Recap (CRISPR): **How Bacteria Defend Against Phages**

Defense Strategies

- **Prevent attachment** → change surface proteins.
  Block DNA entry → membrane proteins stop injection.
  Stop replication → block phage DNA copying.
  Restriction–Modification (RM) → enzymes cut foreign DNA, protect own DNA.
  Cell suicide → kill itself to stop phage spread.
  CRISPR-Cas → adaptive immune system that remembers past phages.

CRISPR-Cas Immune System

What is CRISPR-Cas?

- A **genetic memory system** that helps bacteria recognize and destroy specific phages.
  CRISPR = repeated DNA sequences with "spacer" DNA from old phage attacks.
  Cas proteins = cutters that destroy matching phage DNA.

Steps in CRISPR Immunity

1. **Spacer Acquisition**
   Cas1–Cas2 capture a piece of invading phage DNA and store it as a spacer.

2. **crRNA Biogenesis**
   The CRISPR region is copied into RNA, then cut into guide RNAs (crRNAs).

3. **Interference**
   Cas protein + crRNA find matching phage DNA and cut it.

PAM Sequence

- CRISPR targets phage DNA only if a **PAM** sequence is present.
  This prevents CRISPR from cutting the bacterium's own DNA.

CRISPR Diversity

- Many types exist (Cas9 is only one example).
  Some use one protein; others use many.
  They cut DNA in different ways.

CRISPR in Genetic Disease Research

1. Studying Diseases

- **Monogenic diseases** (one gene): CRISPR can insert/remove mutations to study them.
  Polygenic diseases (many genes): CRISPR can edit **multiple genes at once**, helping study complex diseases like diabetes or schizophrenia.

2. Disease Modeling

- **Cell models** – Edit cells in lab to see how mutations affect them.
  Animal models – Edit animals (e.g., mice) to mimic human disease.
  Xenografts – Transplant human tissue into animals for testing.
  Chimeras – Animals containing human cells to study diseases more realistically.

3. Direct Gene Editing for Therapy

- Fix disease-causing mutations (e.g., sickle cell anemia).
  Edit a patient's own cells → correct mutation → return cells to patient.
  Potential future: edit embryos to prevent diseases (ethical concerns).

4. Challenges

- **Delivery** of CRISPR into the right cells.
  Off-target effects – unintended DNA cuts.
  Ethics – embryo editing, equity, long-term risks.

Off-Target Effects (Simple)

- CRISPR may accidentally cut similar DNA sequences → unwanted mutations.
  Must be minimized for safe therapy.

Engineering Plants for Desired Traits

Vocabulary (Simplified)

- **Selective breeding** – choosing the best plants to reproduce.
  Mutation – change in DNA.
  Mutagenesis – creating random mutations.
  Transgenic – plant has DNA from another organism.
  GMO – genetically modified plant (may include foreign DNA).

Traditional Methods

Selective Breeding

- Humans pick plants with good traits (big fruits, disease resistance).
  Works slowly over many generations.

Mutation Breeding

- Use radiation/chemicals to cause random mutations.
  Faster but unpredictable.

Modern Genetic Methods - Transgenic Plants (GMOs)

- Add genes from other organisms (e.g., Bt toxin gene from bacteria).
  Benefits: pest resistance, less pesticide use.

CRISPR Genome Editing

- Makes **precise** DNA changes without adding foreign DNA.
  Faster and more targeted than older methods.

Advanced CRISPR Applications in Plants

- **Bioremediation** – plants that clean pollutants (e.g., heavy metals).
  Better photosynthesis → more carbon capture.
  Biofuel production – plants modified to produce more oils/sugars.

- **Bio-materials** – create plants that produce strong fibers or polymers.
  Personalized nutrition – crops tailored for specific dietary needs.
  Climate resilience – plants resistant to heat, drought, salinity.

Ethical & Environmental Concerns

- **Unintended effects** on ecosystems.
  Loss of biodiversity if only a few edited varieties dominate.
  Gene flow – edited genes spreading to wild plants.
  Access/equity – ensuring small farmers can benefit too.

Designing an effective guide RNA (gRNA) for CRISPR requires careful consideration of several factors to ensure high on-target activity and minimal off-target effects. The design process is typically aided by specialized online software tools.

Key Design Principles

- **Target Specificity:** The gRNA sequence should be unique in the genome to prevent off-target mutations. This is assessed by performing a genome-wide search for similar sequences and using off-target scoring algorithms.
- **Presence of a PAM Site:** The target sequence must be immediately adjacent to a Protospacer Adjacent Motif (PAM) sequence. For the commonly used *Streptococcus pyogenes* Cas9 (SpCas9), the PAM sequence is 5'-NGG-3', where N is any nucleotide. The gRNA does not include the PAM sequence itself.
- **On-Target Efficiency:** The guide should be highly likely to cut at the intended site. On-target efficiency is predicted by algorithms that consider the sequence composition and position of nucleotides.
- **GC Content:** An optimal GC content (typically 40-80%) contributes to gRNA stability and efficient binding to the target DNA.
- **Sequence Length:** The standard length for the target-specific portion of the gRNA is 20 nucleotides for SpCas9. Shorter, truncated gRNAs (17-18 nt)

can sometimes reduce off-target effects while maintaining efficiency in some systems.

- **Secondary Structure:** The gRNA should avoid forming complex secondary structures or self-dimerization, as this can interfere with Cas protein binding and function.
- **Chromatin Accessibility:** The target site should be in an accessible region of chromatin (euchromatin) for the Cas protein to bind effectively.

Experimental Goal Considerations

The specific goal of the experiment influences the design strategy:

- **Gene Knockout (NHEJ):** Target a common exon early in the gene's coding sequence to increase the chance of an out-of-frame mutation and a complete loss of protein function.
- **Gene Knock-in or Point Mutation (HDR):** The gRNA cleavage site must be very close (~30 nucleotides) to the desired edit location or the repair template homology arms for efficient homology-directed repair (HDR).
- **CRISPR Activation (CRISPRa) or Interference (CRISPRi):** Guide RNAs are designed to target specific regions upstream or nearby the transcription start site (TSS) to modulate gene expression.

Recommended Workflow

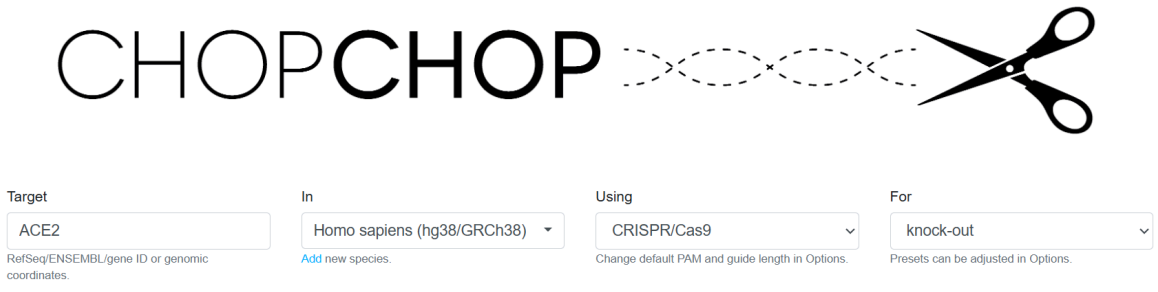
1. **Define the experimental goal** (knockout, knock-in, etc.).
2. **Identify potential gRNA sequences** near the target site that are adjacent to a PAM sequence using online design tools.
3. **Evaluate top candidates** using the on-target and off-target scores provided by the tools. Select guides with high on-target and low off-target scores.
4. **Consider using multiple gRNAs** for a single target to increase efficiency.
5. **Validate the selected gRNAs experimentally** in your specific cell system or organism to confirm efficiency and specificity.

For the most efficient design, it is highly recommended to use current bioinformatics tools such as **Benchling**, **CRISPOR**, **CHOPCHOP**, or the **Broad Institute GPP sgRNA Designer**, as they use sophisticated algorithms and large datasets to score potential gRNAs.

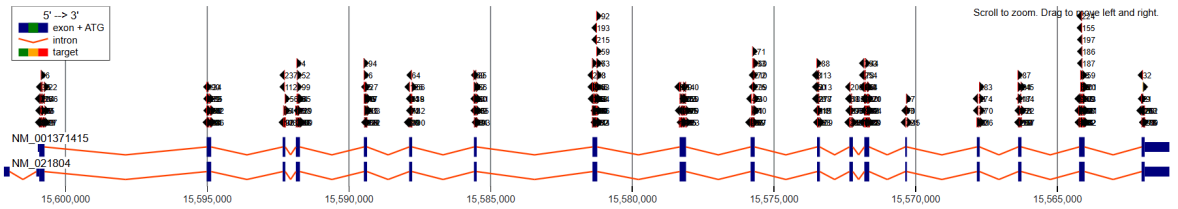
Gene editing techniques, such as CRISPR/Cas9, are used to create precise changes (like knockouts or specific point mutations) in the *ACE2* gene in cell lines and animal models. This allows scientists to:

- **Understand Normal Function:** By deactivating the gene (ACE2 knockout), researchers can study its essential roles in regulating blood pressure, heart function, kidney function, and placental development.
- **Model Diseases:** Editing the gene helps create models for studying conditions associated with ACE2 dysfunction, such as hypertension, heart failure, and diabetic nephropathy.
- **Investigate Genetic Variations:** Researchers can introduce specific single nucleotide polymorphisms (SNPs) found in the human population to understand how these natural variants affect disease susceptibility and severity, including outcomes in COVID-19 patients.

