EnGen® SpRY Cas9 – A universal tool for DNA cleavage *in vitro*

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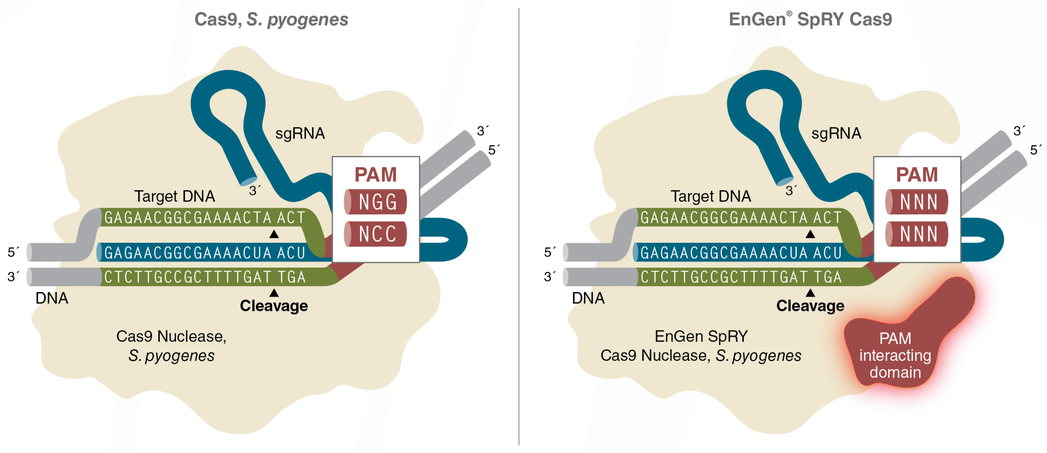
Abstract

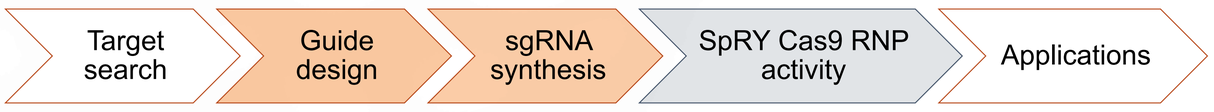
The protospacer adjacent motif (PAM) is required to be present next to a target DNA sequence for CRISPR-Cas9 to distinguish target and non-target. The short DNA sequence requirement limits the number of genomic regions available for targeting and it can be a hurdle to design tools for genome editing.

An engineered *S. pyogenes* Cas9 (Spy Cas9) variant called SpRY has been reported as nearly PAM-less, requiring an NRN>NYN PAM *in vivo* or NNN *in vitro*, instead of the NGG required by wildtype Spy Cas9 (1, 2). *Christie et al.* have developed a method called SpRYgest for universal *in vitro* DNA cleavage in cloning applications with SpRY Cas9 (2).

We demonstrate a simple workflow for SpRYgest using EnGen® sgRNA synthesis followed by *in vitro* cleavage with EnGen® SpRY Cas9 RNPs. We confirm that EnGen® SpRY Cas9 ribonucleoproteins (RNPs) can cleave more than 130 target sequences with various PAMs that are incompatible with wildtype Spy Cas9.



