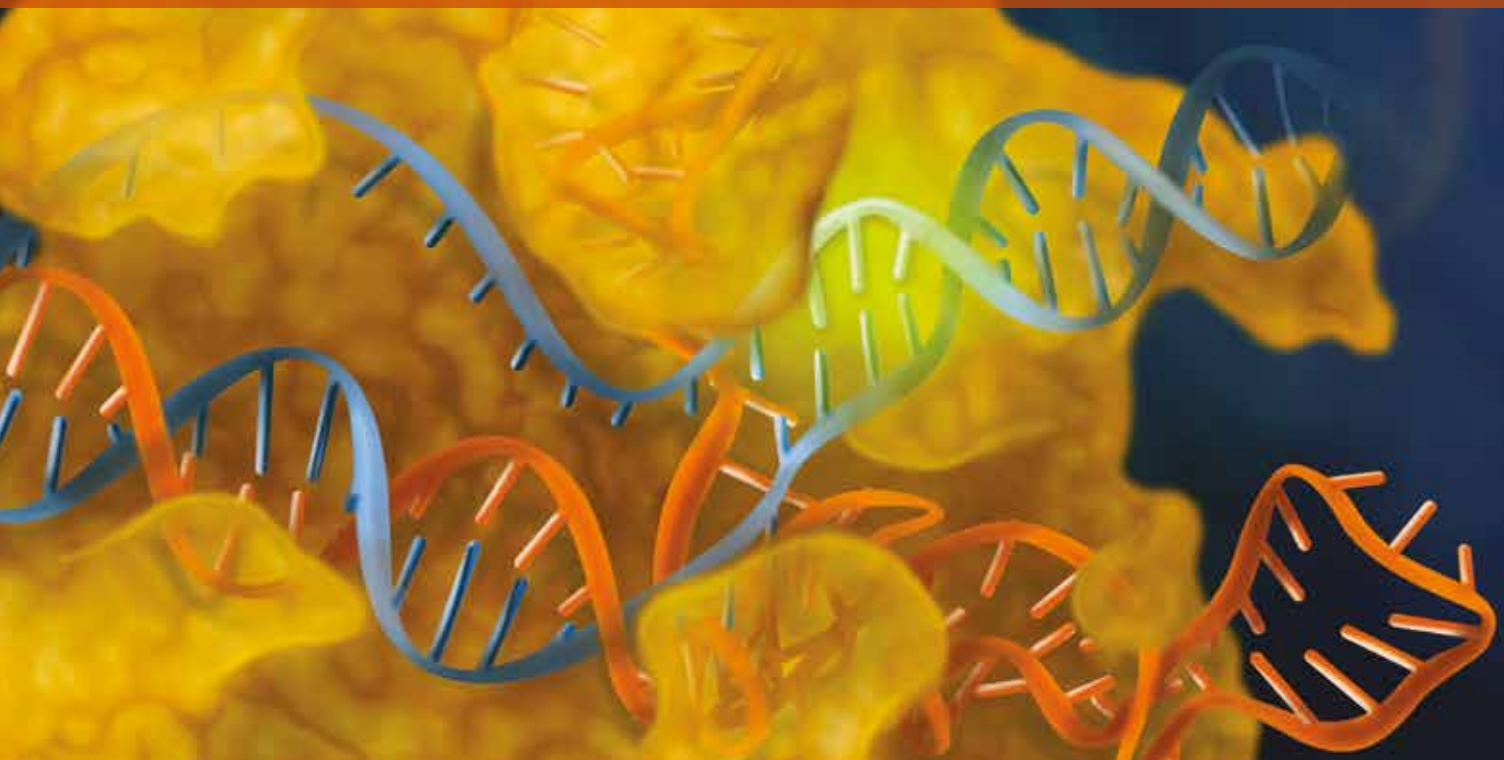
Now includes EnGen® Spy Cas9 HF1
& EnGen SpRY Cas9

