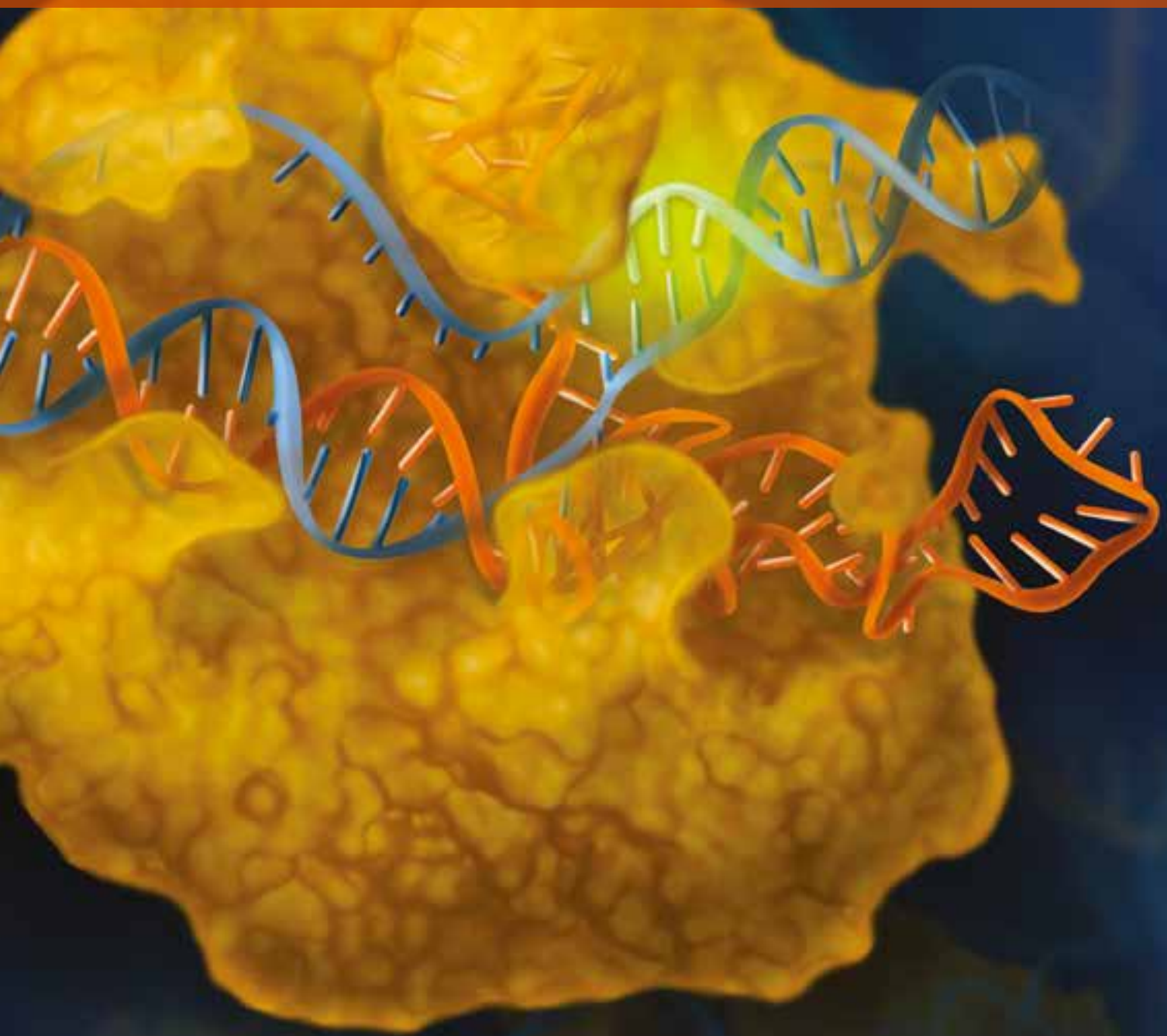
Update 2016

Genome Editing

TOOLS FOR CRISPR/CAS9 APPLICATIONS



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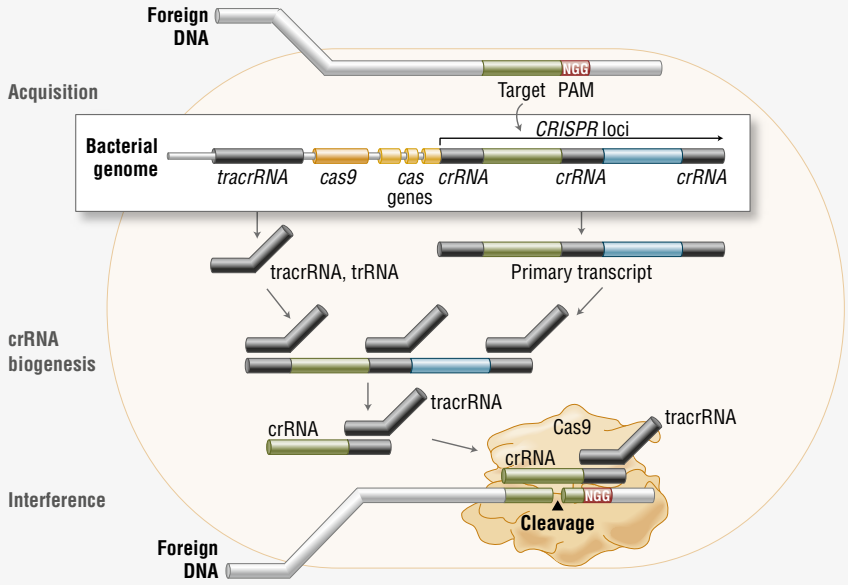
Genome Editing: Tools for CRISPR/Cas9 Applications

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. These genome editing techniques were applied concurrently with other approaches to manipulate gene function, including homologous recombination and RNA interference. RNAi, in particular, became a laboratory staple enabling inexpensive and high-throughput interrogation of gene function. However, the utility of RNAi is hampered by providing only temporary inhibition of gene function and unpredictable off-target effects. 