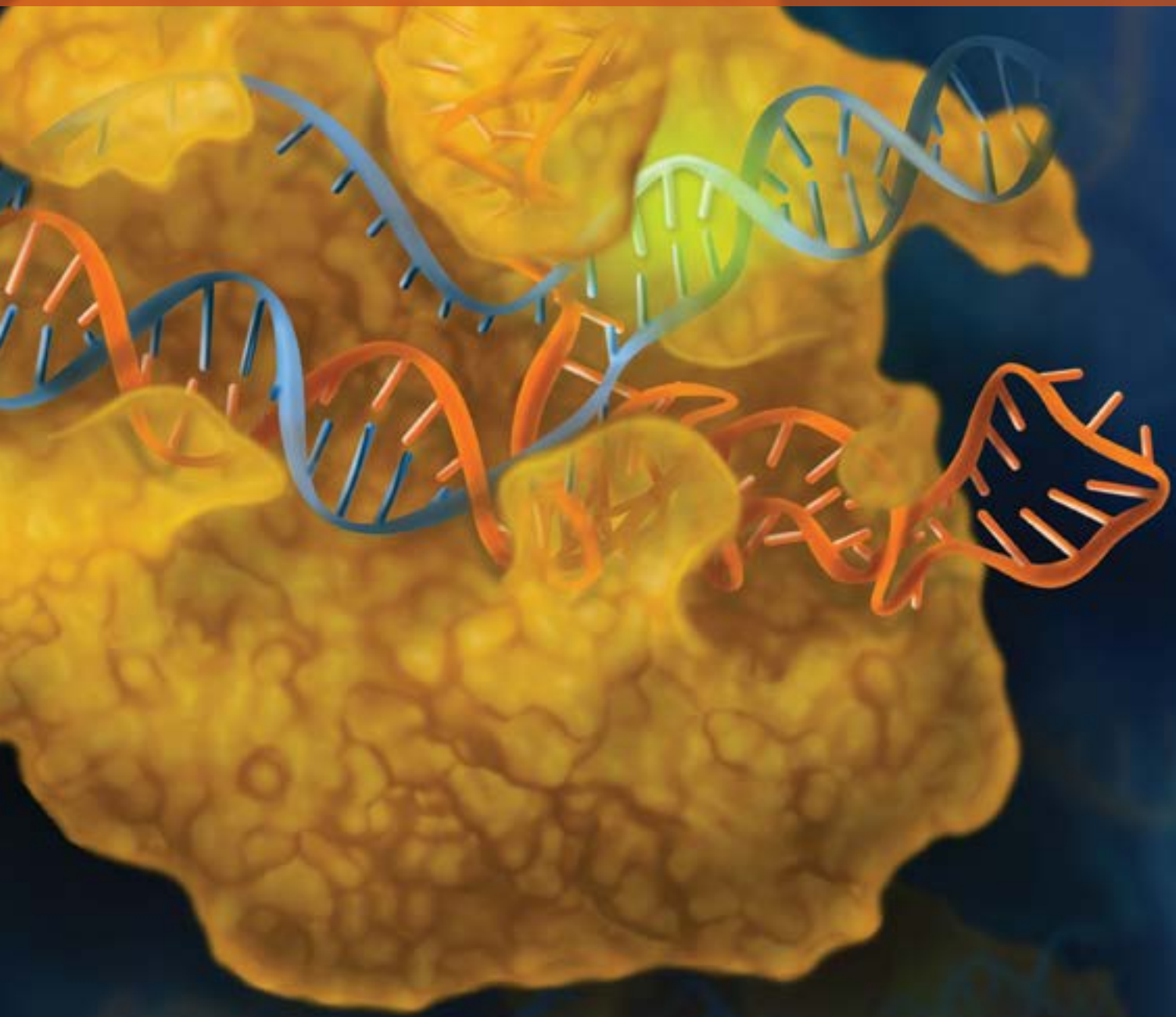
Genome Editing

TOOLS FOR CRISPR/CAS APPLICATIONS

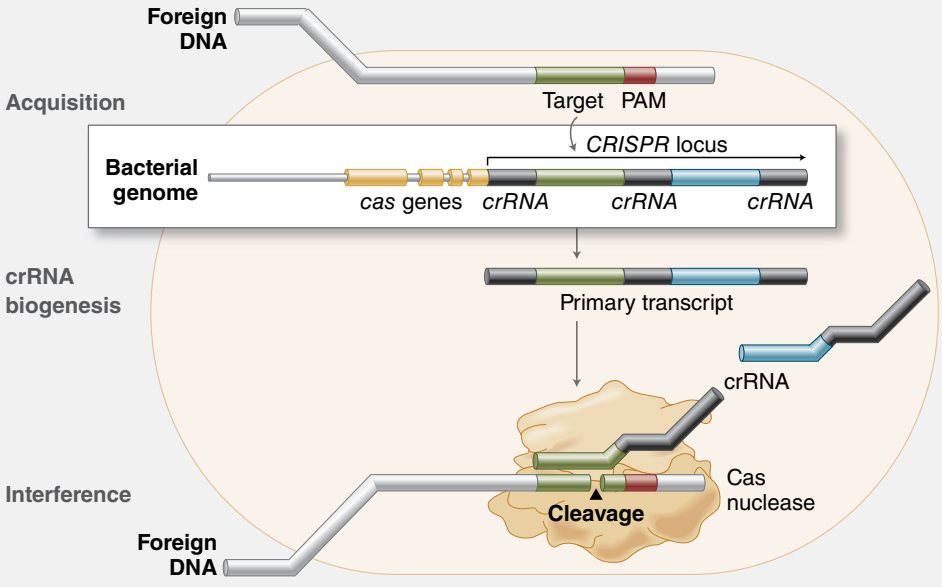


Genome Editing: Tools for CRISPR/Cas Applications

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 (2,3).

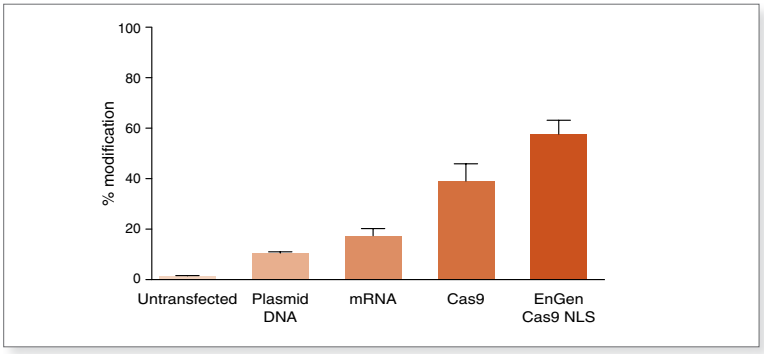
Direct Introduction of Cas RNP Complexes

The highest efficiency strategy for genome engineering with CRISPR/Cas is direct introduction of Cas9/guide RNA complexes (4-9) or Cas12a (Cpf1)/guide RNA complexes. This method further simplifies CRISPR/Cas workflows and has been reported to increase mutagenic