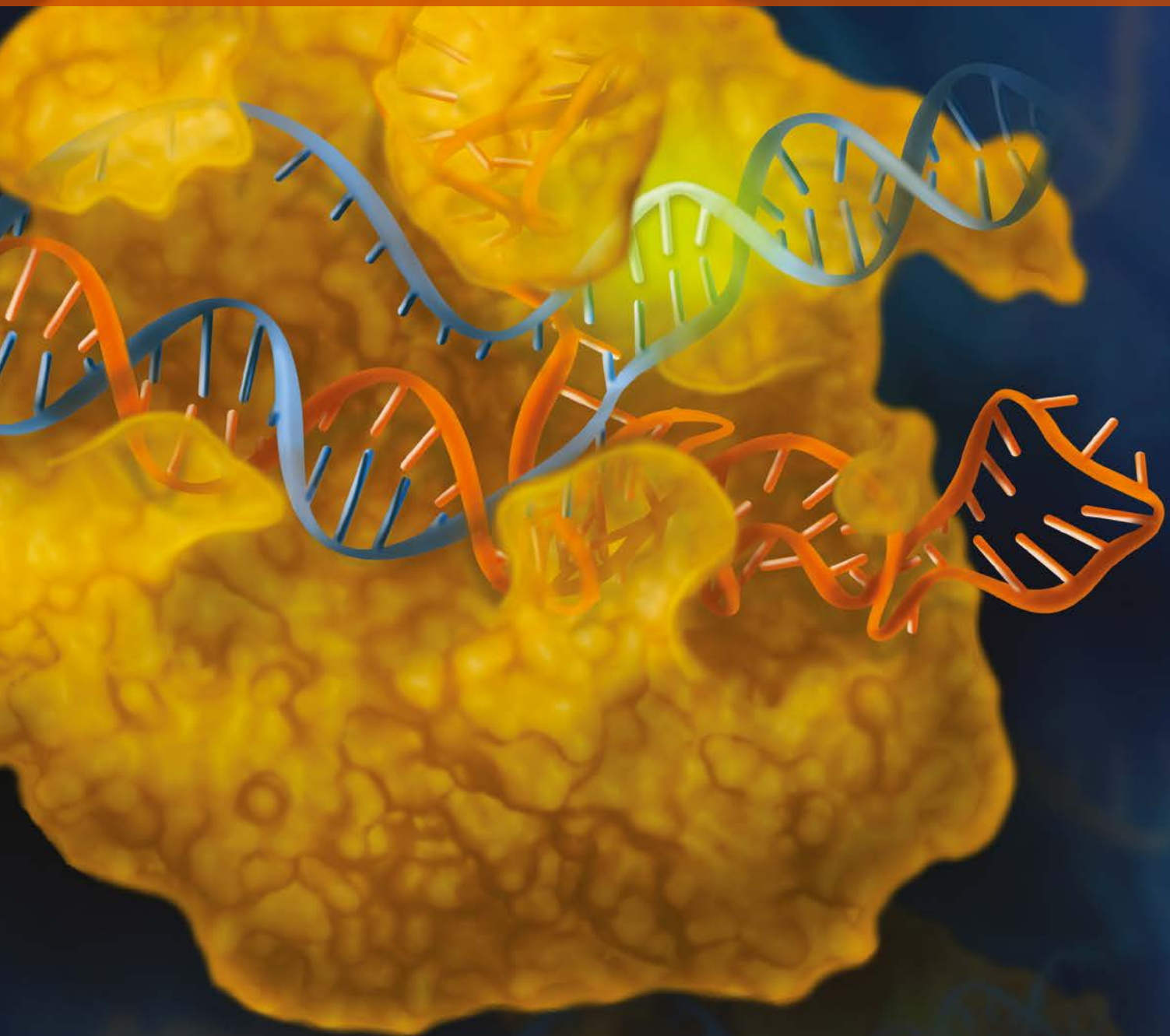
Now includes EnGen® Spy Cas9 HF1

Genome Editing

TOOLS FOR CRISPR/CAS APPLICATIONS



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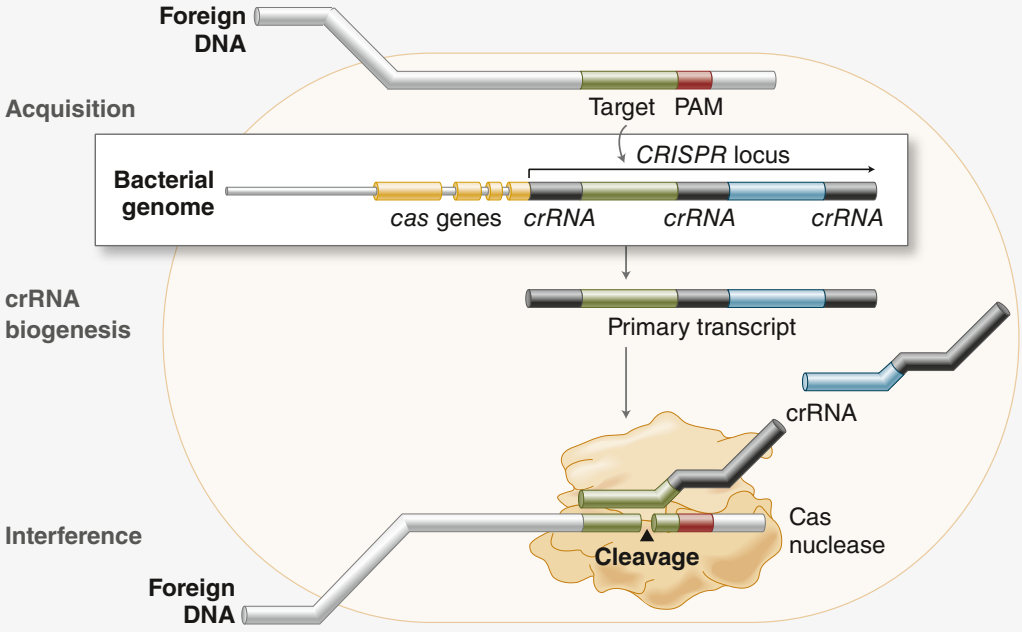
Genome Editing: Tools for CRISPR/Cas Applications

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.)

DOWNLOAD THE NEB AR APP*

Learn more about genome editing.

*see back cover for details

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.

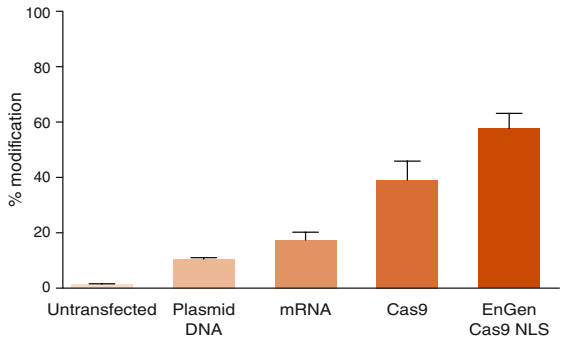
Direct Introduction of Cas RNP Complexes

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* and *S. pyogenes* variants, and Cas12a nuclease (Cpf1), *Lachnospiraceae* bacterium ND2006 with nuclear localization signals (NEB #M0654, NEB #M0667, NEB #M0646, NEB #M0653) as standalone enzymes to support direct introduction of Cas RNP complexes.