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/Cas9 *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, Cas9 endonuclease complexed with a crRNA and separate tracrRNA cleaves foreign DNA containing a 20-nucleotide crRNA complementary sequence adjacent to the PAM sequence. (Figure not drawn to scale.)

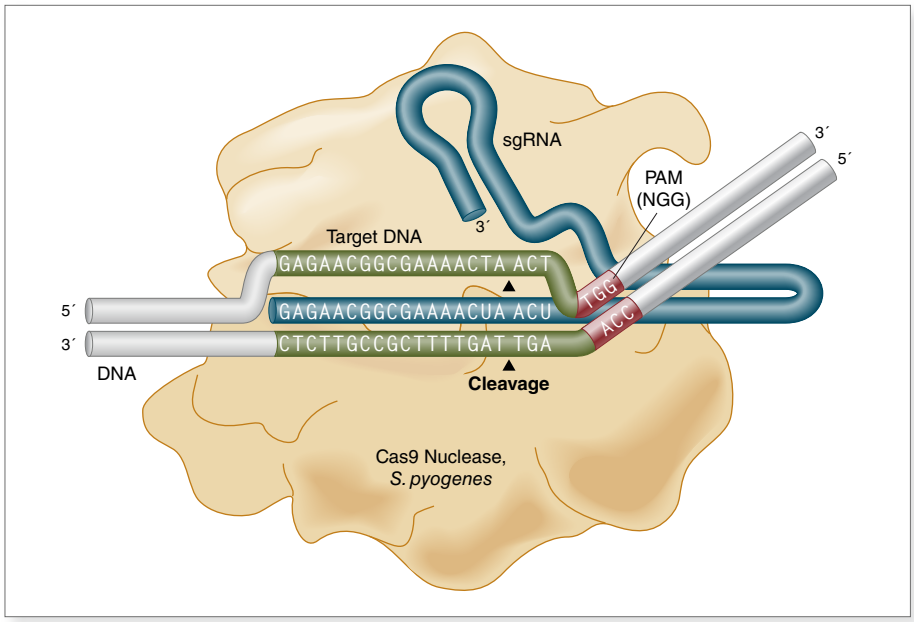
CRISPR/Cas9 Genome Editing

The simplicity of the CRISPR nuclease system, with only three components (Cas9, crRNA and tracrRNA) makes this system attractive for laboratory use. By combining the crRNA and tracrRNA into a synthetic single guide RNA (sgRNA), a further simplified two-component system can be used to introduce targeted double-stranded breaks in genomic DNA (1). 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).