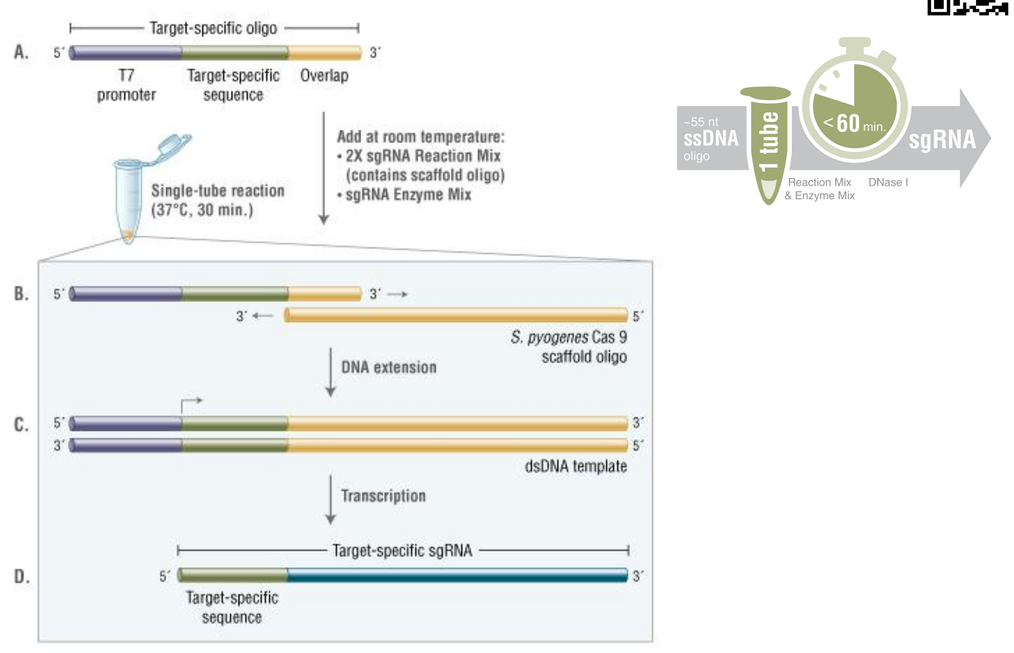
Workflow



Rapid generation of EnGen® sgRNA

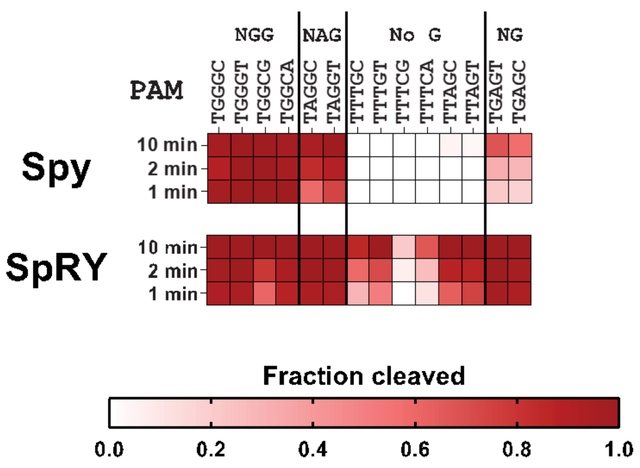
EnGen sgRNA Template
Oligo Designer

EnGen® sgRNA Synthesis Kit, *S. pyogenes*



NEB offers a seamless workflow for sgRNA design and *in vitro* synthesis for *S. pyogenes* Cas9 (Spy Cas9) and variants including SpRY Cas9.

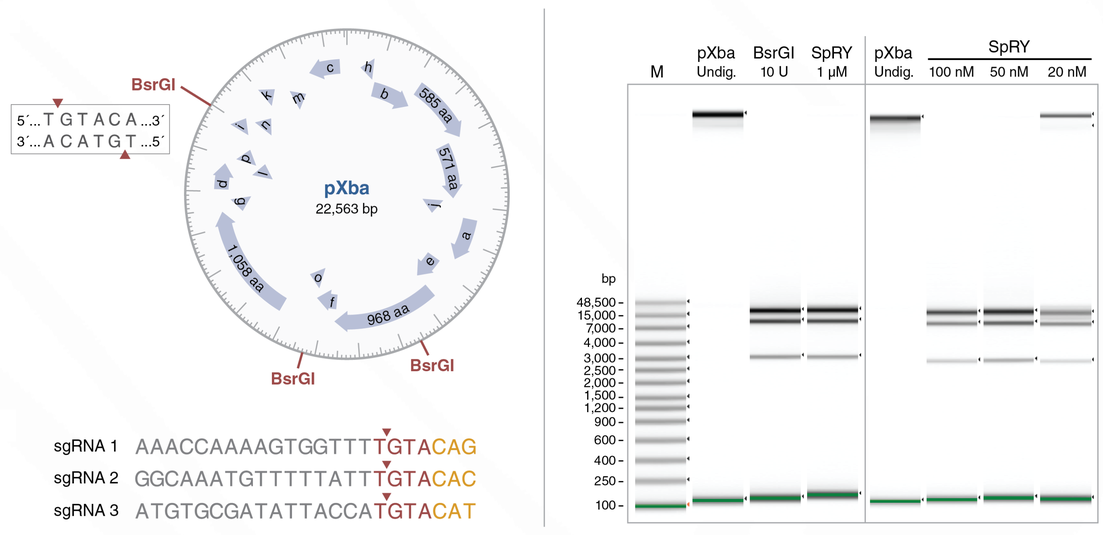
EnGen® SpRY Cas9: RNP activity *in vitro*



5'-FAM labeled dsDNA substrates containing various PAMs adjacent to a target sequence were incubated with either EnGen® Spy Cas9 or EnGen® SpRY Cas9 RNPs at 37°C over a time course.