Ordering Information

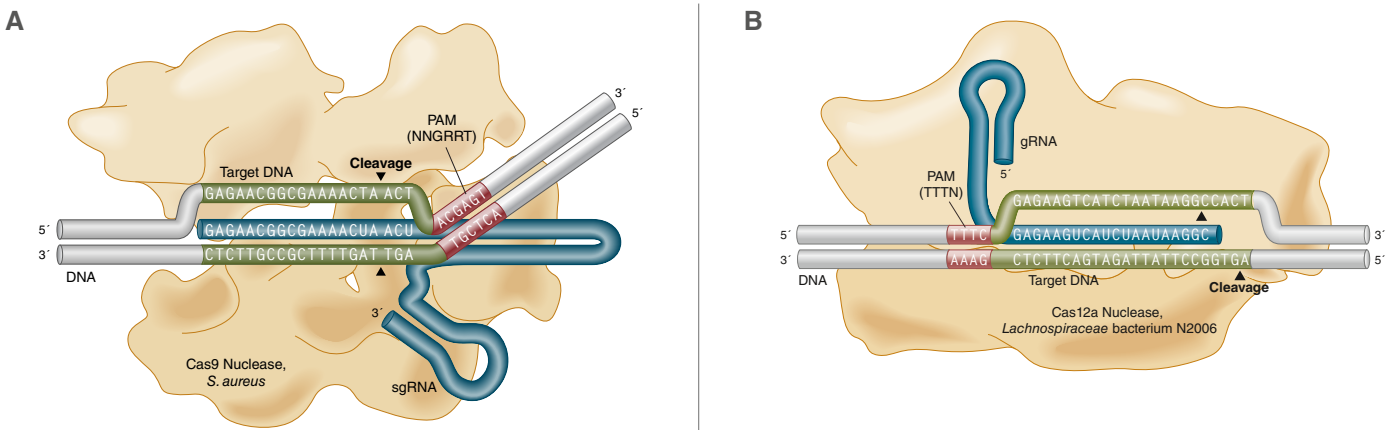
PRODUCT	NEB #
EnGen Spy Cas9 HF1	M0667T/M
Cas9 Nuclease, *S. pyogenes*	M0386S/T/M
EnGen Spy Cas9, NLS	M0646T/M
EnGen Spy Cas9 Nickase	M0650S/T
EnGen Spy dCas9 (SNAP-tag®)	M0652S/T
EnGen Sau Cas9	M0654S/T
EnGen Lba Cas12a (Cpf1)	M0653S/T

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.

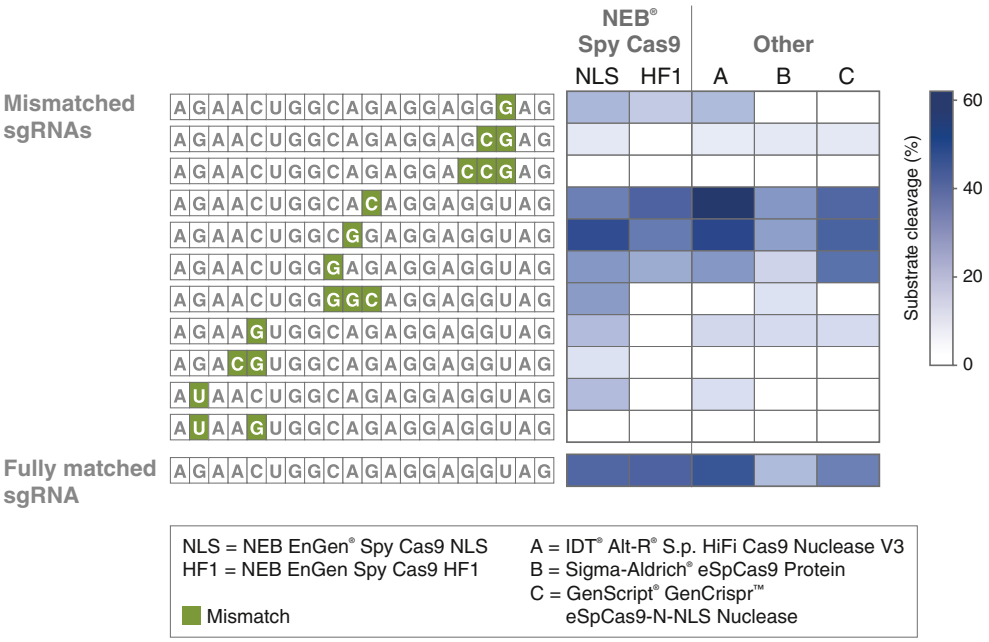
Schematic representation of Cas9 Nuclease, S. aureus (A) and Lba Cas12a, Lachnospiraceae bacterium N2006 (B) sequence recognition and DNA cleavage



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..