TOOLS & RESOURCES

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

Schematic representation of Cas9 Nuclease, *S. pyogenes* sequence recognition and DNA cleavage



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, Csn1 = a CRISPR-associated protein containing two nuclease domains, 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

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

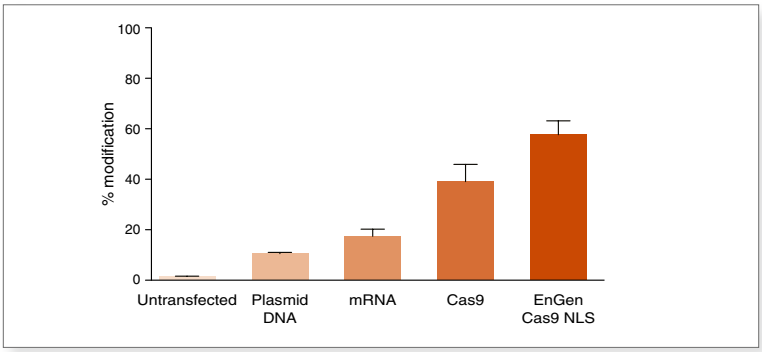
Direct Introduction of Cas9/sgRNA Complexes

The highest efficiency strategy for genome engineering with CRISPR/Cas9 is direct introduction of Cas9/guide RNA complexes (4-9). This method further simplifies CRISPR/Cas9 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* – with nuclear localization signals (NEB #M0646) and without (NEB #M0386) – as a standalone enzyme to support direct introduction of Cas9/sgRNA complexes.

Ordering Information

PRODUCT	NEB #
EnGen Cas9 Nuclease, NLS, *S. pyogenes*	M0646T/M
Cas9 Nuclease, *S. pyogenes*	M0386S/T/M

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.

Rapid Generation of sgRNA

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.

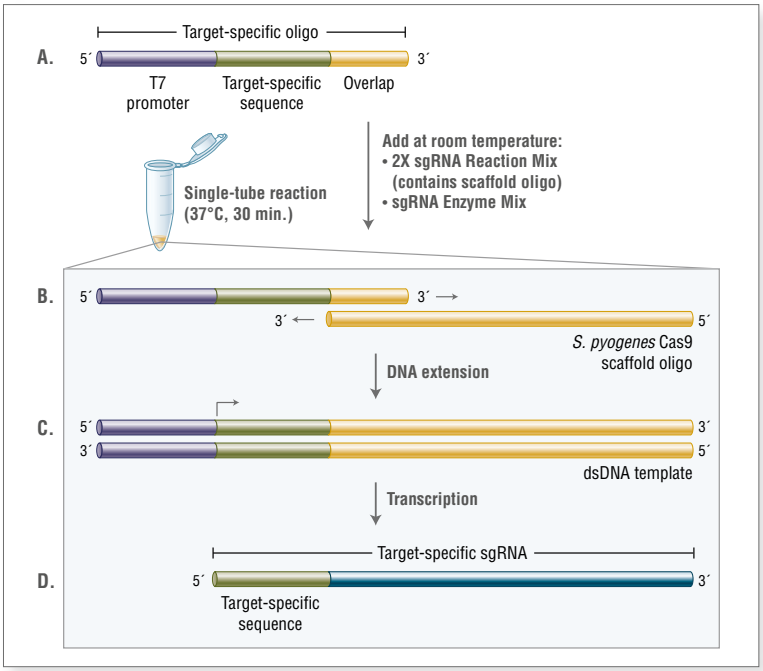
Ordering Information

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



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.