Genome Editing

TOOLS FOR CRISPR/CAS APPLICATIONS



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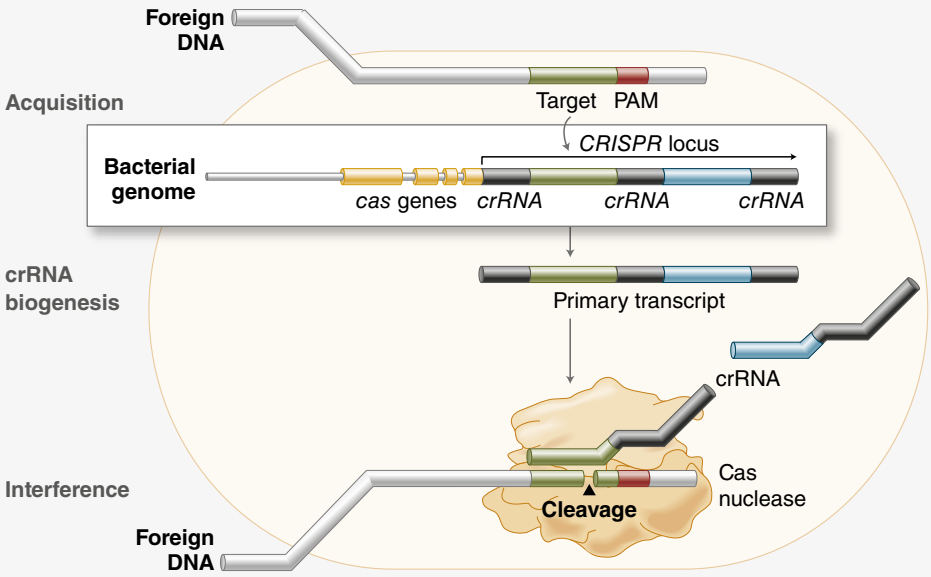
Genome Editing: Tools for CRISPR/Cas Applications

Genome editing is enabled by the development of tools to make precise, targeted changes to the genome of living cells. Recent approaches to targeted genome modification – zinc-finger nucleases (ZFNs) and transcription-activator like effector nucleases (TALENs) – enable researchers to generate mutations by introducing double-stranded breaks to activate repair pathways. These approaches are costly and time consuming to engineer, limiting their widespread use, particularly for large scale, high-throughput studies. Recently, methods based on a bacterial CRISPR-associated protein-9 nuclease (Cas9) from *Streptococcus pyogenes* have generated considerable excitement.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) genes are essential for adaptive immunity in select bacteria and archaea, enabling the organisms to respond to and eliminate invading genetic material.

CRISPR/Cas *in vivo*: Bacterial Adaptive Immunity



In the acquisition phase, foreign DNA is incorporated into the bacterial genome at the CRISPR locus. The CRISPR locus is then transcribed and processed into crRNA during crRNA biogenesis. During interference, Cas endonuclease complexed with crRNA cleaves foreign DNA containing a crRNA complementary sequence adjacent to the PAM sequence. (Figure not drawn to scale.)

CRISPR/Cas Genome Editing

The simplicity of the CRISPR nuclease system (nuclease and guide RNA), makes this system attractive for laboratory use. Breaks activate repair through error prone Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR). In the presence of a donor template with homology to the targeted locus, the HDR pathway may operate, allowing for precise mutations to be made. In the absence of a template, NHEJ is activated, resulting in insertions and/or deletions (indels), which disrupt the target locus (1,2).

TOOLS & RESOURCES

Visit **www.neb.com/GenomeEditing** to find our up-to-date listing of products and protocols to support this application.

Direct Introduction of Cas RNP Complexes

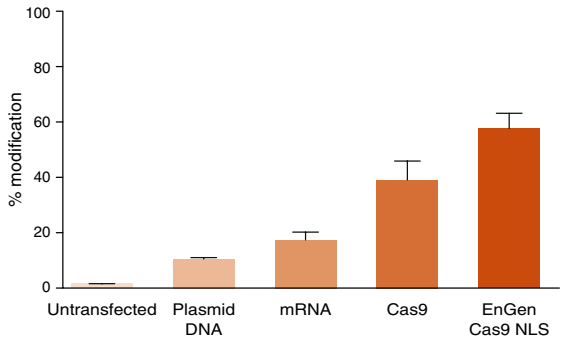
The highest efficiency strategy for genome engineering with CRISPR/Cas is direct introduction of Cas9/guide RNA complexes (3–8) or Cas12a (Cpf1)/guide RNA complexes. This method further simplifies CRISPR/Cas workflows and has been reported to increase mutagenic activity (3–5) and reduce off-target editing events (3,4).

NEB® provides purified Cas9 Nuclease, *S. aureus, S. equinis* and *S. pyogenes* variants, and Cas12a nuclease (Cpf1), *Lachnospiraceae* bacterium ND2006 with nuclear localization signals as standalone enzymes to support direct introduction of Cas RNP complexes.