activity (4–6) and reduce off-target editing events (4,5). NEB® provides purified Cas9 Nuclease, *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

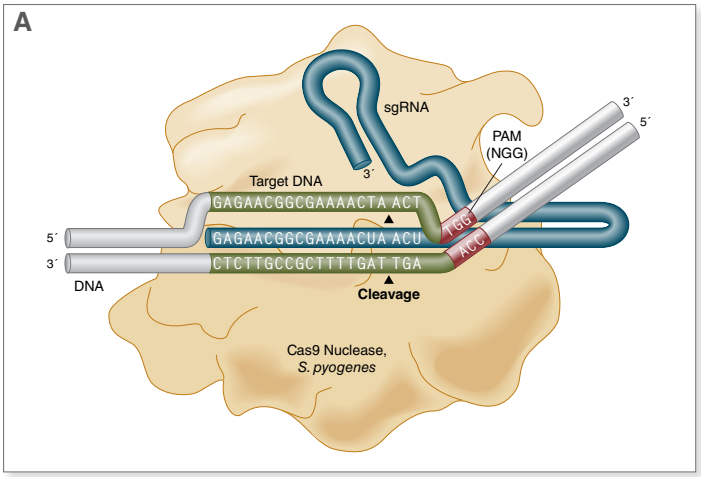
PRODUCT	VWR CAT. NO.	SIZE
EnGen Cas9 Nuclease, NLS, *S. pyogenes*	103218-918	400 pmol
	103218-920	2,000 pmol
Cas9 Nuclease, *S. pyogenes*	102877-594	70 pmol
	103218-912	300 pmol
	102902-446	600 pmol
EnGen Spy Cas9 Nickase	76196-384	70 pmol
	76196-386	400 pmol
EnGen Spy dCas9 (SNAP-tag®)	76196-390	70 pmol
	76196-392	400 pmol
EnGen Lba Cas12a (Cpf1)	76199-124	70 pmol
	76199-126	2,000 pmol