https://chopchop.cbu.uib.no/



ACE2



Download results: Please select one

View in UCSC genome browser

Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
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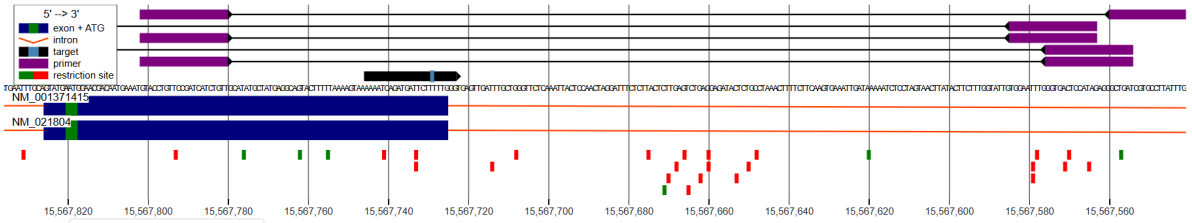
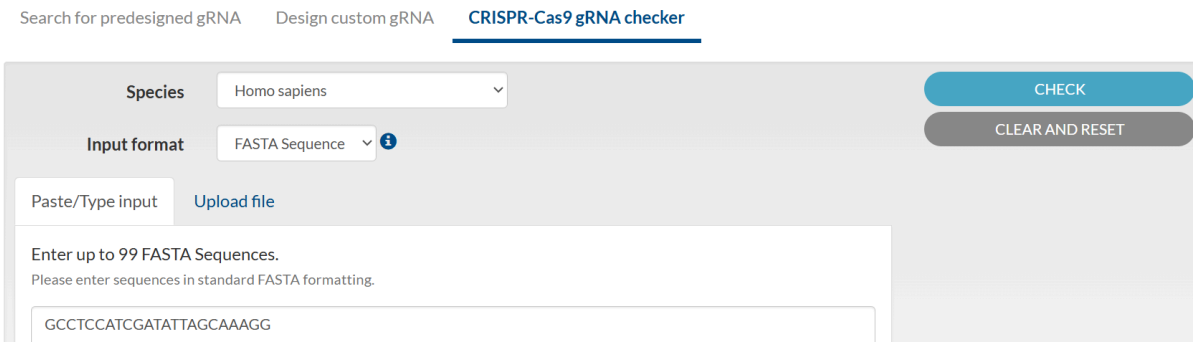


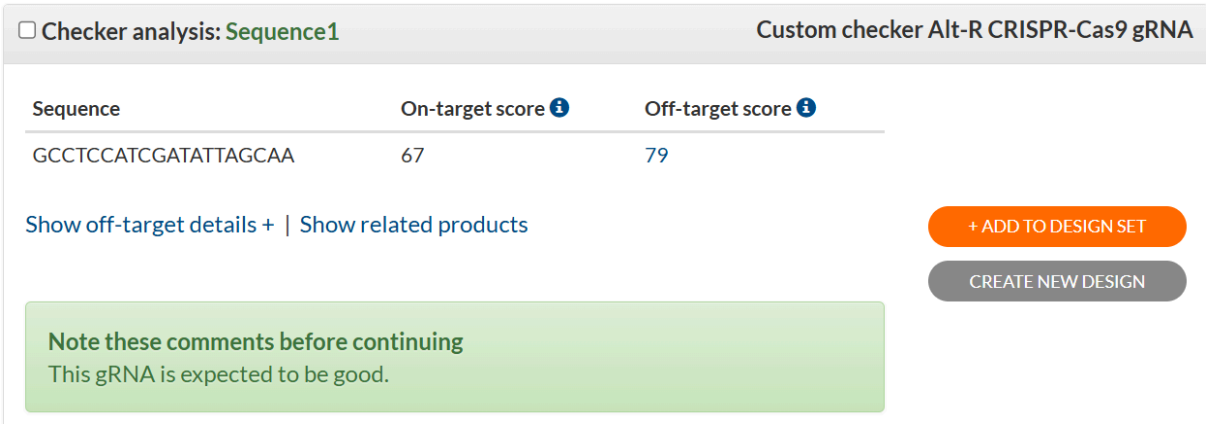
Download: Please select one

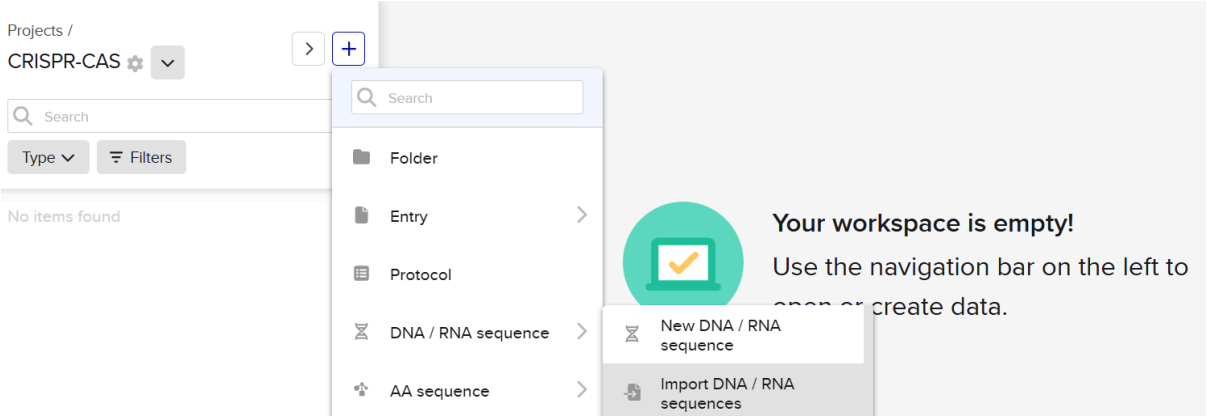
Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	chrX:15567781-15567803	TACCTGTTCCGATCATCTGTTG	60.0	0	chrX:15567555-15567577	GATCAGCCCTCTATGGAGTCAC	60.1	0	0	248

https://www.idtdna.com/site/order/designtool/index/CRISPR_PREDESIGN



Do not enter the NGG/PAM site





CREATE NEW | UPLOAD FILES | IMPORT FROM DATABASE | SELECT CHROMOSOMAL REGION

⚠ **Imports from iGEM may not work due to limitations with their parts registry. Try uploading GenBank files instead.**

Sequence

ACE2

Enter a species name for *ACE2* to search through Ensembl. If no exact matches are found, species will be ignored.

Genome

GRCh38 | Search | Clear

Create DNA / RNA sequence

This search is made possible thanks to work by Yates et. al. and Zerbino et. al.

Gene
ENSG00000130234

Species
GRCh38 (homo_sapiens)

Ensembl version
115
We'll import your data from the most recent Ensembl version.

Location
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☑ Import in sense orientation

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ACE2-201 (ENST00000252519)

Transcript
ACE2-201 (EN...

Import as
Genomic sequence | cDNA
You're about to import a genomic sequence with transcript-specific annotations.