Ordering Information

PRODUCT	NEB #
EnGen Spy Cas9 HF1	M0667T/M
EnGen SpRY Cas9	M0669TM
EnGen Spy Cas9, NLS	M0646T/M
Cas9 Nuclease, *S. pyogenes*	M0386S/T/M
EnGen Spy Cas9 Nickase	M0650S/T
EnGen Spy dCas9 (SNAP-tag®)	M0652S/T
EnGen Sau Cas9	M0654S/T
EnGen Seq1 Cas9	M0668T
EnGen Lba Cas12a (Cpf1)	M0653S/T

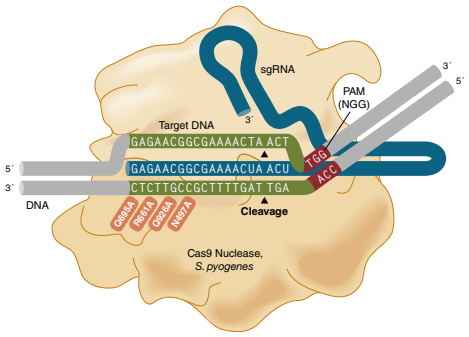
Increased genome editing efficiency using Cas9 RNP delivery



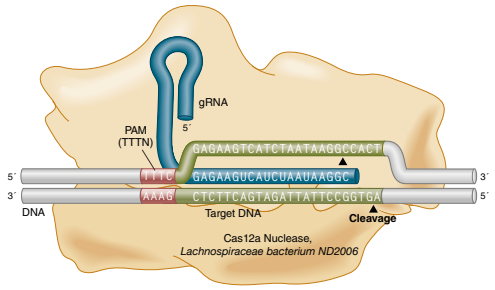
Cas9 and sgRNA targeting a human gene were delivered to HEK293 cells by transfection. Transfected plasmid DNA contained expression cassettes for 2x NLS (N- and C-terminal) Cas9 and sgRNA. Plasmid DNA was delivered using TransIT-X2 (Mirus). Transfected mRNA was modified with pseudouridine and 5-methylcytosine and encoded 2x NLS (N- and C-terminal) Cas9. sgRNA was co-transfected with the mRNA using TransIT-mRNA. Cas9 RNPs were delivered in reverse transfections using Lipofectamine RNAiMAX (Life Technologies) using 10 nanomolar final concentration of ribonucleoprotein (RNP). Cas9 has no NLS in the protein sequence. EnGen Cas9 has N- and C-terminal NLSs. The efficiency of editing was determined using T7 Endonuclease I assay and is expressed as % modification.

NEB's CRISPR Nuclease toolbox (selected products)

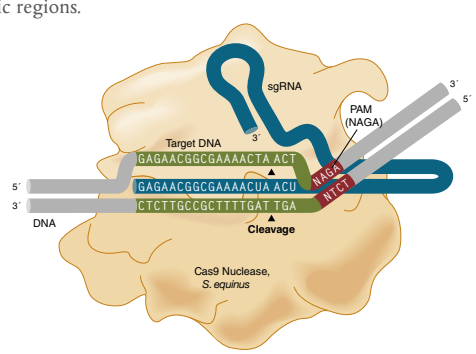
EnGen Spy Cas9 HF1, *Streptococcus pyogenes*
High-fidelity, quadruple substitution variant of EnGen Spy Cas9 NLS from *Streptococcus pyogenes* with reduced non-specific DNA cleavage



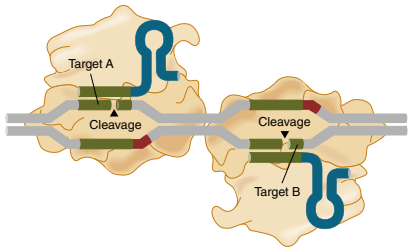
EnGen Lba Cas12a, *Lachnospiraceae* bacterium ND2006
AT-rich PAM, extended temperature range



EnGen Seq1 Cas9, *Streptococcus equinus*
5´- NAGA -3´ PAM sequence allows targeting of additional genomic regions.