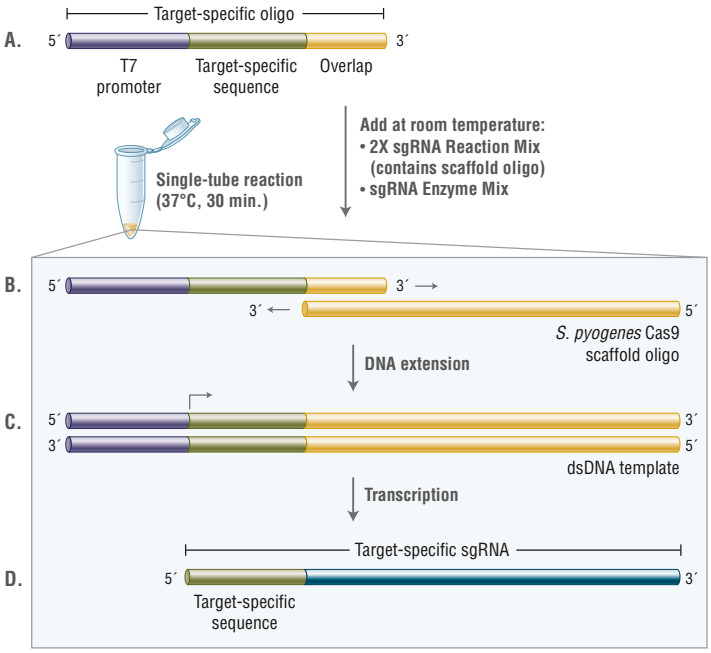
Ordering Information

PRODUCT	NEB #
EnGen sgRNA Synthesis Kit	E3322S

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.

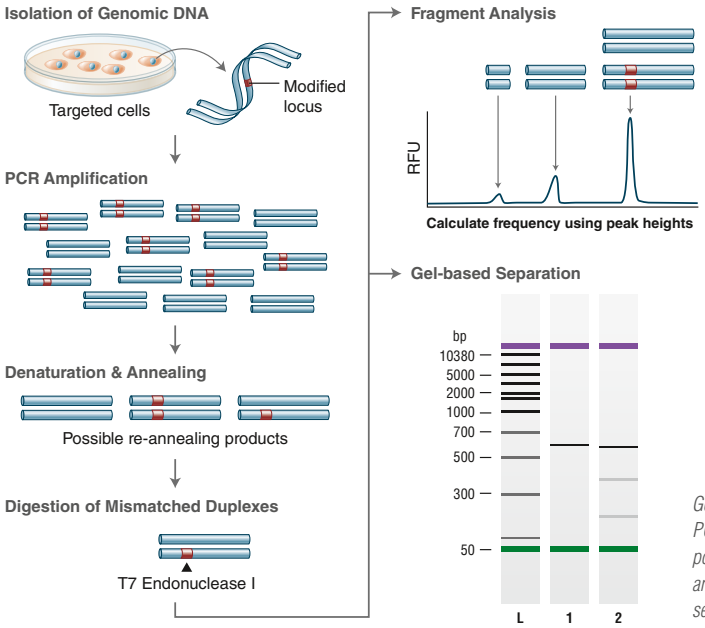
D. The RNA polymerase contained in the EnGen sgRNA Enzyme Mix recognizes the dsDNA of the T7 promoter and initiates transcription, resulting in a target-specific sgRNA.

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

Evaluating Targeting Efficiency with the EnGen Mutation Detection Kit and T7 Endonuclease I

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. The EnGen Mutation Detection Kit provides optimized reagents for performing robust T7 Endonuclease-based detection of genome editing events.

Workflow for EnGen Mutation Detection Kit



Genomic DNA is amplified with primers bracketing the modified locus. PCR products are then denatured and re-annealed yielding three classes of possible structures. Duplexes containing a mismatch greater than one base are digested by T7 Endonuclease I. The DNA is then electrophoretically separated and fragment analysis is used to estimate targeting efficiency.