Upstream bases 0
Downstream bases 0

Project folder*
CRISPR-CAS

Close | Import


Project
Entry
Protocol
DNA / RNA sequence
AA sequence
Oligo
Assembly
CRISPR
Entity from schema
Mixture
More
CRISPR guides
HR template
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RELEVANT ITEMS
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Create
Analyze
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Create PDF
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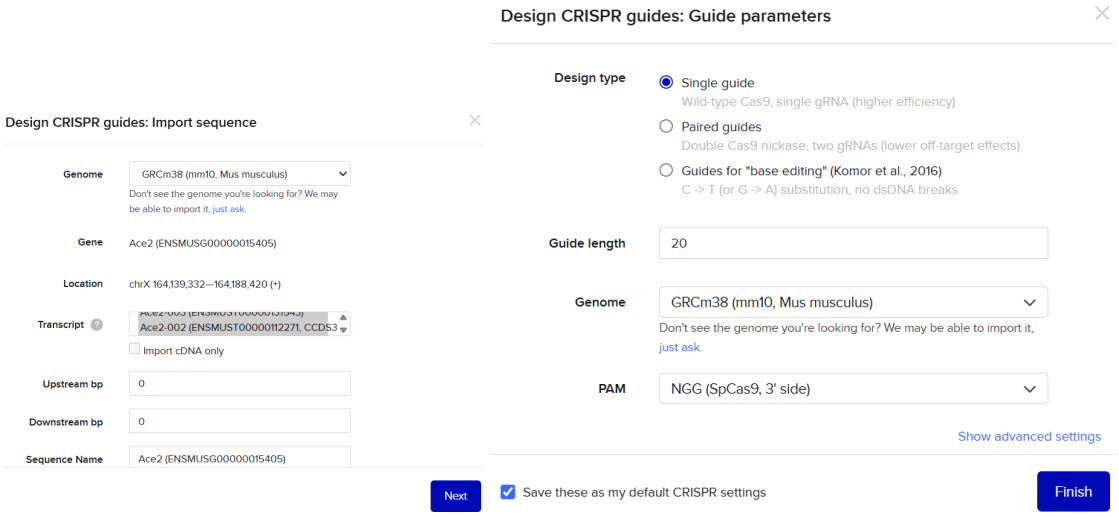
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Transcript
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Import cDNA only
Upstream bp
0
Downstream bp
0
Sequence Name
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Next
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Design type
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Wild-type Cas9, single gRNA (higher efficiency)
Paired guides
Double Cas9 nickase, two gRNAs (lower off-target effects)
Guides for "base editing" (Komor et al., 2016)
C -> T (or G -> A) substitution, no dsDNA breaks
Guide length
20
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NGG (SpCas9, 3' side)
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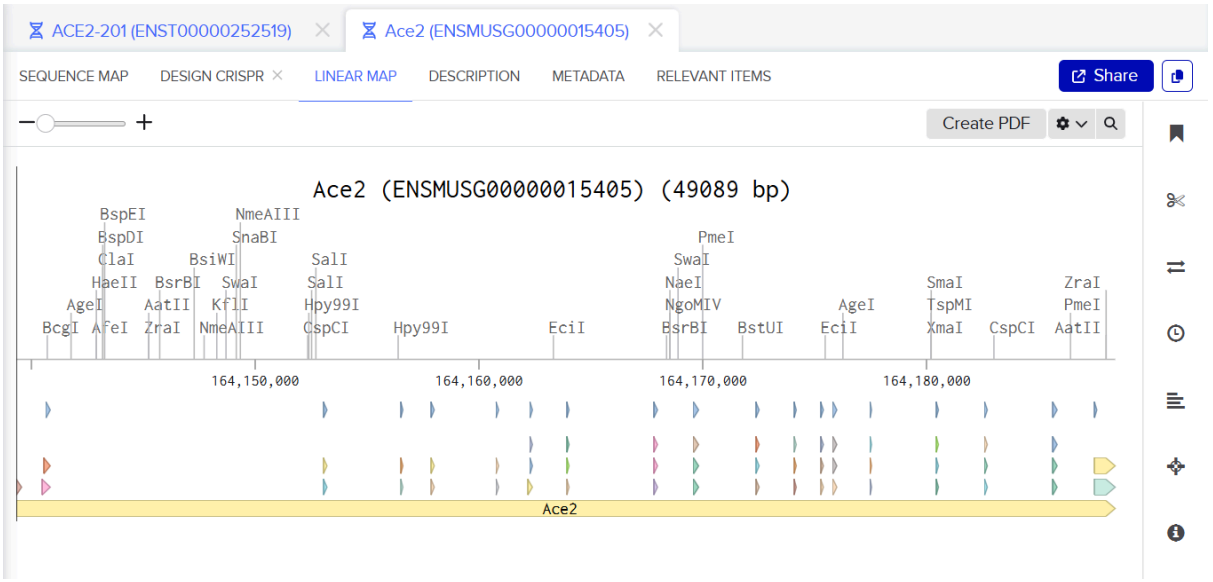
Save these as my default CRISPR settings
Finish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SnaBI
SwaI
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PmeI
AatII
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164,160,000
164,170,000
164,180,000
Ace2

For a typical gene knockout experiment, the best exon to target is generally located within the **first third of the gene's protein-coding sequence (CDS)** and is an **exon that is common to all or most splice variants**.

Why this location is best:

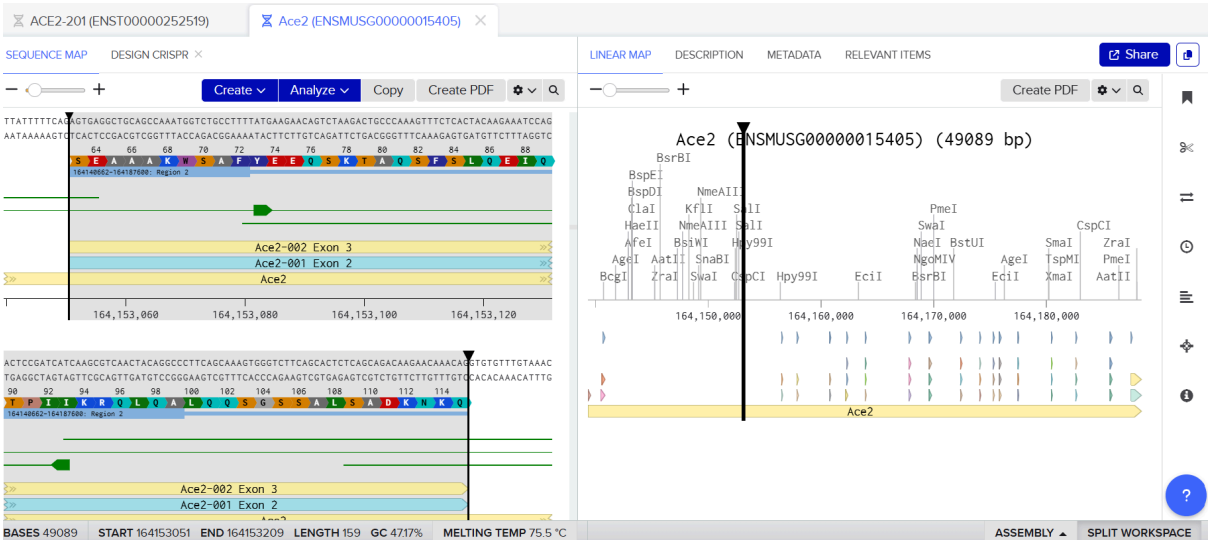
- **Maximizes the chance of a functional knockout:**
    - Targeting the 5' end ensures that any frameshift mutations (insertions or deletions, or indels) caused by the non-homologous end joining (NHEJ) repair pathway will result in a truncated, non-functional protein.
    - An indel early in the gene is likely to introduce a premature stop codon, which can trigger nonsense-mediated decay (NMD) of the mRNA, further ensuring no functional protein is produced.
    - Avoiding the extreme N-terminus (first exon) helps mitigate the potential for the cell to use an alternative start codon downstream, which could result in a partially functional protein.
    - Avoiding the C-terminus ensures that a large portion of the protein is affected, rather than just the final, potentially non-essential, part.
- **Ensures all isoforms are targeted:** Choosing an exon present in all or most transcript variants (isoforms) means that the knockout will be effective across all forms of the protein, leading to a complete loss-of-function phenotype.
- **Minimizes compensatory mechanisms:** Targeting an "asymmetrical" exon (one whose length is not a multiple of 3 base pairs) ensures that even if the cell tries to skip the exon to restore the reading frame, the skipping itself will still cause a frameshift in the remaining sequence, maintaining the knockout effect.

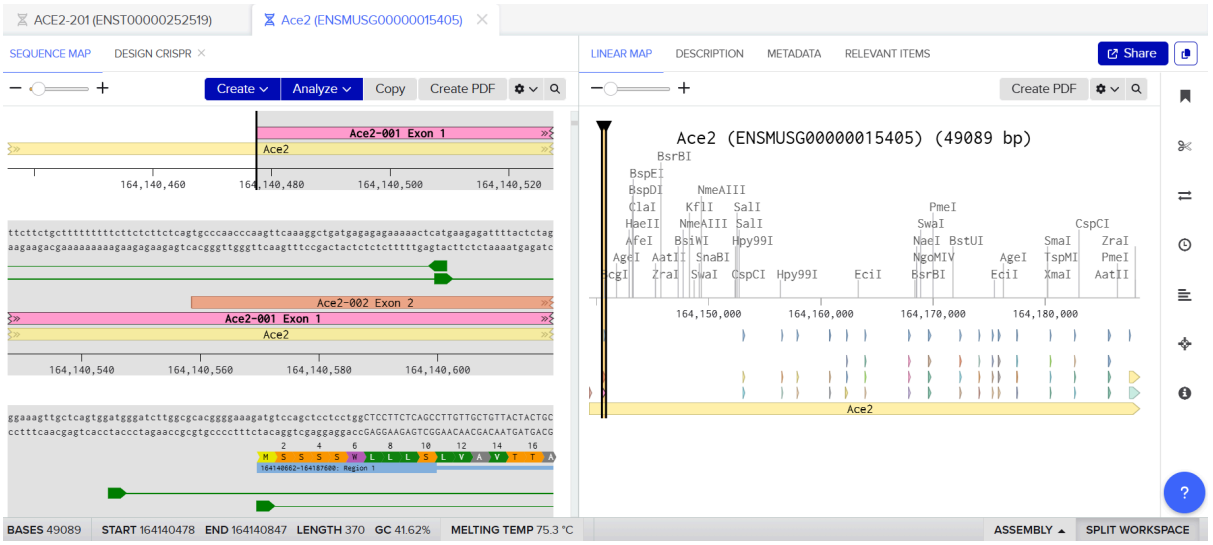
Other key design considerations:

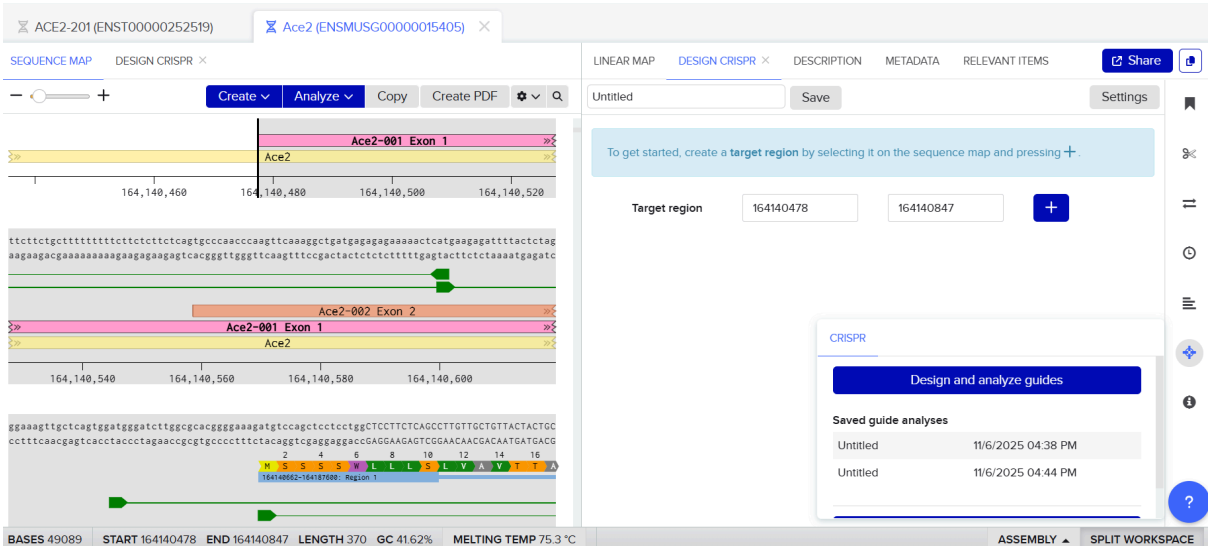
- **On-target activity and off-target effects:** Beyond location, the specific 20-nucleotide gRNA sequence must be optimized for high on-target efficiency and minimal off-target binding elsewhere in the genome.