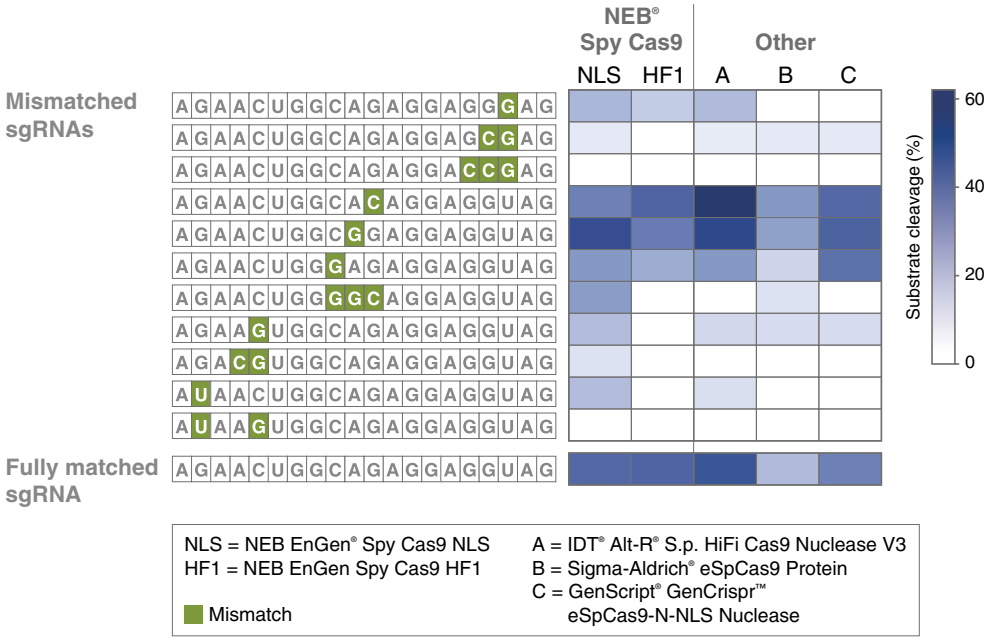
EnGen Spy Cas9 Nickase
In vitro nicking of dsDNA. Increased genome editing specificity *in vivo*, requiring adjacent targets.



Reducing Off-Target DNA Cleavage

EnGen Spy Cas9 HF1 is a high-fidelity, quadruple substitution (N497A/R661A/Q695A/Q926A) variant of EnGen Spy Cas9 NLS from *Streptococcus pyogenes* with reduced non-specific DNA cleavage. Spy Cas9 is an RNA-guided endonuclease that catalyzes site-specific cleavage of double stranded DNA. The single guide RNA (sgRNA) targets Cas9 to the region immediately upstream of a 5´-NGG-3´ protospacer adjacent motif (PAM) producing a double stranded break 3 bases upstream of the PAM (9). EnGen Spy Cas9 HF1 contains Simian virus 40 (SV40) T antigen nuclear localization sequence (NLS) on the N- and C-termini of the protein.

EnGen Spy Cas9 HF1 demonstrates increased sensitivity to mismatches between guide RNA and DNA targets *in vitro*



Comparison of the tolerance of mismatches between the guide RNA sequence and target DNA sequence of EnGen Spy Cas9 NLS, EnGen Spy Cas9 HF1, and other commercially available high fidelity Cas9 variants. One of several guide RNAs encoding a single, double, or triple mismatch with a fluorescently labeled dsDNA substrate were allowed to form a ribonucleoprotein (RNP) complex with each of five Cas9 variants. A fully matched guide RNA was included as a control. The RNPs were incubated with the substrate at a 2:1 ratio at 37°C for 5 minutes. The percent substrate cleavage for each RNP complex was measured by capillary electrophoresis. Results were graphed as a heat map with white representing no cleavage and increasing intensity of blue indicating increasing percent cleavage. The guide RNA sequence is indicated in each row, with mismatches denoted in green. The DNA protospacer sequence is 5´– AGAACTGGCAGAGGAGGTAG – 3´ and the protospacer adjacent motif (PAM) is 5´– TGG – 3´. EnGen Spy Cas9 HF1 demonstrates increased sensitivity to mismatches by showing the greatest ratio of on-target cleavage to average cleavage of off-targets.

Rapid Generation of sgRNA for Spy Cas9

The EnGen sgRNA Synthesis Kit simplifies the generation of microgram quantities of custom sgRNAs in an hour or less by combining template synthesis and transcription. The single-tube reaction is easy to set up and requires a single ~55 nt ssDNA target-specific oligonucleotide, which is combined with the Reaction Mix and Enzyme Mix included in the kit. sgRNAs are suitable for use in downstream applications, including CRISPR/Cas9-based genome editing and *in vitro* DNA cleavage. This single-reaction format offers ease-of-use and eliminates separate DNA amplification and template clean up steps. This kit is compatible with EnGen Spy Cas9 NLS, Cas 9 Nuclease, *S. pyogenes*, EnGen Spy Cas9 Nickase, EnGen Spy dCas9 (SNAP-tag) and EnGen Spy Cas9 HF1..

