATTAATGC AGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGC AACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT
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p.mEvolvR -Δ-LZ3	CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA TCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGA AAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTC CCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACG CTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTG GGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGT TTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTC TGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGC AACTCGGTTCGCCGCATACACTATTCTCAGAATGACTTGGTGAGTA CTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAG AGAATTATGCAGTGCTGCCATAACCATGAGTGATAAACTGCGGC CAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGC TTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGG GAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACC ACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATTAAC GGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGA TGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCC GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGT TCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCC GTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATG AACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGC ATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGA TTTAAAACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTT TTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTTCGTTCC ACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAG ATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACC ACCGCTACCAGCGGTGGTTTGTGTTGCCGGATCAAGAGCTACCAACT CTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAAT ACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT CTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGT GGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCA AGACGATAGTTACCGGATAAAGCGCAGCGGTTCGGGCTGAACGGGG GGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAA CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCC GAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGG AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGT ATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGAAAAACGC CAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTG CTCACATGTTCTTTCTGCGTTATCCCCTGATTCTGTGGATAACCGT ATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGACCCGAACG ACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCC AATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAATTAATGC AGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGC AACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT
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p.mEvolvR -Δ-SpRY	CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA TCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGA AAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTC CCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACG CTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTG GGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGT TTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTC TGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGC AACTCGGTGCGCCGCATACACTATTCTCAGAATGACTTGGTGAGTA CTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAG AGAATTATGCAGTGCTGCCATAACCATGAGTGATAAACTGCGGC CAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGC TTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGG GAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACC ACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATTAAC GGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGA TGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCC GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGT TCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCC GTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATG AACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGC ATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGA TTTAAAACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTT TTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTTCGTTCC ACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAG ATCCTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACC ACCGCTACCAGCGGTGGTTTGTGTTGCCGGATCAAGAGCTACCAACT CTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAAT ACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT CTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGT GGCTGCTGCCAGTGCGGATAAGTCGTGTCTTACCGGGTTGGACTCA AGACGATAGTTACCGGATAAAGCGCAGCGGTGCGGGCTGAACGGGG GGTTCGTGCACACAGCCCAGCTTGAGCGAACGACCTACACCGAA CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCC GAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGG AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGT ATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGAAAAACGC CAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTG CTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGT ATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGACCCGAACG ACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCC AATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAATTAATGC AGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGC AACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT
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Ampicillin resistance cassette-Cole1 origin of replication-hU6 promoter-
 gRNA sequence-Nme2Cas9 gRNA scaffold-CMV promoter-NLS-
 mCherry-GSGM-eNme2.NR-nCas9(D16A)-NLS-Poll5MA-stop-SV40
 polyA

p.mEvolvR -Δ- NNGSlug	CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA TCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGA AAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTC CCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACG CTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTG GGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGT TTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTC TGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGC AACTCGGTGCGCCGATACACTATTCTCAGAATGACTTGGTGAGTA CTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAG AGAATTATGCAGTGCTGCCATAACCATGAGTGATAAACTGCGGC CAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGC TTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGG GAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACC ACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATTAAC GGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGA TGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCC GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGT TCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCC GTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATG AACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGC ATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGA TTTAAAACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTT TTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCC ACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAG ATCCTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACCC ACCGCTACCAGCGGTGGTTTGTGTTGCCGGATCAAGAGCTACCAACT CTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAAT ACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACT CTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGT GGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCA AGACGATAGTTACCGGATAAAGCGCAGCGGTGCGGGCTGAACGGGG GGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAA CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCC GAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCCG AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGT ATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGAAAAACGC CAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTG CTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGT ATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGACCCGAACG ACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCC AATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAATTAATGC AGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGC AACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT
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GTTGTGTGACTCTGGTAACTAGAGATCCCTCAGACCCTTTTAGTCA
GTGTGGAAAATCTCTAGCAGTAGTAGTTCATGTCATCTTATTATTC
AGTATTTATAACTTGCAAAGAAATGAATATCAGAGAGTGAGAGGA
ACTTGTATTATTCAGCTTATAATGGTTACAAATAAAGCAATAGCAT
CACAAATTTACAAATAAAGCATTTTTTTTCACTGCATTCTAGTTGT
GGTTTGTCCAACTCATCAATGTATCTTATCATGTCTGGCTCTAGCT
ATCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTT
CCGCCCATTCTCCGCCCATGGCTGACTAATTTTTTTTATTATGCA

	GAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGA GGAGGCTTTTTTGGAGGCCT Ampicillin resistance cassette-ColE1 origin of replication-RSV promoter- 5' truncated HIV-1 LTR-Psi packaging signal-Rev response element - cPPT-TetOn promoter-NLS-Slug-nCas9(D10A)-NLS-Poli5MA-stop-hU6 promoter-gRNA sequence-SlugCas9 gRNA scaffold-EF1α core promoter-NLS-mCherry-T2A-rtTA3g-3'LTR (ΔU3)-SV40 polyA
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