Bioinformatics tools (like Benchling, CHOPCHOP, or Synthego's design tool) should be used for this analysis.

- **Chromatin accessibility**: The target region should be in an accessible region of the chromatin (euchromatin) for the Cas9 protein complex to bind efficiently.
- **GC content**: Aim for a GC content of 40-60% for stable gRNA-DNA binding.
- **Multiple gRNAs**: Designing and testing multiple gRNAs for a single gene (e.g., 2 or 3) is recommended to increase the success rate of achieving a complete knockout.



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164140847
CRISPR
Design and analyze guides
Saved guide analyses
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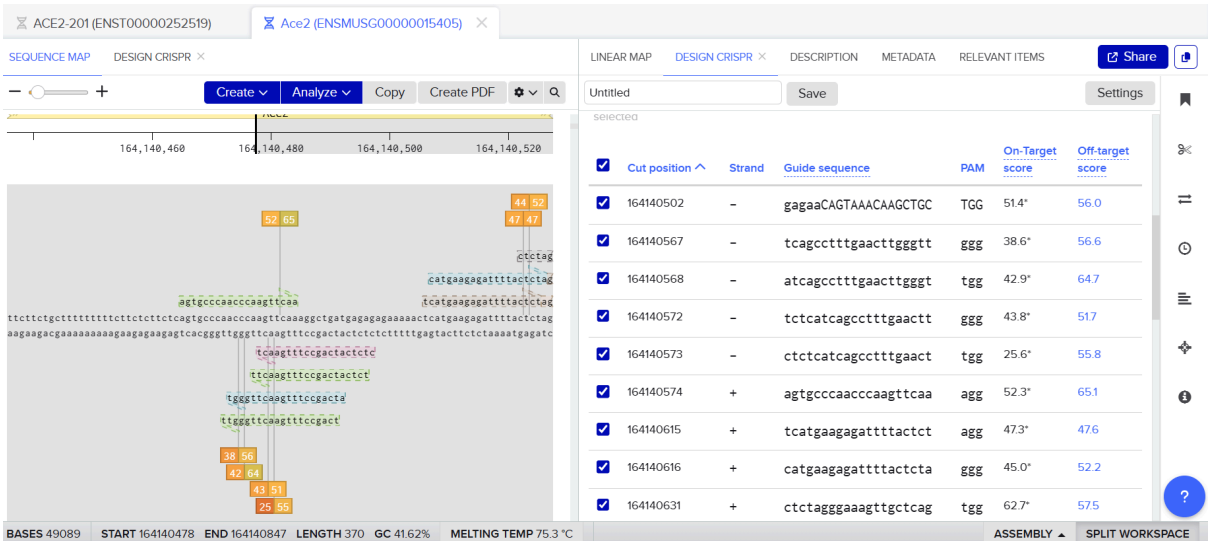
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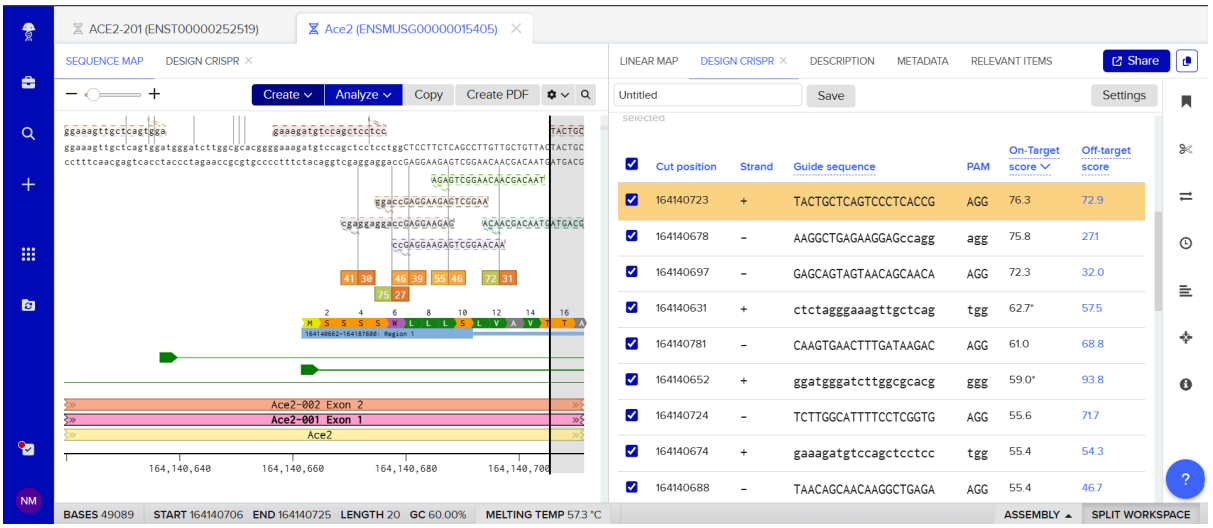
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164140631 + ctctagggaaagttgctcag tgg 62.7* 57.5
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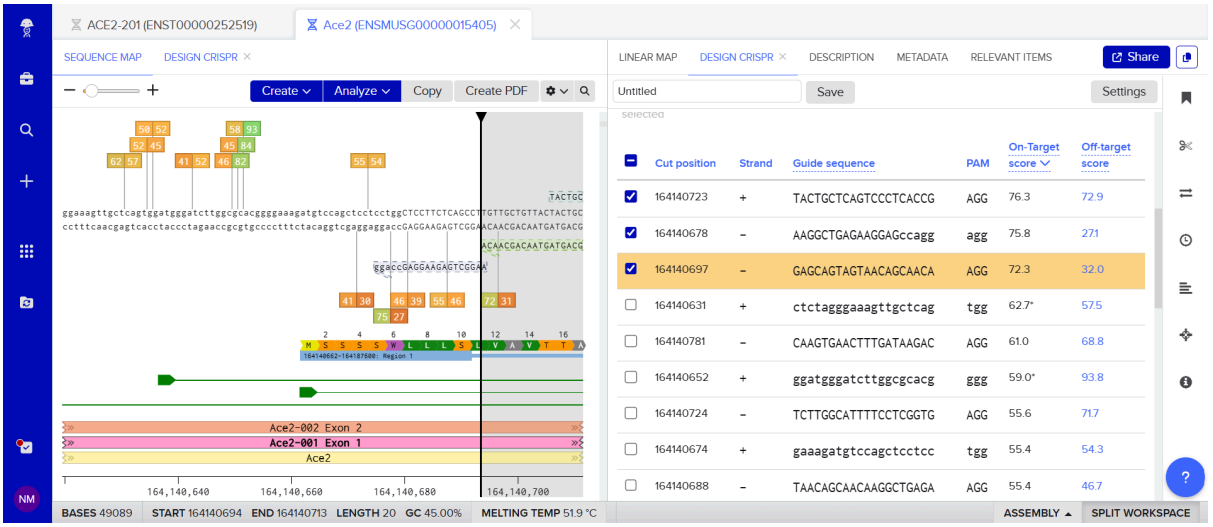
Check if this is hitting the intronic or exon region. **Routine Knockout:** Researchers typically use **2 to 4 high-quality gRNAs per gene** to ensure a high success rate and minimize the chance of false negatives.



- **Multiplexing:** For targeting multiple genes simultaneously or generating large genomic deletions, advanced cloning methods (like Golden Gate Assembly) allow for the delivery of up to **10, or even up to 30, different gRNAs** in a single vector, although efficiency may decrease with higher numbers.
- **Genome-wide Screens:** Pooled CRISPR screens use libraries containing tens of thousands of unique gRNAs to target every gene in the genome, with typically 3-6 different gRNAs per gene represented in the library pool.

- **Target Region Constraints:** For specific applications like CRISPR activ

On-Target Sites

- **Definition:** The specific, user-defined DNA sequence (typically 20 nucleotides long for Cas9) immediately upstream of a Protospacer Adjacent Motif (PAM, e.g., NGG for *S. pyogenes* Cas9) that the gRNA is designed to target.