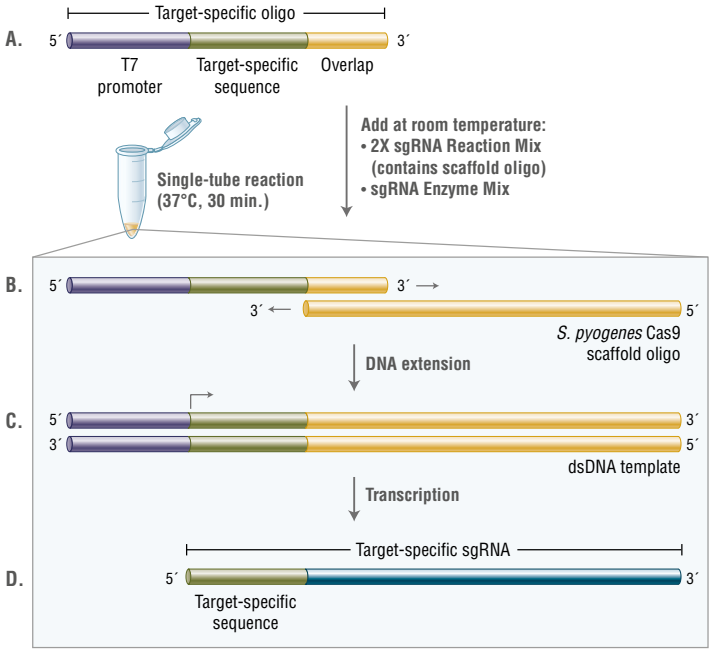
Ordering Information

PRODUCT	NEB #
EnGen sgRNA Synthesis Kit	E3322V/S

Need help configuring target-specific DNA oligos?

Try our **EnGen sgRNA Template Oligo Designer** (accessible through NEBioCalculator® at NEBiocalculator.neb.com)

EnGen sgRNA Synthesis Kit overview



Try Monarch RNA Cleanup Kits for purification of sgRNA after synthesis.

Learn more at **www.neb.com/MonarchRNACleanup**.

> *"This kit is really easy to use and will save us plenty of time in making sgRNAs! Thanks for the streamlined method!"*
>
> – Postdoctoral Researcher, Harvard University

A. The target-specific oligo contains the T7 promoter sequence, ~20 nucleotides of target-specific sequence and a 14-nucleotide overlap sequence complementary to the S. pyogenes Cas9 Scaffold Oligo, supplied in the reaction mix. Target-specific oligos are mixed with the EnGen 2X sgRNA Reaction Mix and the EnGen sgRNA Enzyme Mix at room temperature.

B. At 37°C the two oligos anneal at the 14-nucleotide overlap region of complementarity.

C. The DNA polymerase contained in the EnGen sgRNA Enzyme Mix extends both oligos from their 3´ ends, creating a dsDNA template.

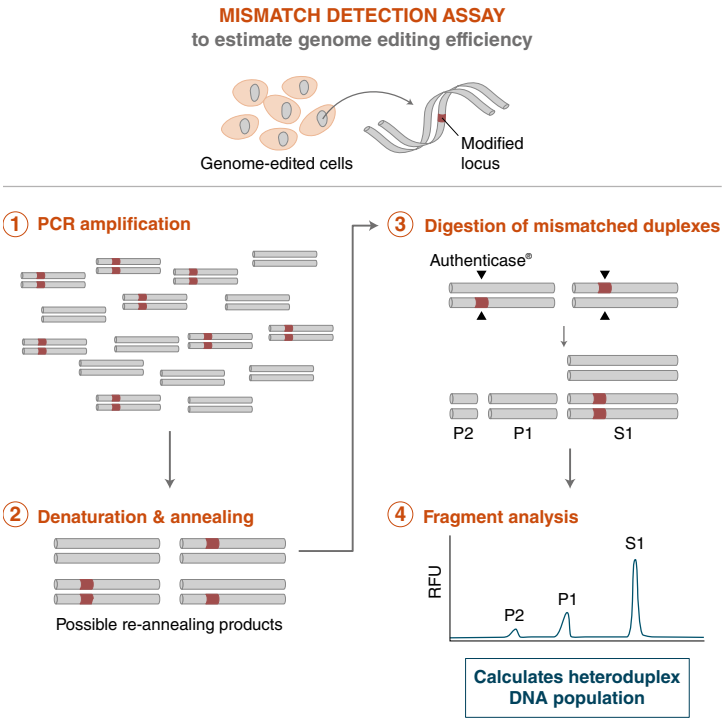
D. The RNA polymerase contained in the EnGen sgRNA Enzyme Mix recognizes the dsDNA of the T7 promoter and initiates transcription, resulting in a target-specific sgRNA.