Ordering Information

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

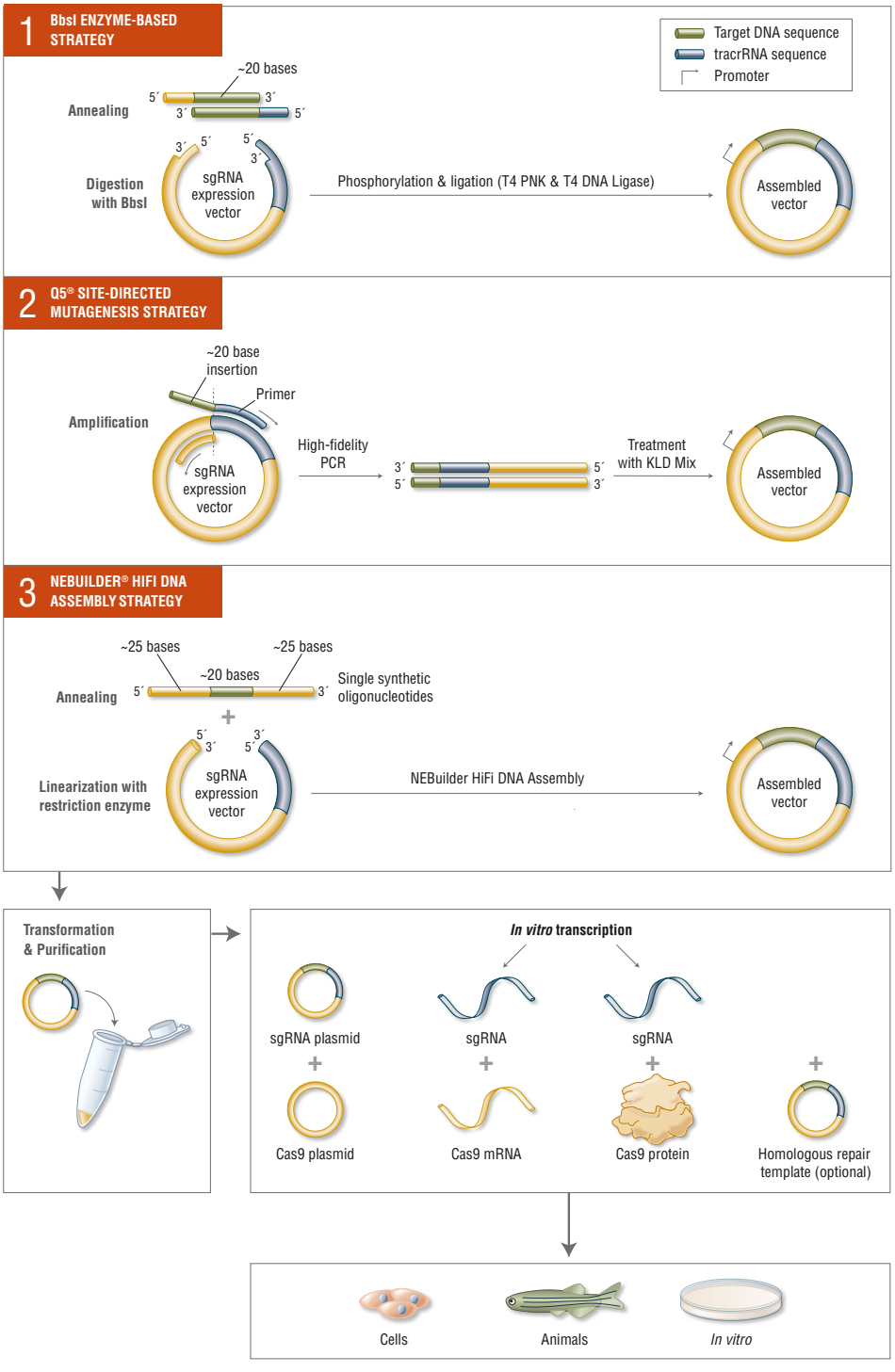
Need to determine targeting efficiencies over 50%?

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

sgRNA Template Construction for CRISPR/Cas Genome Editing

Cas9 experiments require the introduction of guide RNAs, in addition to Cas9 nuclease. sgRNAs contain a 20-base sequence, specific to the target DNA, upstream of an invariant scaffold sequence. sgRNAs can be delivered as an RNA made *in vitro*, or by delivering an expression cassette in which the sgRNA is transcribed from an upstream promoter. For researchers using plasmid-based expression of sgRNA in target cells, or sgRNAs made *in vitro* from plasmid templates, NEB provides tools to support a number of strategies to quickly change the 20-bp targeting sequence of sgRNA templates.