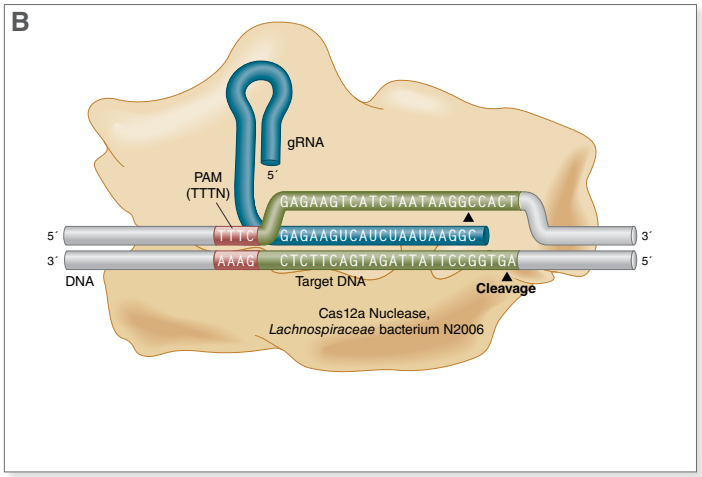
Increased genome editing efficiency using Cas9 RNP delivery



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. pyogenes* (A) and Lba Cas12a, *Lachnospiraceae* bacterium N2006 (B) sequence recognition and DNA cleavage





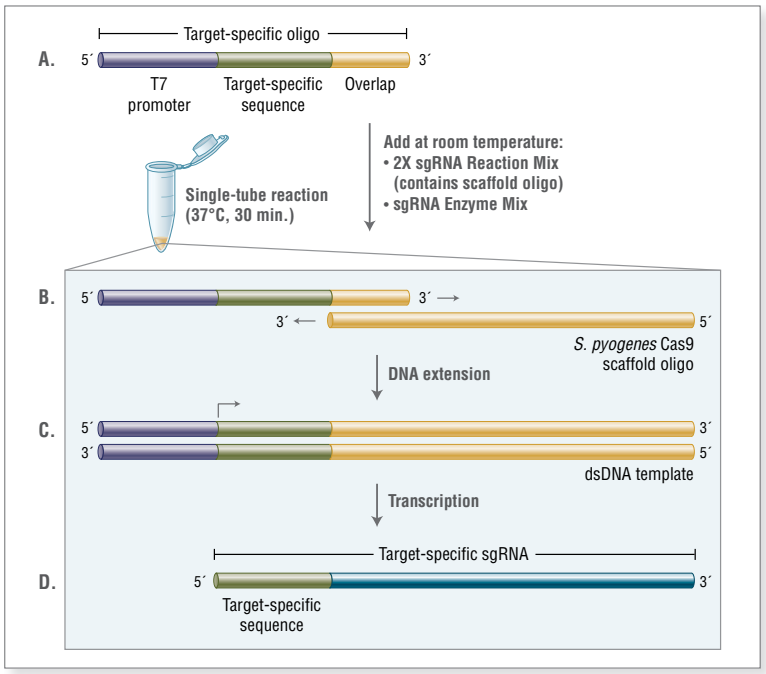
Rapid Generation of sgRNA for Spy Cas9

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 Cas9 NLS, EnGen Spy Cas9 Nickase, and EnGen Spy dCas9 (SNAP-tag).

Ordering Information

PRODUCT	VWR CAT. NO.	SIZE
EnGen sgRNA Synthesis Kit	103218-922	20 reactions

EnGen sgRNA Synthesis Kit overview



"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.

GLOSSARY

Like any new technique, genome editing with CRISPR/Cas9 comes with a list of acronyms and abbreviations. This list should help you to familiarize yourself with the language associated with CRISPR/Cas9 studies.