All steps occur in a single reaction during a 30-minute incubation at 37°C.

Evaluating Targeting Efficiency with Mutation Detection Kits and Assays

A widely used method to identify mutations is the T7 Endonuclease I mutation detection assay (10,11). This assay detects heteroduplex DNA that results from the annealing of a DNA strand, including desired mutations, with a wild-type DNA strand. Multiple options are available for performing robust detection of genome editing events including the T7 Endonuclease-based EnGen Mutation Detection Kit and Authenticase, which provides improved detection of single-and Two-basepair mutations.

Authenticase Mismatch Detection Assy



Authenticase can replace T7 Endonuclease I in the mismatch detection assay used to assess the efficiency of genome editing (S1 is the starting material. P1 and P2 are products of Authenticase digestion).

Ordering Information

PRODUCT	NEB #
Authenticase®	M0689S/L
EnGen Mutation Detection Kit	E3321S
T7 Endonuclease I	M0302S/L
Q5® Hot Start High-Fidelity 2X Master Mix	M0494S/L/X

Need to determine targeting efficiencies over 50%?

Visit **www.neb.com/Cas9locusmod** to find out how.

Learn more about genome editing vocabulary through the NIST Genome Editing Lexicon **www.nist.gov/programs-projects/nist-genome-editing-lexicon**

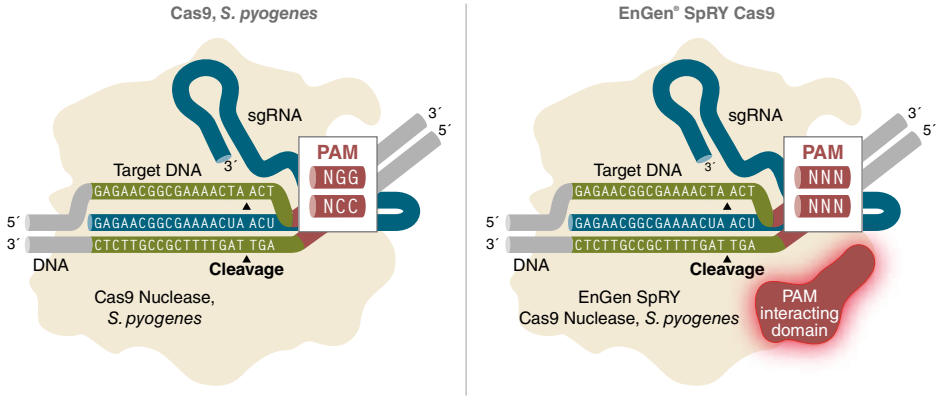
EnGen SpRY Cas9 Nuclease

EnGen SpRY Cas9 from Streptococcus pyogenes is an engineered RNA-guided DNA endonuclease that catalyzes site-specific cleavage of double-stranded DNA (dsDNA). Targeting requires an ~100 nucleotide single guide RNA (sgRNA) with complementarity to the 20 nucleotide region immediately upstream of a protospacer adjacent motif (PAM) on the dsDNA substrate. Unlike the canonical 5´-NGG-3´ PAM of wild-type Spy Cas9, SpRY Cas9 is essentially PAMless in vitro, requiring a PAM of 5´-NNN-3´ (1,2). DNA cleavage by EnGen SpRY Cas9 produces a double-stranded break occurring 3 nucleotides upstream of the PAM. EnGen SpRY Cas9 contains Simian virus 40 (SV40) T antigen nuclear localization sequence (NLS) on the C-terminus of the protein.

BENEFITS

- Eliminate sequence constraints for dsDNA targeting with non-specific PAM (5´-NNN-3´ PAM)
- Digest large plasmids in cloning workflows successfully
- Use in conjunction with the EnGen sgRNA Synthesis Kit, *S. pyogenes* (NEB #E3322), EnGen Mutation Detection Kit (NEB #E3321) and NEBuilder® HiFi DNA Assembly Master Mix (NEB #E2621)