- **Goal:** Maximize the binding and cleavage efficiency at this specific site to ensure the desired genetic modification (e.g., knockout or knock-in) occurs effectively.
- **Design Considerations:**
    - **Location:** For a gene knockout, targeting an early exon is often preferred to ensure a functional protein is not produced. For other applications (knock-in, activation/repression), the location might be more constrained, requiring a balance between location and sequence efficiency.
    - **Efficiency Score:** Bioinformatics tools provide an on-target activity score (usually 0-1), with higher scores indicating a greater likelihood of a successful edit.
    - **Chromatin Accessibility:** The target region should be in an accessible chromatin state for the Cas9 complex to bind efficiently.

Off-Target Sites

- **Definition:** Other locations in the genome that have enough sequence similarity (partial homology) to the gRNA target sequence (including the PAM) that the Cas9 complex can still bind and cleave, even with a few mismatches.
- **Concern:** Off-target activity can lead to unintended, non-specific genetic modifications, which can confound experimental results, cause unwanted phenotypes, or, in a therapeutic context, pose serious safety risks (e.g., disrupting a tumor-suppressor gene).
- **Design Considerations to Minimize Risk:**
    - **Specificity Score:** Design tools provide an off-target score, with higher scores typically indicating a lower potential for off-target activity (fewer potential off-target sites or fewer close matches).
    - **Sequence Uniqueness:** The chosen 20nt target sequence should be as unique as possible within the entire genome.

- **Mismatch Location:** Mismatches are better tolerated at the 5' end of the gRNA sequence (distal to the PAM) than at the 3' end (proximal to the PAM or "seed" region), so a good gRNA will avoid off-target sequences with perfect matches in the seed region.
- **gRNA Length/Modifications:** Using a slightly shorter gRNA (e.g., 17-18 nt) or incorporating specific chemical modifications can increase specificity.
- **Use of High-Fidelity Cas9:** Utilizing engineered high-fidelity Cas9 variants (like SpCas9-HF1 or HypaCas9) that are less tolerant of mismatches can significantly reduce off-target effects.
- **Validation:** Potential off-target sites predicted by in silico tools should be verified experimentally (e.g., via targeted deep sequencing or whole-genome sequencing) to confirm their activity or absence.

https://www.ncbi.nlm.nih.gov/nuccore/EU899414.1

Escherichia coli strain TW14359 flagellar biosynthesis protein FlhB (ECs2590) gene, complete cds

GenBank: EU899414.1

FASTA Graphics

Go to:

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  AUTHORS   Leopold,S.R., Magrini,V., Holt,N.J., Shaikh,N., Mardis,E.R.,
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  TITLE     A precise reconstruction of the emergence and constrained
            radiations of Escherichia coli O157 portrayed by backbone
            concatenomic analysis
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 106 (21), 8713-8718 (2009)
   PUBMED   19439656
REFERENCE   2  (bases 1 to 1149)
  AUTHORS   Leopold,S.R., Magrini,V., Holt,N.J., Shaikh,N., Mardis,E.R.,
            Cagno,J., Oguro,Y., Iguchi,A., Hayashi,T., Mellmann,A., Karch,H.,
            Besser,T.E., Sawyer,S.A., Whittam,T.S. and Tarr,P.I.
```

https://www.snapgene.com/plasmids/crispr_plasmids/pET28a_Cas9-Cys

SnapGene

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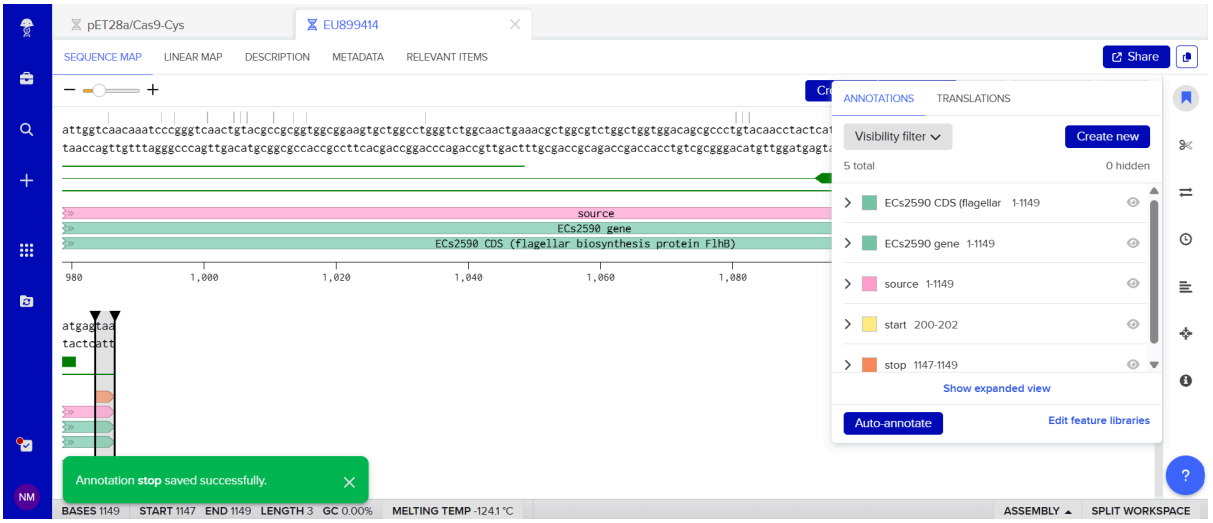
pET28a Cas9-Cys

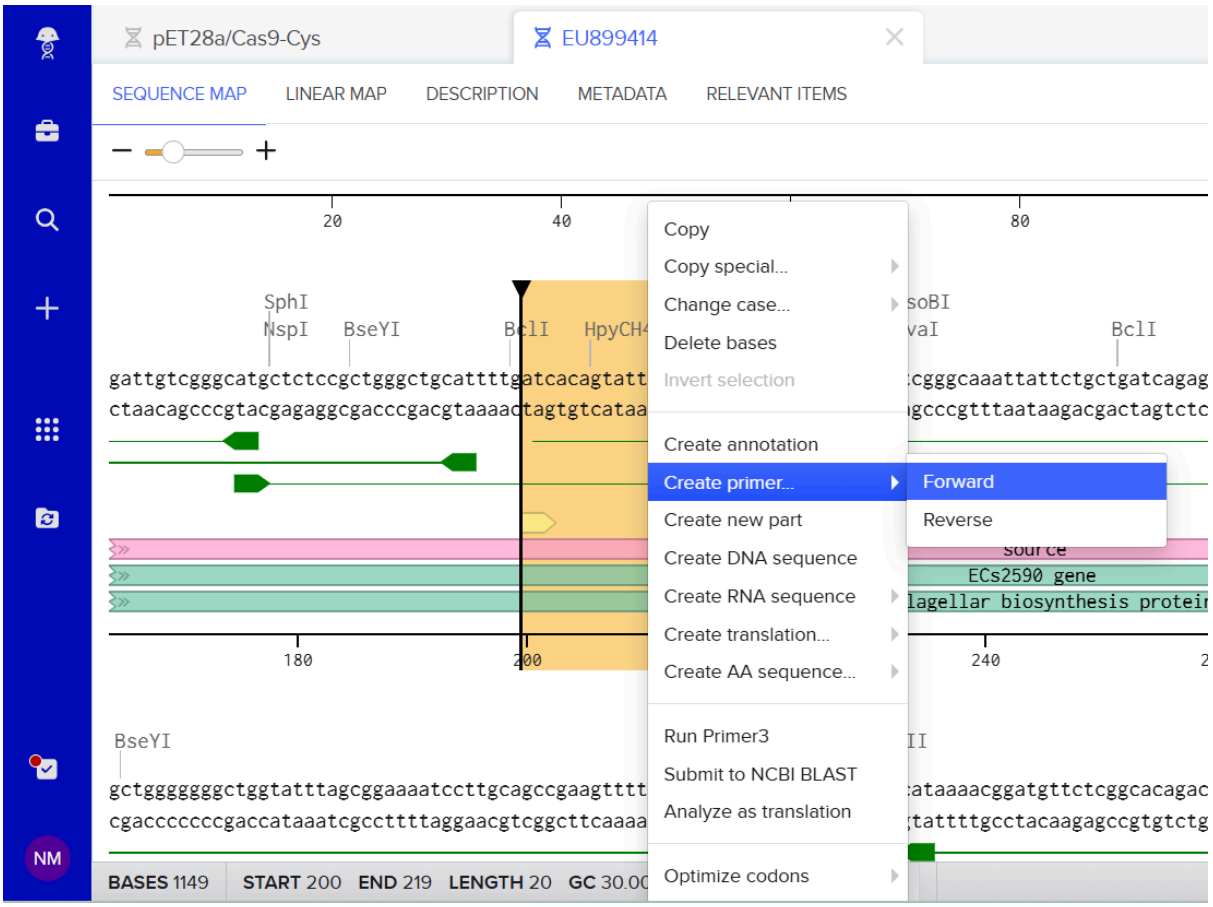
Bacterial plasmid for regulated expression of S. pyogenes Cas9 with a C-terminal cysteine.

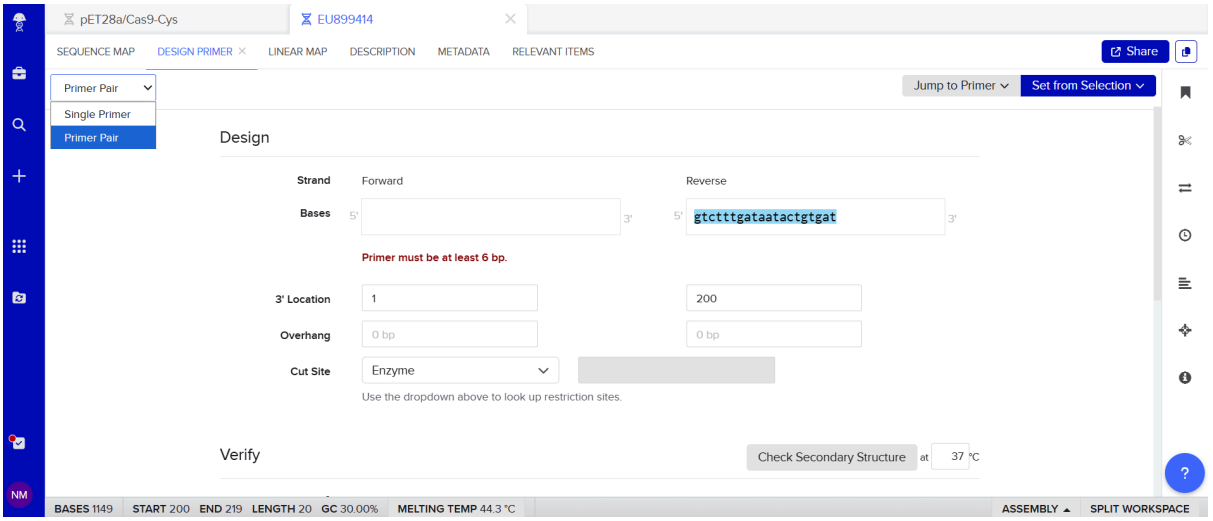
Sequence Author: Kim Lab / Addgene #53261

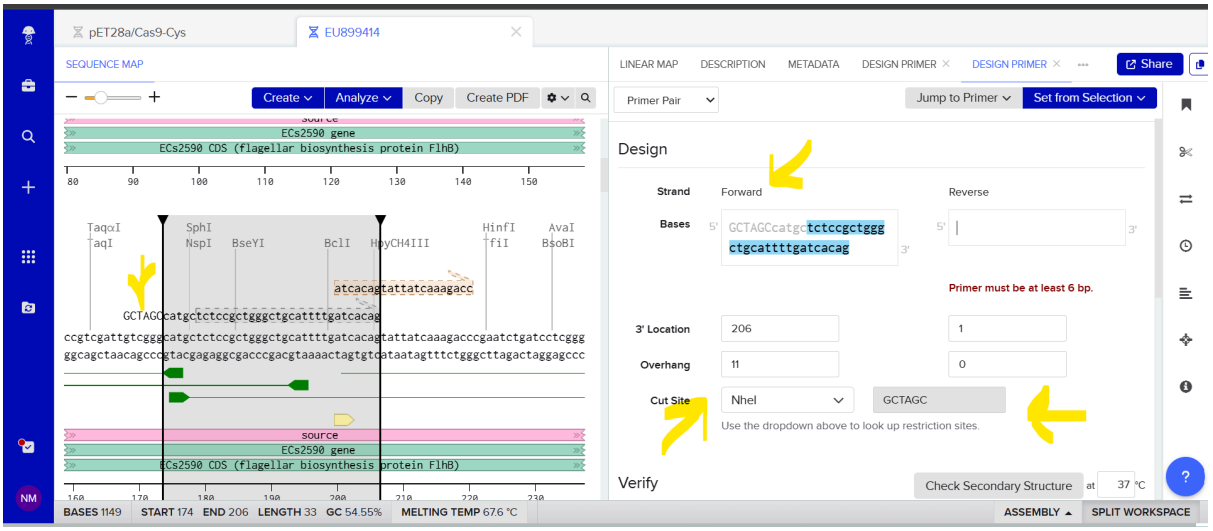
Open in SnapGene | Download SnapGene

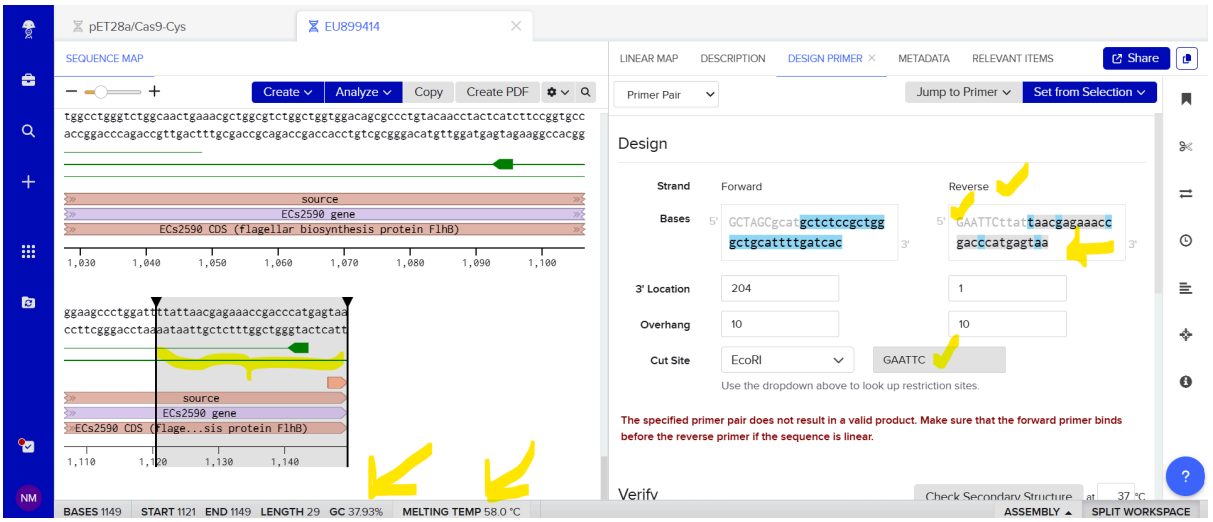
Download Plasmid

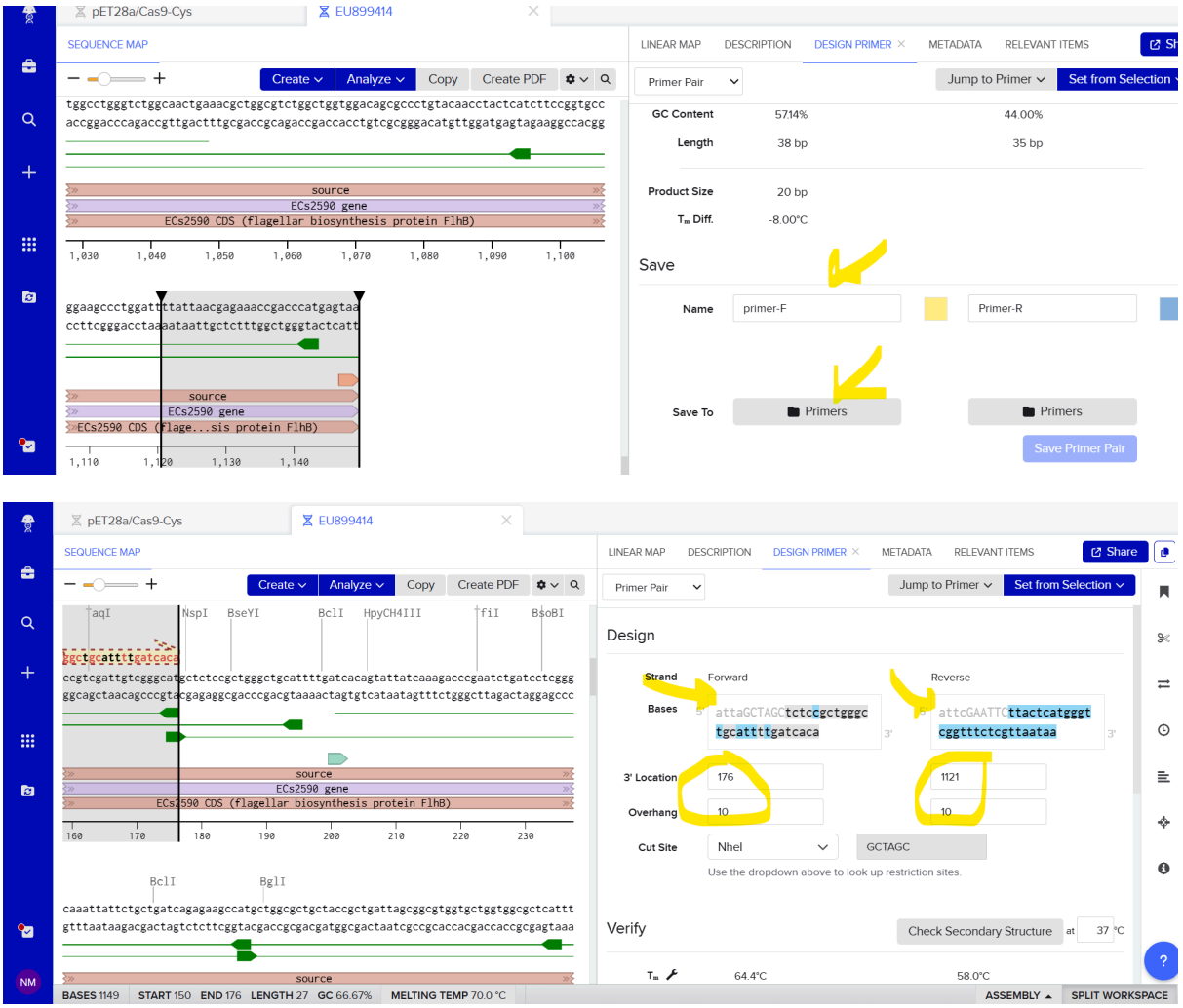

pET28a/Cas9-Cys
EU899414
SEQUENCE MAP
LINEAR MAP
DESCRIPTION
METADATA
RELEVANT ITEMS
Share
ANNOTATIONS
TRANSLATIONS
Visibility filter
Create new
5 total
0 hidden
ECs2590 CDS (flagellar 1-1149
ECs2590 gene 1-1149
source 1-1149
start 200-202
stop 1147-1149
Show expanded view
Auto-annotate
Edit feature libraries
source
ECs2590 gene
ECs2590 CDS (flagellar biosynthesis protein FlhB)
Annotation stop saved successfully.
BASES 1149 START 1147 END 1149 LENGTH 3 GC 0.00% MELTING TEMP -124.1 °C
ASSEMBLY
SPLIT WORKSPACE


pET28a/Cas9-Cys
EU899414
SEQUENCE MAP
LINEAR MAP
DESCRIPTION
METADATA
RELEVANT ITEMS
SphI
NspI
BseYI
BclI
HpyCH4
BclI
Copy
Copy special...
Change case...
Delete bases
Invert selection
Create annotation
Create primer...
Create new part
Create DNA sequence
Create RNA sequence
Create translation...
Create AA sequence...
Run Primer3
Submit to NCBI BLAST
Analyze as translation
Optimize codons
Forward
Reverse
ECs2590 gene
BseYI
BASES 1149
