SaCas9 and AsCas12a (AsCpf1) are as potent and more specific than SpCas9

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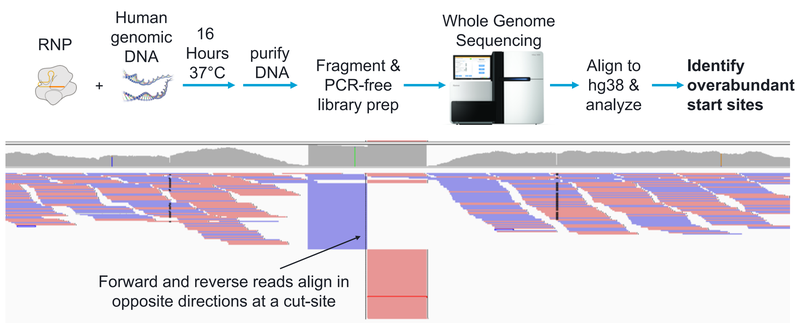
Introduction

- This is an off-target study comparing SpCas9, SaCas9, and AsCas12a (AsCpf1).
- Digenome-Seq[1] was used to assess specificity because it is highly sensitive and uses a completely reconstituted system enabling assessment of on- and off-target cutting relative to ribonucleoprotein (RNP) concentration in a straightforward fashion.
- 25 'matched sites'[2] in the human genome were used in the study. Matched sites are sequences in the genome which have protospacer adjacent motif (PAM) sequences for multiple enzymes. Thus the same target site can be interrogated for all the enzymes, reducing bias due to the target and guide sequences.
- A subset of 10 sites were analyzed in a concentration-response using Digenome-Seq and T cell editing, via RNP nucleofection, to determine whether and how on- and off-target cutting was related to RNP concentration.
- This rigorous methodology can be used generically for comparative specificity assessments.

Digenome-Seq method



25 target sites for each enzyme (75 RNPs total) were assayed using multiplex Digenome-Seq[3] (5 RNPs / reaction). RNPs were incubated with human genomic DNA at 1,000 nM concentration each for 16 hours at 37°C. WGS data was aligned to hg38 and cut sites identified using the algorithm[4] with a template score >= 15. To deconvolute the multiplexed RNPs, cut sites were assigned to target sites after alignment, having 6 or less mismatches plus gaps.

Selection of matched target sites

Matched Target Site (20-Ns):
TTTVNNNNNNNNNNNNNNNNNNNN**NGGRRT**

↓

Human genome: hg38

↓

Applied filters:
1) Removed sites on chromosomes labeled as: alt, random, X, Y, M
2) GC% >35% and <70% in target sequence
3) No more than 3 identical bases in a row in target sequence
4) Removed sites with more than 1 guide site in the genome
5) No repetitive element within 500bp of either side of guide

↓

11,092 sites returned

↓

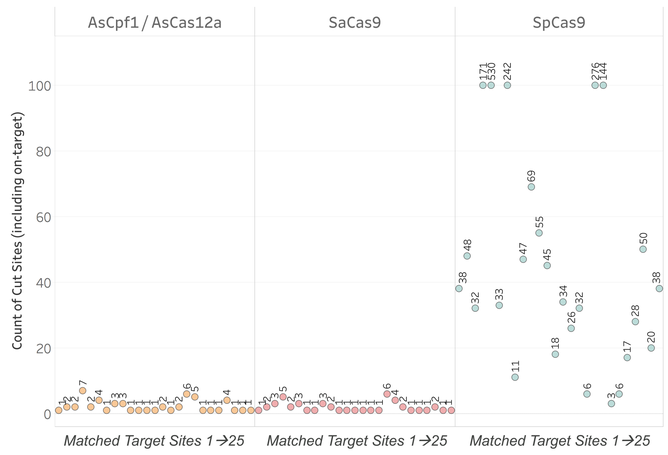
List randomized, working from top down
1) No common variants >0.1% in guide or PAM
2) Target sites are at least 10kb apart
3) Inspection of target region from previous WGS runs to ensure >15x coverage and no SNPs / indels / other variants
4) Successfully design PCR primers to amplify target region

↓

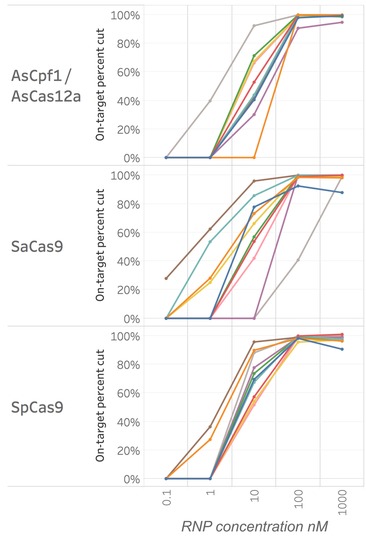
25 sites chosen

Results

SpCas9 has 10x–100x more off-target cut sites than AsCas12a and SaCas9 at 1 µM RNP