Cas = CRISPR-associated genes

Cas9, Cas12a = a CRISPR-associated protein that is programmed by small RNAs to cleave DNA

crRNA = CRISPR RNA

dCAS9 = nuclease-deficient Cas9

DSB = Double-Stranded Break

gRNA = guide RNA

HDR = Homology-Directed Repair

HNH = an endonuclease domain named for characteristic histidine and asparagine residues

Indel = Insertion and/or deletion

NHEJ = Non-Homologous End Joining

PAM = Protospacer-Adjacent Motif

RNP = ribonucleoprotein

RuvC = an endonuclease domain named for an *E. coli* protein involved in DNA repair

sgRNA = single guide RNA

tracrRNA = trans-activating crRNA

TALEN = Transcription-Activator Like Effector Nuclease

ZFN = Zinc-Finger Nuclease

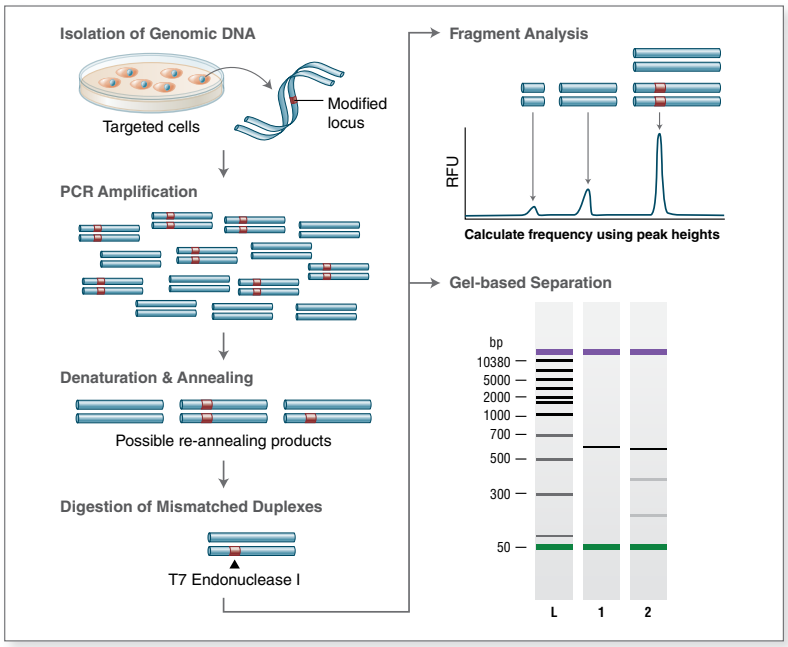
Evaluating Targeting Efficiency with the EnGen Mutation Detection Kit and T7 Endonuclease I

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.

Ordering Information

PRODUCT	VWR CAT. NO.	SIZE
EnGen Mutation Detection Kit	103049-174	25 reactions
T7 Endonuclease I	101228-382	250 units
	101228-380	1,250 units
Q5 Hot Start High-Fidelity 2X Master Mix	102500-142	100 reactions
	102500-140	500 reactions

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.