START 200
END 219
LENGTH 20
GC 30.00

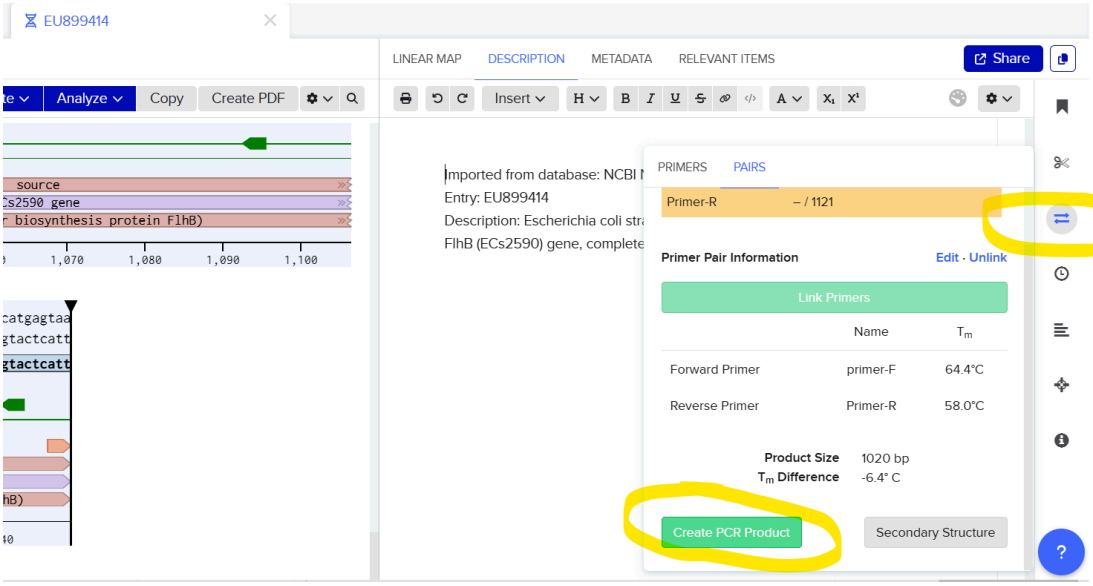

pET28a/Cas9-Cys
EU899414
SEQUENCE MAP
DESIGN PRIMER
LINEAR MAP
DESCRIPTION
METADATA
RELEVANT ITEMS
Share
Primer Pair
Single Primer
Primer Pair
Jump to Primer
Set from Selection
Design
Strand
Forward
Reverse
Bases
5'
3'
gtctttgataatactgtgat
Primer must be at least 6 bp.
3' Location
1
200
Overhang
0 bp
0 bp
Cut Site
Enzyme
Use the dropdown above to look up restriction sites.
Verify
Check Secondary Structure
at
37 °C
BASES 1149
START 200
END 219
LENGTH 20
GC 30.00%
MELTING TEMP 44.3 °C
ASSEMBLY
SPLIT WORKSPACE


pET28a/Cas9-Cys
EU899414
SEQUENCE MAP
Create
Analyze
Copy
Create PDF
ECs2590 gene
ECs2590 CDS (flagellar biosynthesis protein FlhB)
80
90
100
110
120
130
140
150
TaqαI
TaqI
SphI
NspI
BseYI
BclI
HpyCH4III
HinfI
fiI
AvaI
BsoBI
atcacagtattatcaaagacc
GCTAGCcatgctctccgctgggctgcattttgatcacag
ccgtcgattgtcgggcatgctctccgctgggctgcattttgatcacagtattatcaaagacccgaatctgatcctcggg
ggcagctaacagcccgtacgagaggcgacccgacgtaaaactagtgtcataatagtttctgggcttagactaggagccc
source
ECs2590 gene
ECs2590 CDS (flagellar biosynthesis protein FlhB)
160
170
180
190
200
210
220
230
BASES 1149
START 174
END 206
LENGTH 33
GC 54.55%
MELTING TEMP 67.6 °C
LINEAR MAP
DESCRIPTION
METADATA
DESIGN PRIMER
DESIGN PRIMER
Share
Primer Pair
Jump to Primer
Set from Selection
Design
Strand
Forward
Reverse
Bases
5'
3'
GCTAGCcatgctctccgctgggctgcattttgatcacag
Primer must be at least 6 bp.
3' Location
206
1
Overhang
11
0
Cut Site
NheI
GCTAGC
Use the dropdown above to look up restriction sites.
Verify
Check Secondary Structure
at
37 °C
ASSEMBLY
SPLIT WORKSPACE

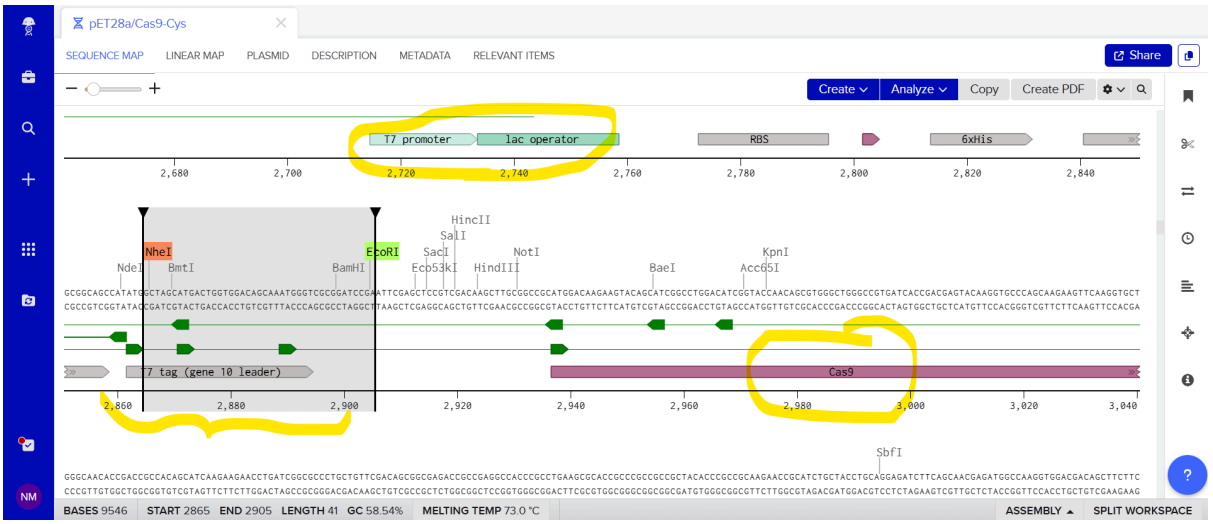

pET28a/Cas9-Cys
EU899414
SEQUENCE MAP
Create
Analyze
Copy
Create PDF
tggcctgggtctggcaactgaaacgctggcgtctggctggtggacagcgccctgtacaacctactcatcttccggtgcc
accggacccagaccgttgactttgcgaccgcagaccgaccacctgtcgcgggacatgttggatgagtagaaggccacgg
source
ECs2590 gene
ECs2590 CDS (flagellar biosynthesis protein FlhB)
1,030
1,040
1,050
1,060
1,070
1,080
1,090
1,100
ggaagccctggatttttattaacgagaaaccgacccatgagtaa
ccttcgggacctaaaataattgctctttggctgggtactcatt
source
ECs2590 gene
ECs2590 CDS (flage...sis protein FlhB)
1,110
1,120
1,130
1,140
BASES 1149
START 1121
END 1149
LENGTH 29
GC 37.93%
MELTING TEMP 58.0 °C