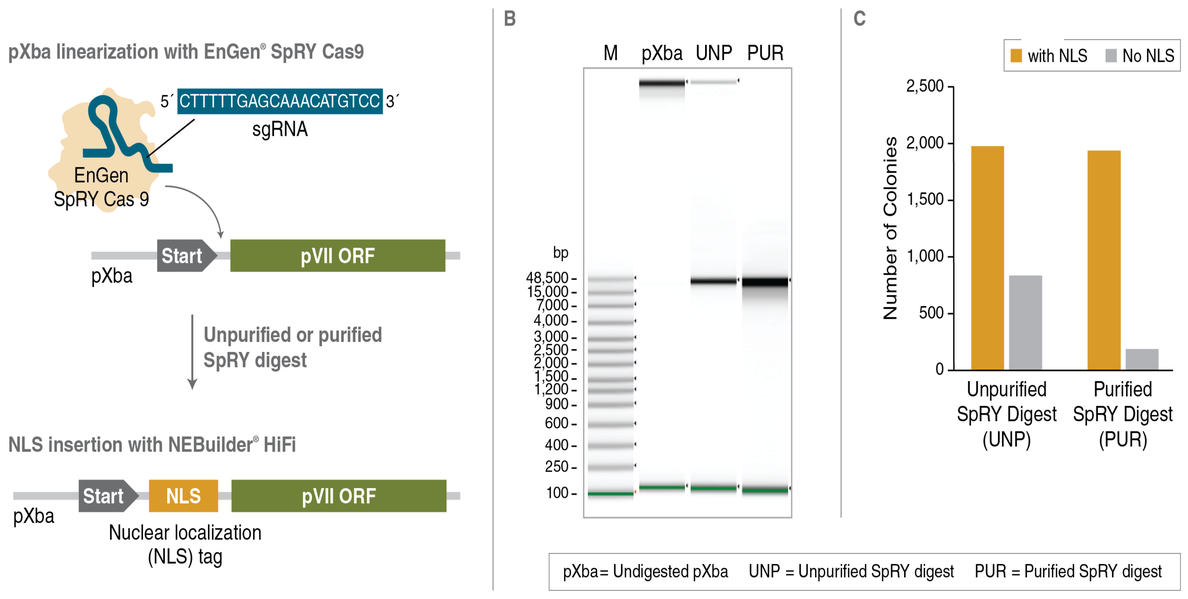
The samples were then subjected to capillary electrophoresis to measure the amount of cleaved versus intact DNA in each sample.

Multiplexed EnGen® SpRY Cas9 RNPs as programmable restriction endonucleases



Plasmid pXba (~22.6 kb) was digested with EnGen® SpRY Cas9 multiplexed with three sgRNAs targeting BsrGI restriction sites in the plasmid (1 hr at 37ºC). Multiplexed EnGen® SpRY Cas9 RNPs showed a specific pattern of cleavage similar to that of a digest with BsrGI restriction enzyme.

Programmed RNPs in an NEBuilder® cloning workflow



pXba was linearized downstream of the start codon of the pVII ORF with programmed EnGen® SpRY Cas9 RNP (1 hr at 37°C). The reactions were purified with Monarch® PCR & DNA Cleanup Kit or left unpurified. A synthetic oligonucleotide encoding a nuclear localization signal (NLS) was successfully inserted via use of NEBuilder® HiFi DNA Assembly Kit.

Conclusions

EnGen® SpRY Cas9 is not constrained by the requirement for an NGG PAM and can produce a double stranded break upstream of any trinucleotide sequence in *in vitro* applications.

Multiplexed EnGen® SpRY Cas9 RNPs digest plasmid DNA at multiple sites simultaneously in one reaction to produce desired fragments.

EnGen® SpRY Cas9 RNPs can be a useful tool for *in vitro* DNA cloning applications, especially in situations where use of traditional restriction enzymes is not compatible.

References

1. **Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants.** *Walton, et al. Science 2020 Apr 17;368(6488):290-6. doi: 10.1126/science.aba8853.*

2. **Precise DNA cleavage using CRISPR-SpRYgests.** *Christie, et al. Nat. Biotechnol. 2023 Mar 41, 409-16. doi: 10.1038/s41587-022-01492-y.*