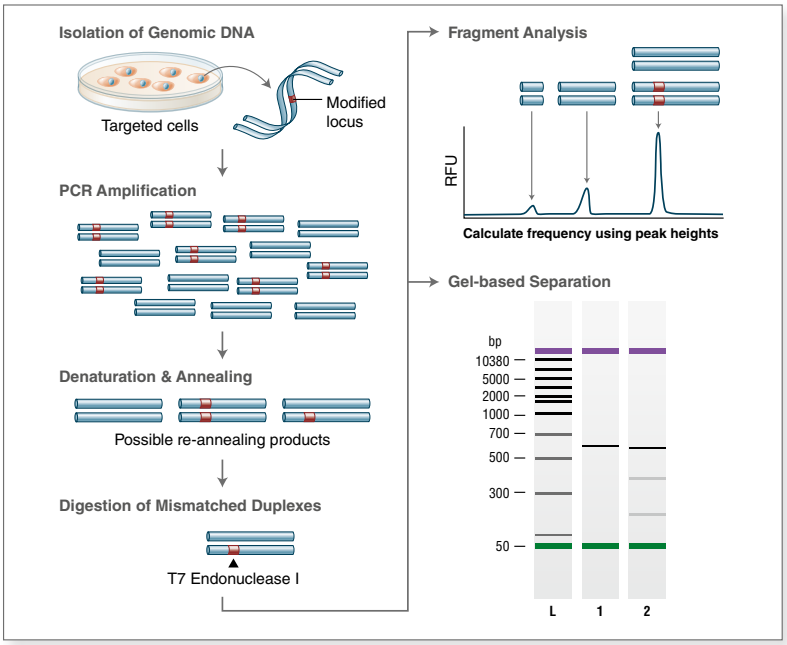
All steps occur in a single reaction during a 30-minute incubation at 37°C.

> *"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

Evaluating Targeting Efficiency with the EnGen Mutation Detection Kit

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.

ONLINE RESOURCES

Plasmid Repositories
- addgene.org

CRISPR-gRNA Design Tools
- deskgen.com
- crispr.mit.edu
- zifit.partners.org/ZiFiT
- e-crisp.org
- chopchop.rc.fas.harvard.edu
- benchling.com

Online Forums
- groups.google.com/forum/#!forum/crispr

Organism-specific Resources
- wormcas9hr.weebly.com
- flyrnai.org

Visit www.neb.com to find:

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

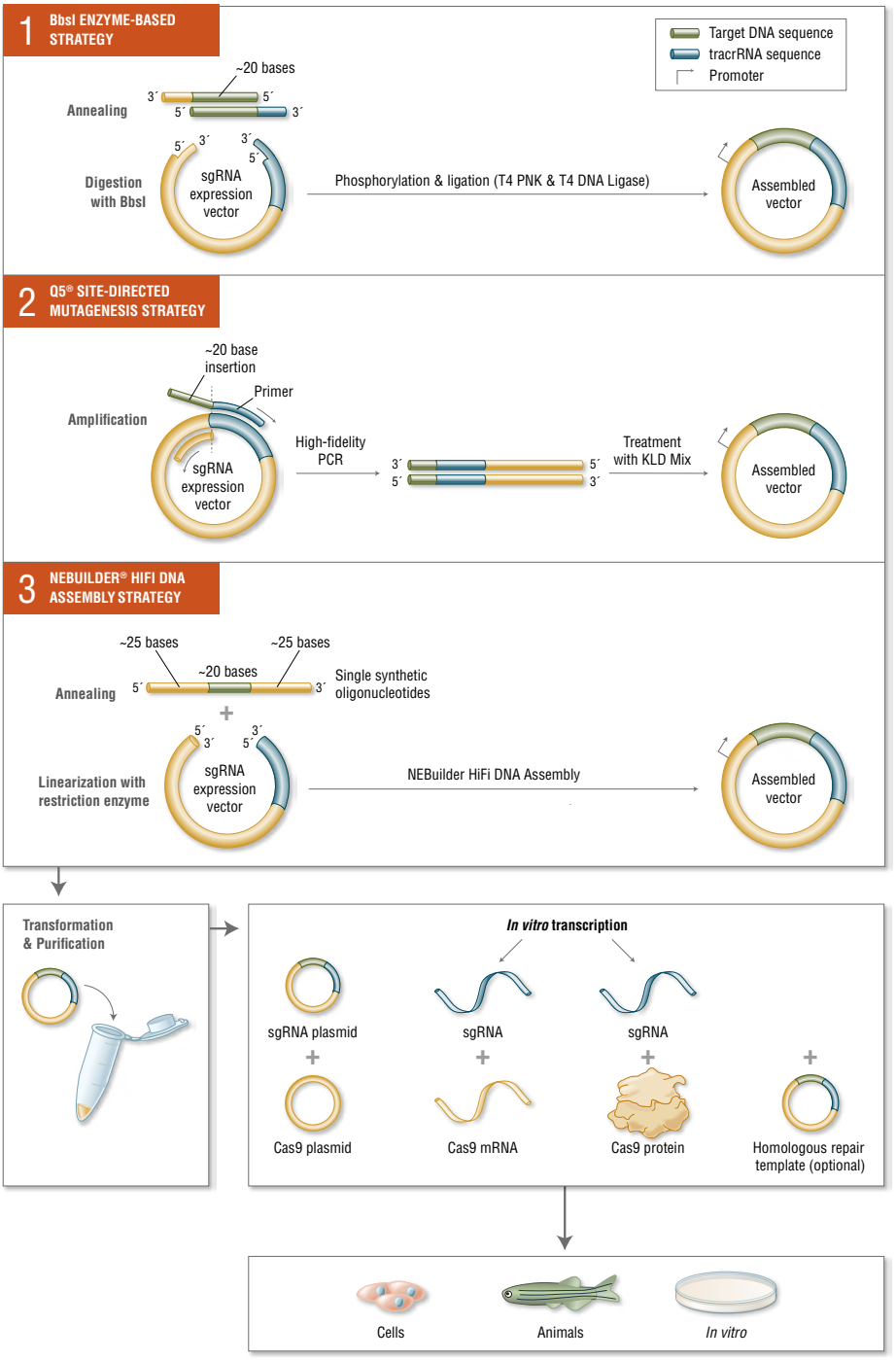
EnGen sgRNA Template Oligo Designer (accessible through **NEBioCalculator®** at **NEBiocalculator.neb.com**)

