An in vitro method for benchmarking of CRISPR-associated endonucleases

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Introduction

Direct delivery of Cas9 or Cpf1 ribonucleoprotein (RNP) complexes to cells by electroporation is emerging as an important delivery mode for *ex vivo* gene editing therapies. Understanding the dose/efficacy relationship of the delivered RNP is a key step in optimizing a therapeutic. An essential requirement in accurately establishing this relationship and determining RNP quality is measuring the *in vitro* activity of a given RNP complex in a standardized manner. Here we describe an *in vitro* assay for measuring the specific activity of CRISPR-associated nucleases on synthetic DNA substrates. We then apply this assay to benchmark the activity of several naturally occurring and engineered CRISPR nucleases. This standardized *in vitro* benchmarking provides critical input to help create the best RNP based therapy.

Specific Activity Method

The specific activity assay substrate (**a**) is a 200 bp synthetic DNA amplicon comprised of genomic DNA target sequences and constant 5' and 3' primer handles. Selected target sites have a 3' NGGRRT PAM with one of the following 5' PAMs 20 bp upstream: TTTV, TTN, NYCV, or TATV, allowing us to compare activities of multiple nucleases using the same synthetic substrate. EC50 values are generated by a dose-response of RNP over 15 nM substrate for 1 hour at 37º C in 30 mM Hepes pH 7.5, 150 mM NaCl, and 2 mM $MgCl_2$. RNP is added to DNA substrate using a Biomek FX^{p} liquid handler. Nuclease activity is quenched by addition of Proteinase K. Fraction of cleaved substrate is measured using the Fragment Analyzer automated CE system (AATI). Analysis is performed with GraphPad Prism using four parameter dose-response.

a

5' — 20mer Target — 3'

Cpf1 variant PAM   Sp/Sau Cas9 PAM

All RNPs were formed at 2:1 in excess of gRNA in 30 mM Hepes pH 7.5, 150 mM NaCl, and 2 mM $MgCl_2$. Guides were supplied by IDT as 2-part alt-R for SpCas9, SauCas9, SpCas9-HF1, and eSpCas9. IDT single guide alt-R were used for AsCpf1, LbCpf1, FnCpf1, AsCpf1-RVR, and AsCpf1 RR. Digital scanning fluorimetry (DSF) was performed on RNPs to ensure RNP complexation.

Conclusions

In vitro matched site benchmarking offers activity comparisons between CRISPR-associated nucleases. Potency measurements on matched substrates indicate no significant differences in activity across nucleases tested. Finally, engineered variants High-fidelity Cas9 and enhanced specificity Cas9 display target discrimination *in vitro*. Benchmarking these nucleases enables understanding the cellular dose-efficacy relationship and helps optimize an ex-vivo RNP therapeutic.