Designing Homologous Repair Templates

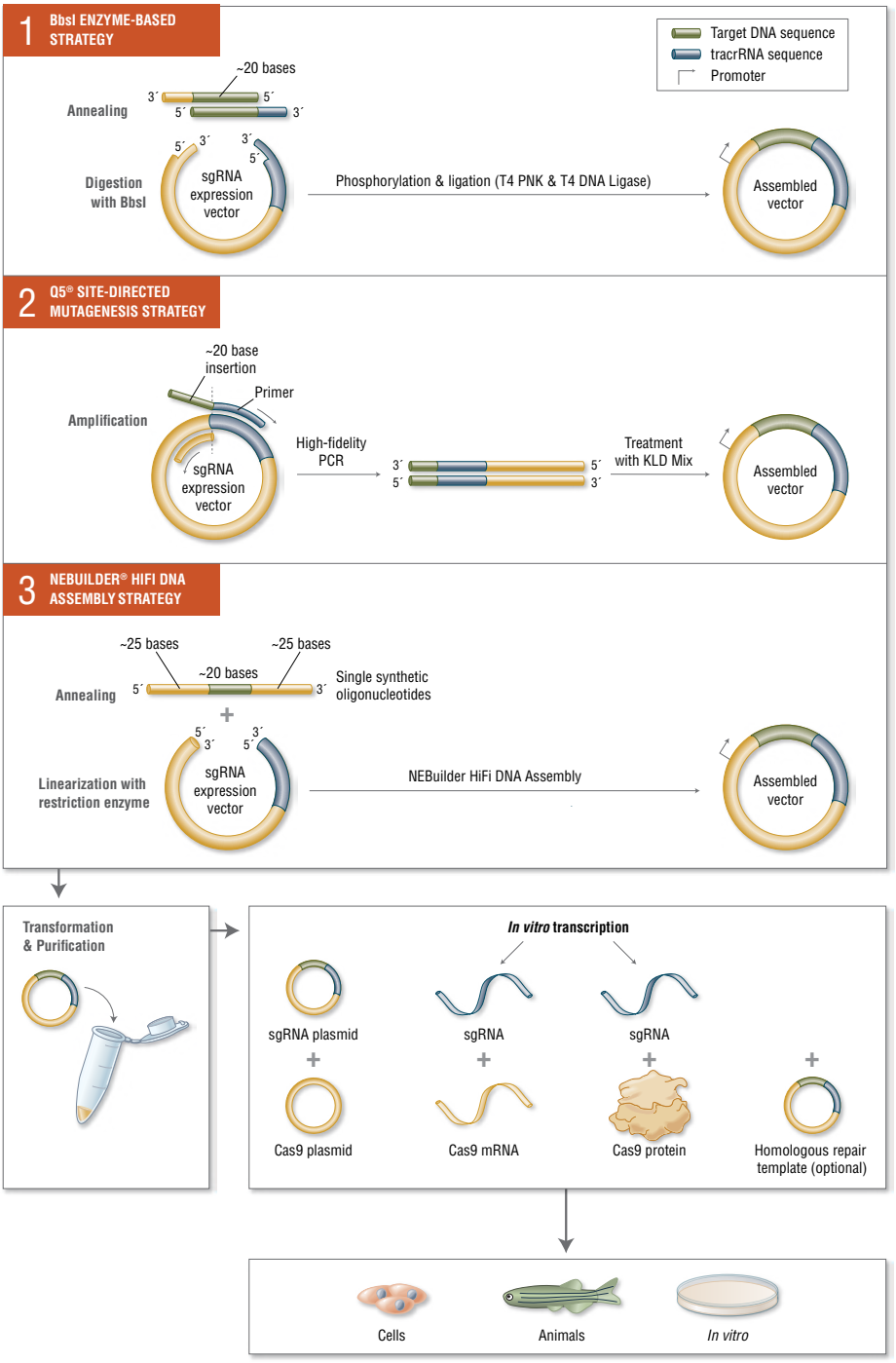
Modifications of Cas9-target sites can be achieved by supplying a homologous repair template in addition to sgRNA and Cas9. Homologous repair templates can be plasmids or oligonucleotides containing regions of homology surrounding the target sequence, that are altered to have the desired mutations. Plasmids can be used to "knock-in" larger insertions, such as selectable markers or fluorescent tags. To construct a plasmid template, the homologous region can be amplified from genomic DNA using a high-fidelity polymerase, and then cloned into your plasmid backbone. NEB suggests Q5 High Fidelity DNA Polymerase products for this amplification, followed by the NEB PCR Cloning Kit. Further modifications can then be introduced using NEBuilder HiFi DNA Assembly Master Mix or by site-directed mutagenesis, using the Q5 Site-Directed Mutagenesis Kit.

Ordering Information

PRODUCT	VWR CAT. NO.	SIZE
Q5 Hot Start High-Fidelity 2X Master Mix	102500-142	100 reactions
	102500-140	500 reactions
NEB PCR Cloning Kit	102877-564	20 reactions
NEBuilder HiFi DNA Assembly Master Mix	102877-804	10 reactions
	102877-802	50 reactions
	102877-806	250 reactions
NEBuilder HiFi DNA Assembly Cloning Kit	102877-808	10 reactions
Q5 Site-Directed Mutagenesis Kit	102855-188	10 reactions
Q5 Site-Directed Mutagenesis Kit (without Competent Cells)	102877-562	10 reactions

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	VWR CAT. NO.	SIZE
BbsI	101229-162	300 units
	101229-160	1,500 units
T4 DNA Ligase	101228-180	20,000 units
	101228-182	20,000 units
	101228-176	100,000 units
	101228-178	100,000 units
T4 Polynucleotide Kinase	101228-174	500 units
	101228-172	2,500 units