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

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

sgRNA Template Construction for CRISPR/Cas9 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 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

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 (NEB #E1202). Further modifications can then be introduced using NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621) or by site-directed mutagenesis, using the Q5 Site-Directed Mutagenesis Kit (NEB #E0554).

Ordering Information

PRODUCT	NEB #
Q5 Hot Start High-Fidelity 2X Master Mix	M0494S/L
NEB PCR Cloning Kit	E1202S
NEBuilder HiFi DNA Assembly Master Mix	E2621S/L/X
NEBuilder HiFi DNA Assembly Cloning Kit	E5520S
Q5 Site-Directed Mutagenesis Kit	E0554S
Q5 Site-Directed Mutagenesis Kit (without Competent Cells)	E0552S

Featured NEB Products Supporting CRISPR Workflows

PRODUCT NAME	CRISPR/CAS9 APPLICATION	NEB #	SIZE
NEW EnGen Cas9 Nuclease NLS, *S. pyogenes*	*in vitro* cleavage of dsDNA. Genome engineering by direct introduction of active nuclease complexes	M0646T/M	400/2,000 pmol
NEW EnGen Mutation Detection Kit	Determination of the targeting efficiency of genome editing protocols	E3321S	25 rxns
NEW EnGen sgRNA Synthesis Kit	Generation of microgram quantities of custom sgRNA	E3322S	20 rxns
Cas9 Nuclease, *S. pyogenes*	*in vitro* cleavage of dsDNA. Genome engineering by direct introduction of active nuclease complexes	M0386S/T/M	70/300/600 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	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
T7 Endonuclease I	Determination of the targeting efficiency of genome editing protocols	M0302S/L	250/1,250 units

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

References

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USA
New England Biolabs, Inc.
Telephone (978) 927-5054
Toll Free (USA Orders) 1-800-632-5227
Toll Free (USA Tech) 1-800-632-7799
Fax (978) 921-1350
www.neb.com

Canada
New England Biolabs, Ltd.
Toll Free: 1-800-387-1095
info.ca@neb.com

China, People's Republic
New England Biolabs (Beijing), Ltd.
Telephone: 010-82378265/82378266
info@neb-china.com

France
New England Biolabs France
Telephone : 0800 100 632
info.fr@neb.com

Japan
New England Biolabs Japan, Inc.
Telephone: +81 (0)3 5669 6191
info@neb-japan.com

Singapore
New England Biolabs, PTE. Ltd.
Telephone +65 6776 0903
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Featured Online 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**)

NEW ENGLAND BioLabs® GmbH

Germany & Austria
New England Biolabs GmbH
Brüningstr. 50, Geb B852
65926 Frankfurt/Main, Germany
Tel: +49/(0)69/305-23140
Fax: +49/(0)69/305-23149

Free Call: 0800/246 5227 (Germany)
Free Call: 00800/246 52277 (Austria)
info.de@neb.com
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