EnGen SpRY Cas9 has no sequence constraints for dsDNA targeting *in vitro*

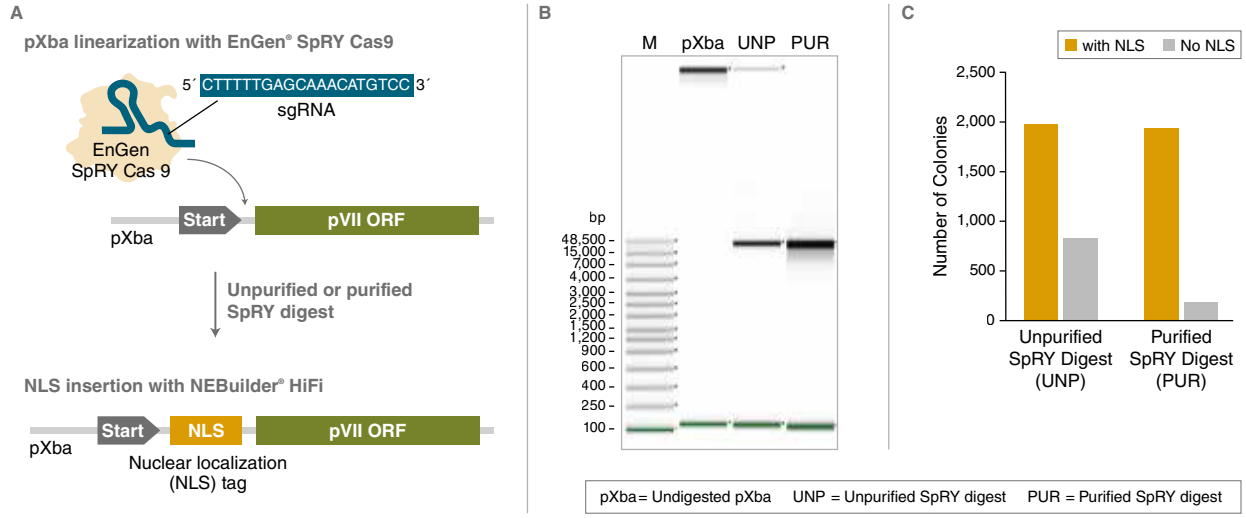


EnGen SpRY Cas9 is a variant of Cas9 nuclease from S. pyogenes with several point mutations within the PAM interacting domain (1). Unlike wildtype Cas9, EnGen SpRY Cas9 is not constrained by the presence of an NGG PAM and can produce a double stranded break three nucleotides upstream of any trinucleotide sequence in in vitro applications.

Ordering Information

PRODUCT	NEB #
EnGen SpRY Cas9	M0669T/M

Streamline large construct cloning workflows with EnGen SpRY Cas9 and NEBuilder HiFi DNA Assembly



1 µg of pXba (22,563 bp) was linearized downstream of the start codon of the pVII ORF with 50 nM EnGen SpRY Cas9 and 50 nM sgRNA in 1X NEBuffer™ r3.1 for 1 hour at 37°C. The reactions were either spin column purified or left unpurified before proceeding to DNA assembly. The NEBuilder HiFi DNA Assembly kit was used to insert an oligonucleotide encoding a nuclear localization signal (NLS) tag to pVII according to the recommended protocol. The number of colonies grown after transformation with DNA assembly reactions and no insert controls were measured to qualitatively assess how many transformants arise from undigested plasmid following EnGen SpRY Cas9 digest. Though not a requirement, purification of linearized pXba prior to DNA assembly reduced the percentage of background colonies.

References:
1. Walton, R.T., et al. (2020) *Science.* 368(6488):290–6.
2. Christie, K.A., et al. (2023) *Nat. Biotechnol.* 41(3):409–16.