Ordering Information

PRODUCT	NEB #
BbsI	R0539S/L
T4 DNA Ligase	M0202S/T/L/M
T4 Polynucleotide Kinase	M0201S/L

PRODUCT	NEB #
Q5 Site-Directed Mutagenesis Kit	E0554S
Q5 Site-Directed Mutagenesis Kit (Without Competent Cells)	E0552S

PRODUCT	NEB #
Q5 Hot Start High-Fidelity 2X Master Mix	M0494S/L
NEBuilder HiFi DNA Assembly Master Mix	E2621S/L/X
NEBuilder HiFi DNA Assembly Cloning Kit	E5520S

PRODUCT	NEB #
EnGen sgRNA Synthesis Kit	E3322S
NEB 5-alpha Competent *E. coli* (High Efficiency)	C2987P/R/I/H/U
NEB 10-beta Competent *E. coli* (High Efficiency)	C3019I/H
HiScribe T7 mRNA Kit with CleanCap® Reagent AG	E2080S
HiScribe™ T7 ARCA mRNA Kit	E2065S
HiScribe T7 ARCA mRNA Kit (with tailing)	E2060S
HiScribe T7 High Yield RNA Synthesis Kit	E2040S
HiScribe T7 Quick High Yield RNA Synthesis Kit	E2050S
RNA Cap Structure Analog 3′-O-Me-m^{7}G(5′) ppp(5′)G	S1411S/L
Vaccinia Capping System	M2080S
Monarch RNA Cleanup Kit	T2040S

Featured NEB Products Supporting CRISPR Workflows

PRODUCT NAME	CRISPR/CAS9 APPLICATION	NEB #	SIZE
NEW EnGen Spy Cas9 HF1	*In vitro* cleavage of dsDNA. Genome engineering by direct introduction of active nuclease complexes.	M0667T/M	400/2,000 pmol
EnGen Spy Cas9 NLS	*In vitro* cleavage of dsDNA. Genome engineering by direct introduction of active nuclease complexes.	M0646T/M	400/2,000 pmol
EnGen Mutation Detection Kit	Determination of the targeting efficiency of genome editing protocols	E3321S	25 rxns
EnGen sgRNA Synthesis Kit	Generation of microgram quantities of custom sgRNA	E3322S	20 rxns
EnGen Spy Cas9 Nickase	*In vitro* nicking of dsDNA. Genome engineering by direct introduction of active nuclease complexes.	M0650S/T	70/400 pmol
EnGen Spy dCas9 (SNAP-tag®)	*In vitro* binding of DNA. Compatible with SNAP-tag substrates for visualization and enrichment.	M0652S/T	70/400 pmol
EnGen Sau Cas9	*In vitro* cleavage of dsDNA. Genome engineering by direct introduction of active nuclease complexes. Recognizes 5´-NNGRRT-3´ PAM.	M0654S/T	70/400 pmol
EnGen Lba Cas12a (Cpfl)	*In vitro* cleavage of dsDNA. Genome engineering by direct introduction of active nuclease complexes. Recognizes 5´-TTTN PAM.	M0653S/T	70/2,000 pmol
Cas9 Nuclease, *S. pyogenes*	*In vitro* cleavage of dsDNA. Genome engineering by direct introduction of active nuclease complexes.	M0386S/T/M	70/400/2,000 pmol
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
Monarch RNA Cleanup Kit	Cleanup of sgRNA and Cas9 mRNA	T2040S/L	10/100 preps

* Visit **Q5PCR.com** for ordering information.

VISIT NEB.COM TO FIND:

Protocols for applications such as sgRNA synthesis and direct introduction of sgRNA/Cas9 complexes

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

NEBuilder® for DNA Assembly (**NEBuilder.neb.com**)

NEBaseChanger® for the Q5 Site-Directed Mutagenesis Kit (**NEBaseChanger.neb.com**)

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Featured Tools

For help with configuring target-specific DNA oligos, try our **EnGen sgRNA Template Oligo Designer** (accessible through **NEBioCalculator®** at **NEBiocalculator.neb.com**)

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

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