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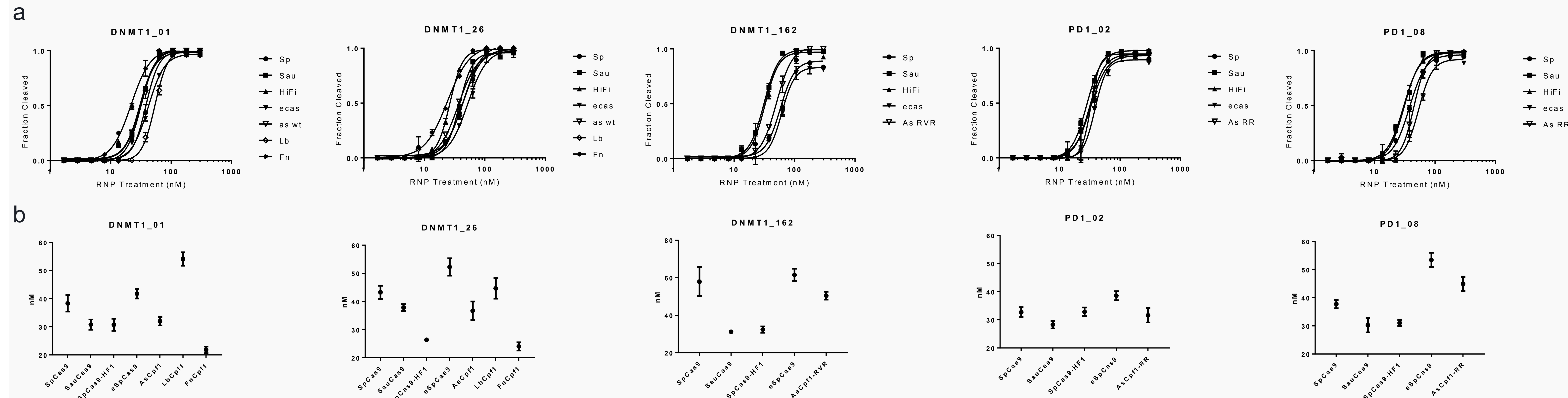
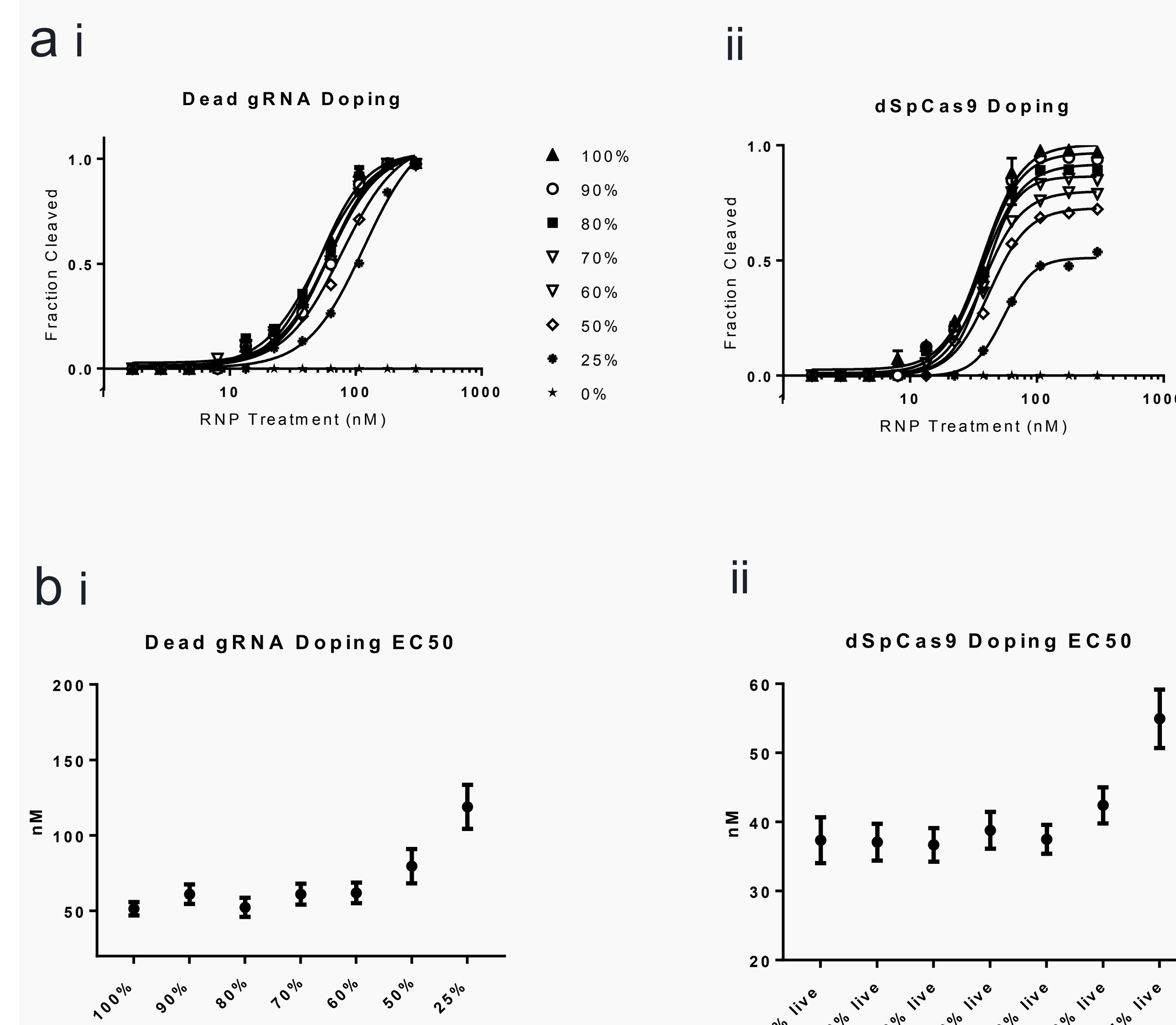
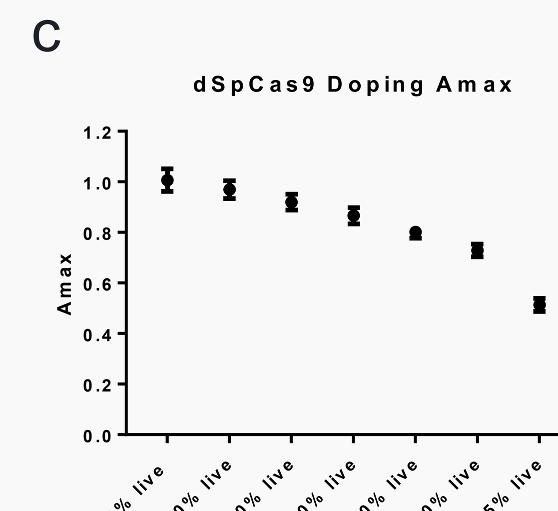
In vitro Benchmarking of CRISPR-associated Nucleases



a) Specific activity benchmarking was performed on the following CRISPR nucleases: SpCas9, SauCas9, High-fidelity (SpCas9-HF1), enhanced specificity (eSpCas9), AsCpf1, LbCpf1, FnCpf1, AsCpf1-RVR, and AsCpf1 RR. N = 2 **b**) 95% CI EC50 values indicate that the variance in activity between enzymes is similar and largely target specific. NGG PAM was used for SpCas9, SpCas9-HF1, and eCas9. NGGRRT for SauCas9. TTTV for AsCpf1, LbCpf1, and FnCpf1. TATV for AsCpf1 RVR. NYCV for AsCpf1 RR.