PRODUCT	VWR CAT. NO.	SIZE
Q5 Site-Directed Mutagenesis Kit	102855-188	10 reactions
Q5 Site-Directed Mutagenesis Kit (Without Competent Cells)	102877-562	10 reactions

PRODUCT	VWR CAT. NO.	SIZE
Q5 Hot Start High-Fidelity 2X Master Mix	102500-142	100 reactions
	102500-140	500 reactions
NEBuilder HiFi DNA Assembly Master Mix	102877-804	10 reactions
	102877-802	50 reactions
	102877-806	250 reactions
NEBuilder HiFi DNA Assembly Cloning Kit	102877-808	10 reactions

PRODUCT	VWR CAT. NO.	SIZE
EnGen sgRNA Synthesis Kit	103218-922	20 reactions
NEB 5-alpha Competent *E. coli* (High Efficiency)	102855-190	96 wells
	103218-956	384 wells
	101417-900	6 x 0.2 ml
	101417-898	20 x 0.05 ml
	103218-958	12 x 8-tube strips
NEB 10-beta Competent *E. coli* (High Efficiency)	101417-920	6 x 0.2 ml
	200067-180	20 x 0.05 ml
HiScribe T7 ARCA mRNA Kit	102902-442	20 reactions
HiScribe T7 ARCA mRNA Kit (with tailing)	102902-440	20 reactions
HiScribe T7 High Yield RNA Synthesis Kit	101641-558	50 reactions
HiScribe T7 Quick High Yield RNA Synthesis Kit	102855-162	50 reactions
RNA Cap Structure Analog 3´-O-Me-m^{7}G(5´) ppp(5´)G	101227-248	1 µmol
	101227-246	5 µmol
Vaccinia Capping System	101641-574	400 units

Featured NEB Products Supporting CRISPR Workflows

PRODUCT	CRISPR/CAS9 APPLICATION	VWR CAT. NO.	SIZE
EnGen Cas9 Nuclease NLS, *S. pyogenes*	*in vitro* cleavage of dsDNA. Genome engineering by direct introduction of active nuclease complexes	103218-918	400 pmol
		103218-920	2,000 pmol
EnGen Mutation Detection Kit	Determination of the targeting efficiency of genome editing protocols	103049-174	25 reactions
EnGen sgRNA Synthesis Kit	Generation of microgram quantities of custom sgRNA	103218-922	20 reactions
NEW EnGen Spy Cas9 Nickase	*In vitro* nicking of dsDNA. Genome engineering by direct introduction of active nuclease complexes	76196-384	70 pmol
		76196-386	400 pmol
NEW EnGen Spy dCas9 (SNAP-tag®)	*In vitro* binding of DNA. Compatible with SNAP-tag substrates for visualization and enrichment.	76196-390	70 pmol
		76196-392	400 pmol
NEW EnGen Lba Cas12a (Cpfl)	*In vitro* cleavage of dsDNA. Genome engineering by direct introduction of active nuclease complexes. Recognizes 5´-TTTN PAM.	76199-124	70 pmol
		76199-126	2,000 pmol
Cas9 Nuclease, *S. pyogenes*	*in vitro* cleavage of dsDNA. Genome engineering by direct introduction of active nuclease complexes	102877-594	70 pmol
		103218-912	300 pmol
		102902-446	600 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	102855-188	10 reactions
		102877-562	10 reactions
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	102877-804	10 reactions
		102877-802	50 reactions
		102877-806	250 reactions
NEBuilder HiFi DNA Assembly Cloning Kit	Single-tube, isothermal generation of the Cas9-sgRNA construct and HDR templates	102877-808	10 reactions
HiScribe T7 ARCA mRNA Kit (with or without tailing)	Generation of Cas9 mRNA with ARCA cap	102902-440	20 reactions
		102902-442	20 reactions
HiScribe T7 High Yield RNA Synthesis Kit	Generation of sgRNA and Cas9 mRNA	101641-558	50 reactions
HiScribe T7 Quick High Yield RNA Synthesis Kit	Generation of sgRNA and Cas9 mRNA	102855-162	50 reactions
T7 Endonuclease I	Determination of the targeting efficiency of genome editing protocols	101228-382	250 units
		101228-380	1,250 units

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