Featured NEB Products Supporting CRISPR Workflows

CRISPR NUCLEASES

NEB #	PRODUCT NAME	VIAL SIZE	CONC.	VOLUME PER VIAL	REACTION BUFFER	PAM SEQUENCE	NLS	TAGS	MUTATIONS (IF APPLICABLE)
M0386S	Cas9 Nuclease, *S. pyogenes*	90 pmol	1 µM	0.090 ML	Buffer r3.1	5´- NGG -3´	No NLS	N-terminal 6X His tag	N/A (wild-type)
M0386M	Cas9 Nuclease, *S. pyogenes*	2500 pmol	20 µM	0.125 ML					
M0386T	Cas9 Nuclease, *S. pyogenes*	500 pmol	20 µM	0.025 ML					
M0646M	EnGen® Spy Cas9 NLS	500 pmol	20 µM	0.125 ML	Buffer r3.1	5´- NGG -3´	SV40 NLS Sequence on N & C termini	N-terminal 6X His tag	N/A (wild-type)
M0646T	EnGen Spy Cas9 NLS	2500 pmol	20 µM	0.025 ML					
M0650S	EnGen Spy Cas9 Nickase	90 pmol	1 µM	0.090 ML	Buffer r3.1	5´- NGG -3´	SV40 NLS Sequence on N & C termini	N-terminal 6X His tag	D10A in the RuvC nuclease domain
M0650T	EnGen Spy Cas9 Nickase	500 pmol	20 µM	0.025 ML					
M0652S	EnGen Spy dCas9 (SNAP-tag®)	90 pmol	1 µM	0.090 ML	Buffer r3.1	5´- NGG -3´	SV40 NLS Sequence on N & C termini	"N-terminal 6X His tag N-terminal SNAP-tag"	D10A in the RuvC and H840A in the HNH nuclease domains
M0652T	EnGen Spy dCas9 (SNAP-tag)	500 pmol	20 µM	0.025 ML					
M0653S	EnGen Lba Cas12a (Cpf1)	70 pmol	1 µM	0.070 ML	Buffer r2.1	5´- TTTV -3´	SV40 NLS Sequence on N & C termini	N-terminal 6X His tag	N/A (wild-type)
M0653T	EnGen Lba Cas12a (Cpf1)	2000 pmol	100 µM	0.020 ML					
M0654T	EnGen Sau Cas9	500 pmol	20 µM	0.025 ML	Buffer r3.1	5´ - NNGRRT - 3´	SV40 NLS Sequence on N & C termini	C-terminal 6X His tag	N/A (wild-type)
M0667M	EnGen Spy Cas9 HF1	500 pmol	20 µM	0.125 ML	Buffer r3.1	5´- NGG -3´	SV40 NLS Sequence on N & C termini	N-terminal 6X His tag	N497A/R661A/Q695A/Q926A
M0667T	EnGen Spy Cas9 HF1	2500 pmol	20 µM	0.025 ML					
M0668T	EnGen Seq1 Cas9	500 pmol	20 µM	0.025 ML	Buffer r3.1	5´- NAGA -3´	SV40 NLS Sequence on N & C termini	C-terminal 6X His tag	N/A (wild-type)
M0669M	EnGen SpRY Cas9	500 pmol	20 µM	0.125 ML	Buffer r3.1	5´- NNN -3´	SV40 NLS Sequence on N & C termini	C-terminal 6X His tag	A61R, L1111R, D1135L, S1136W, G1218K, E1219Q, N1317R, A1322R, R1333P, R1335Q, T1337R
M0669T	EnGen SpRY Cas9	2500 pmol	20 µM	0.025 ML					

FEATURED PRODUCTS SUPPORTING CRISPR WORKFLOWS

PRODUCT NAME	CRISPR/CAS9 APPLICATION	NEB #	SIZE
Q5 Site-directed Mutagenesis Kit (with or without competent cells)	Insertion of target sequence into the Cas9-sgRNA construct and modification of HDR templates	E0554S/E0552S	10 rxns
Q5 High-fidelity DNA Polymerases	High-fidelity construct generation for use with CRISPR workflows	Multiple*	Multiple*
NEBuilder HiFi DNA Assembly Master Mix	Single-tube, isothermal generation of the Cas9-sgRNA construct and HDR templates	E2621S/L/X	10/50/250 rxns
NEBuilder HiFi DNA Assembly Cloning Kit	Single-tube, isothermal generation of the Cas9-sgRNA construct and HDR templates	E5520S	10 rxns
HiScribe T7 ARCA mRNA Kit (with or without tailing)	Generation of Cas9 mRNA with ARCA cap	E2060S/E2065S	20 rxns
HiScribe T7 High Yield RNA Synthesis Kit	Generation of sgRNA and Cas9 mRNA	E2040S	50 rxns
HiScribe T7 Quick High Yield RNA Synthesis Kit	Generation of sgRNA and Cas9 mRNA	E2050S	50 rxns
HiScribe T7 mRNA Kit with CleanCap® Reagent AG	Generation of Cas9 mRNA with CleanCap Reagent AG	E2080S	20 rxns
T7 Endonuclease I	Determination of the targeting efficiency of genome editing protocols	M0302S/L	250/1,250 units
NEW Authenticase	Determination of the targeting efficiency of genome editing protocols	M0689S/L	250/1,250 units
Monarch RNA Cleanup Kit	Cleanup of sgRNA and Cas9 mRNA	T2040S/L	10/100 preps

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For help with designing primers for DNA assembly, try **NEBuilder® DNA Assembly Tool** (**NEBuilder.neb.com**)

Did you know that many of these products can be purchased in large volumes and custom formats? Learn more at **www.neb.com/customizedsolutions**