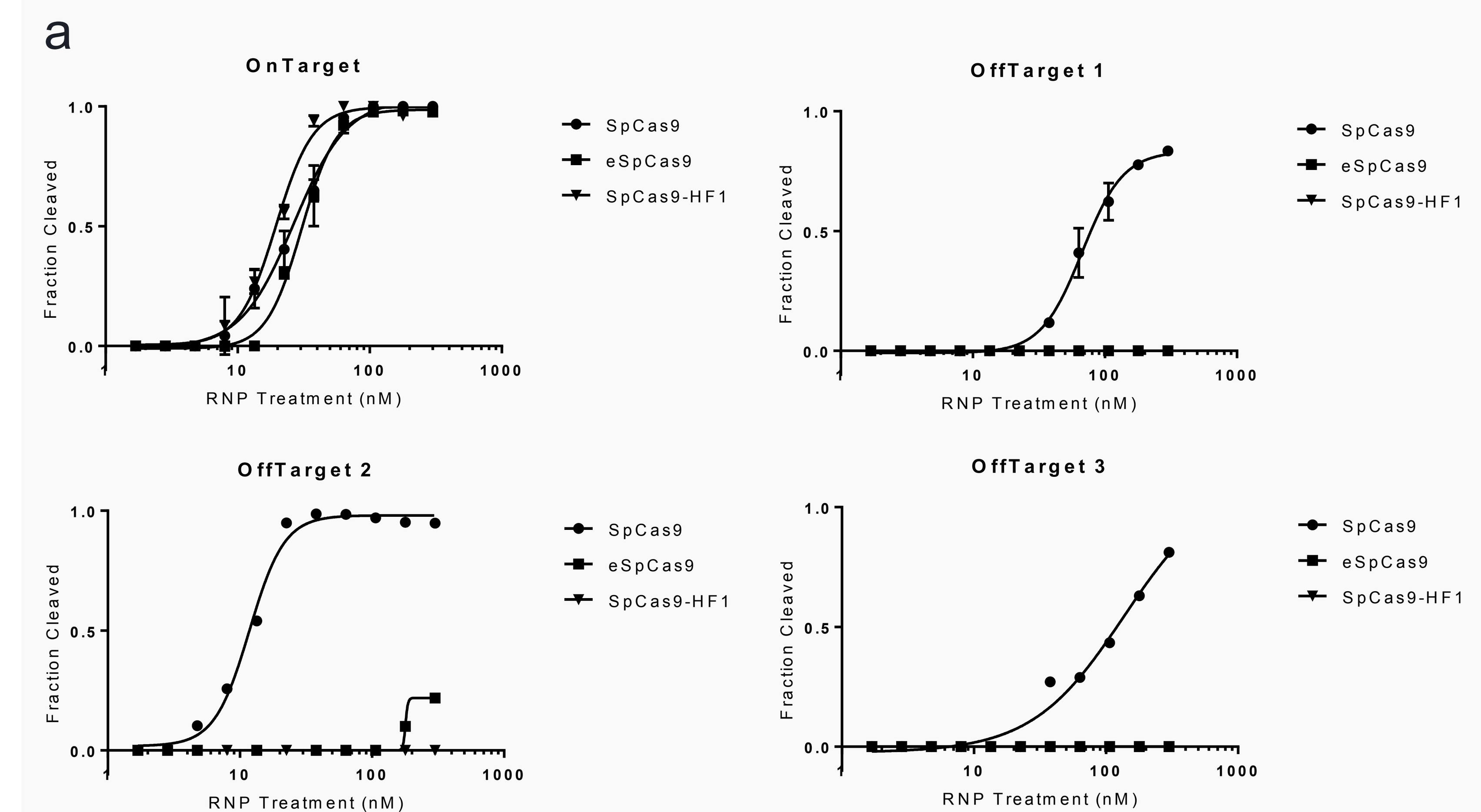
Assay Characterization and Sensitivity





a) Assay sensitivity was determined by doping in of **i.** dead (orthogonal) gRNA and **ii.** dSpCas9 **b**) 95% confidence interval values indicate EC50 values are significantly different at 50% **i.** dead gRNA and **ii.** dSpCas9 **c**) Decrease in Amax suggests dSpCas9 binds to substrate DNA inhibiting SpCas9 activity.

Off-target Analysis of Engineered Variants



a) SpCas9, SpCas9-HF1, and eSpCas9 show comparable on-target activity. Rationally engineered SpCas9-HF1 and eSpCas9 have almost no activity against off-targets *in vitro*.

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