On-target activity and potency are similar between the three enzymes in Digenome-Seq

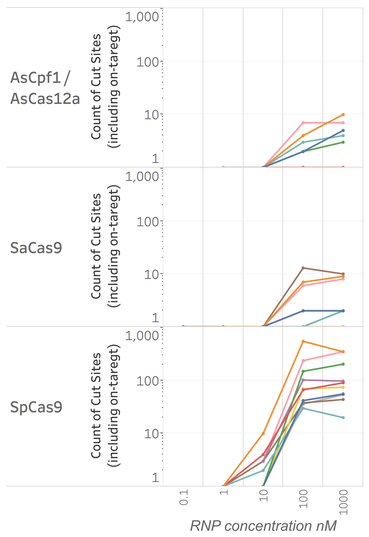


10 guide RNAs for each enzyme were sub-selected (30 RNPs total) and assayed using multiplex Digenome-Seq[2] in a second experiment. As before, RNPs were incubated with human genomic DNA at concentrations from 1000 nM to 0.1 nM each, for 16 hours at 37°C.

On-target cutting percent is defined as the percent of read starts divided by the total number of reads.

Each color point/line refers to a distinct target site.

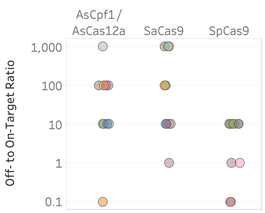
Off-targets are concentration-dependent and SaCas9 & AsCas12a have larger specificity windows



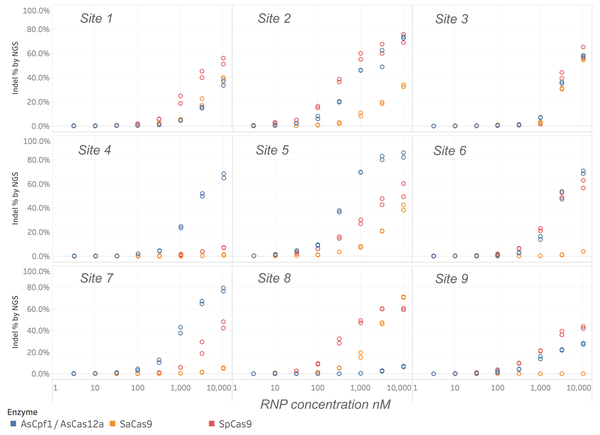
The same 10 target sites were assessed for off-target cutting in concentration response.

The y-axis plots the count of unique off-target cut sites. Target sites are represented by color.

Below is the off- to on-target ratio. The lowest concentration of an off-target detected divided by the lowest concentration of the on-target detected.



Concentration-response in primary human T cells shows editing is enzyme- and target site-specific



Nine target sites were assessed for on-target editing in primary human T cells by RNP nucleofection. RNP concentration at the time of nucleofection is on the x-axis and percent editing (indels at expected cut site) measured by targeted PCR-NGS, on the y-axis. Each trellis panel represents a different target site.

Conclusions

- SpCas9 had 10x-100x more cut sites than SaCas9 and AsCas12a assayed with Digenome-Seq at saturating RNP concentrations (1000 nM). The average SpCas9 had 78 off-target cut sites, while the SaCas9 had 0.9 and AsCas12a had 1.2.
- On-target cutting was found to be concentration-dependent.
  - Half-maximal biochemical potency was similar for all 3 enzymes at ~3 to 30 nM.
  - Half-maximal cellular potency was ~300 nM to 3,000 nM, although more variable with some target sites showing striking differences between enzymes.
- Off-target cutting was also concentration-dependent with the "off-to-on" target ratio often reaching 100-1000x for SaCas9 and AsCas12a but not exceeding 10 for SpCas9.
- This study points towards the use of SaCas9 and AsCas12a for potential therapeutic interventions where large specificity windows are more attainable.

References

[1] Kim, D. et al. Nat. Methods 12, 237–243 (2015).
[2] Kleinstiver, B. P. et al. Nat. Biotechnol. 34, 869–874 (2016).
[3] Kim, D., Kim, S., Kim, S., Park, J. & Kim, J.-S. Genome Res. 26, 406–415 (2016).
[4] Maeder, M. L. et al. Nat. Med. 25, 229–233 (2019).

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Disclosures

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