LINEAR MAP
DESCRIPTION
DESIGN PRIMER
METADATA
RELEVANT ITEMS
Share
Primer Pair
Jump to Primer
Set from Selection
Design
Strand
Forward
Reverse
Bases
5'
3'
GCTAGCgcatgctctccgctgg
gctgcattttgatcac
GAATTCttattaacgagaaaacc
gacccatgagtaa
3' Location
204
1
Overhang
10
10
Cut Site
EcoRI
GAATTC
Use the dropdown above to look up restriction sites.
The specified primer pair does not result in a valid product. Make sure that the forward primer binds before the reverse primer if the sequence is linear.
Verify
Check Secondary Structure
at
37 °C
ASSEMBLY
SPLIT WORKSPACE

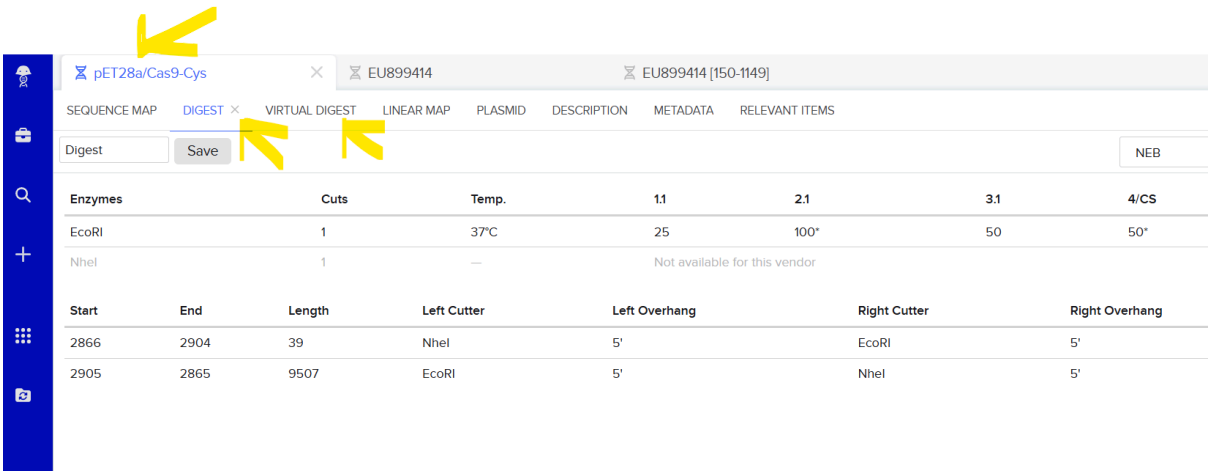


A leader sequence (atta/attc) in primer design is a small number of additional base pairs added to the 5' end of a primer to facilitate its function, most commonly to help a restriction enzyme cut more efficiently when cloning. This sequence of 3-6 nucleotides typically includes a restriction enzyme site and is positioned before the region of the primer that will bind to the DNA template.


EU899414
LINEAR MAP
DESCRIPTION
METADATA
RELEVANT ITEMS
Share
Analyze
Copy
Create PDF
Insert
source
Cs2590 gene
r biosynthesis protein FlhB)
1,070
1,080
1,090
1,100
Imported from database: NCBI
Entry: EU899414
Description: Escherichia coli str
FlhB (ECs2590) gene, complete
PRIMERS
PAIRS
Primer-R
– / 1121
Primer Pair Information
Edit · Unlink
Link Primers
Name
Tm
Forward Primer
primer-F
64.4°C
Reverse Primer
Primer-R
58.0°C
Product Size
1020 bp
Tm Difference
-6.4° C
Create PCR Product
Secondary Structure

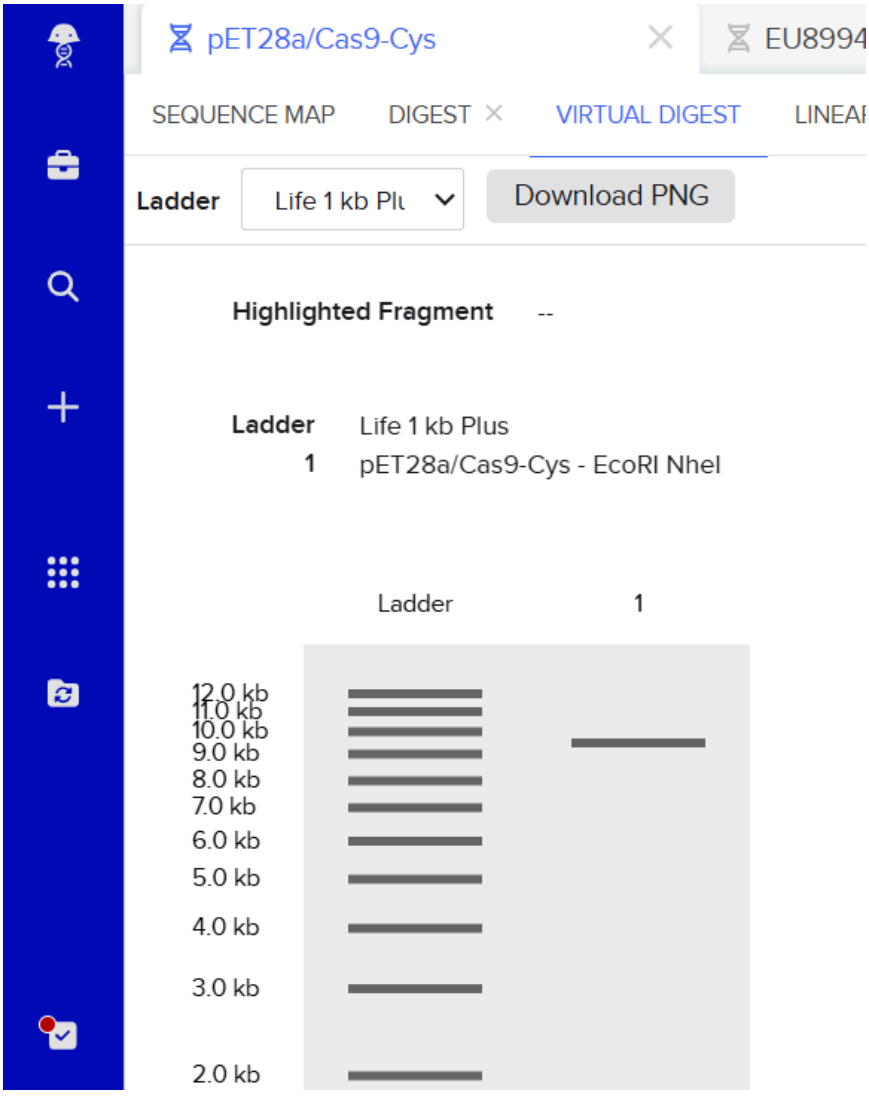

pET28a/Cas9-Cys
SEQUENCE MAP
LINEAR MAP
PLASMID
DESCRIPTION
METADATA
RELEVANT ITEMS
Share
Create
Analyze
Copy
Create PDF
NcoI
RBS
6xHis
NheI
EcoRI
BamHI
SacI
SalI
HincII
NdeI
BmtI
Eco53kI
thro...ite
T7 tag (gene 10 leader)
pET28a/Cas9-Cys (9546 bp)
BASES 9546
INSERT 2905
ASSEMBLY
SPLIT WORKSPACE

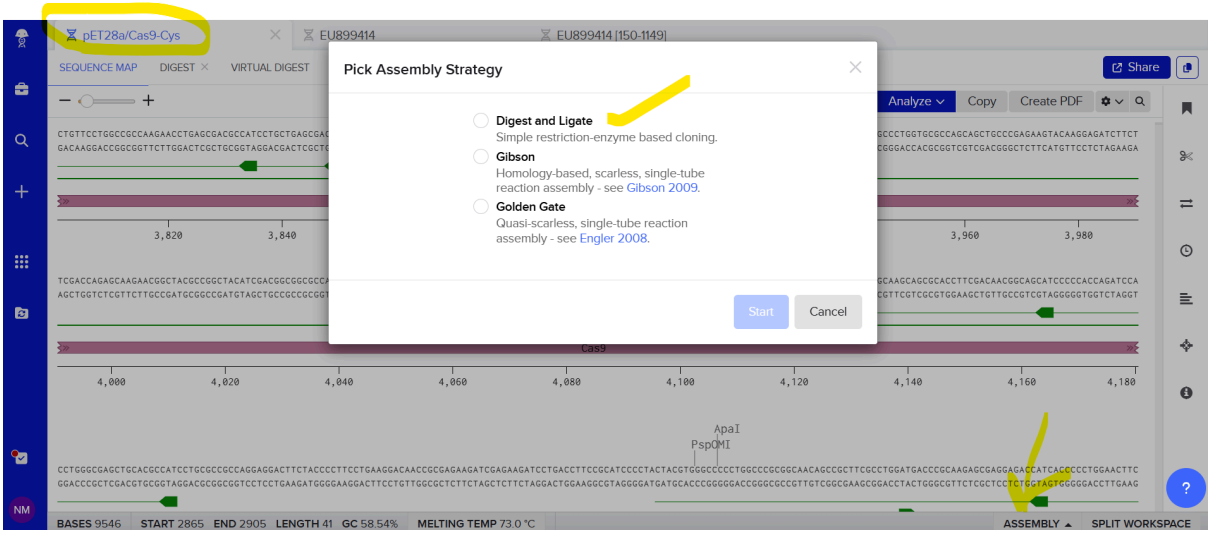

pET28a/Cas9-Cys
SEQUENCE MAP
LINEAR MAP
PLASMID
DESCRIPTION
METADATA
RELEVANT ITEMS
Share
Create
Analyze
Copy
Create PDF
T7 promoter
lac operator
RBS
6xHis
NheI
EcoRI
BamHI
SacI
HincII
SalI
NotI
HindIII
BaeI
KpnI
Acc65I
T7 tag (gene 10 leader)
Cas9
SbfI
BASES 9546
START 2865
END 2905
LENGTH 41
GC 58.54%
MELTING TEMP 73.0 °C
ASSEMBLY
SPLIT WORKSPACE

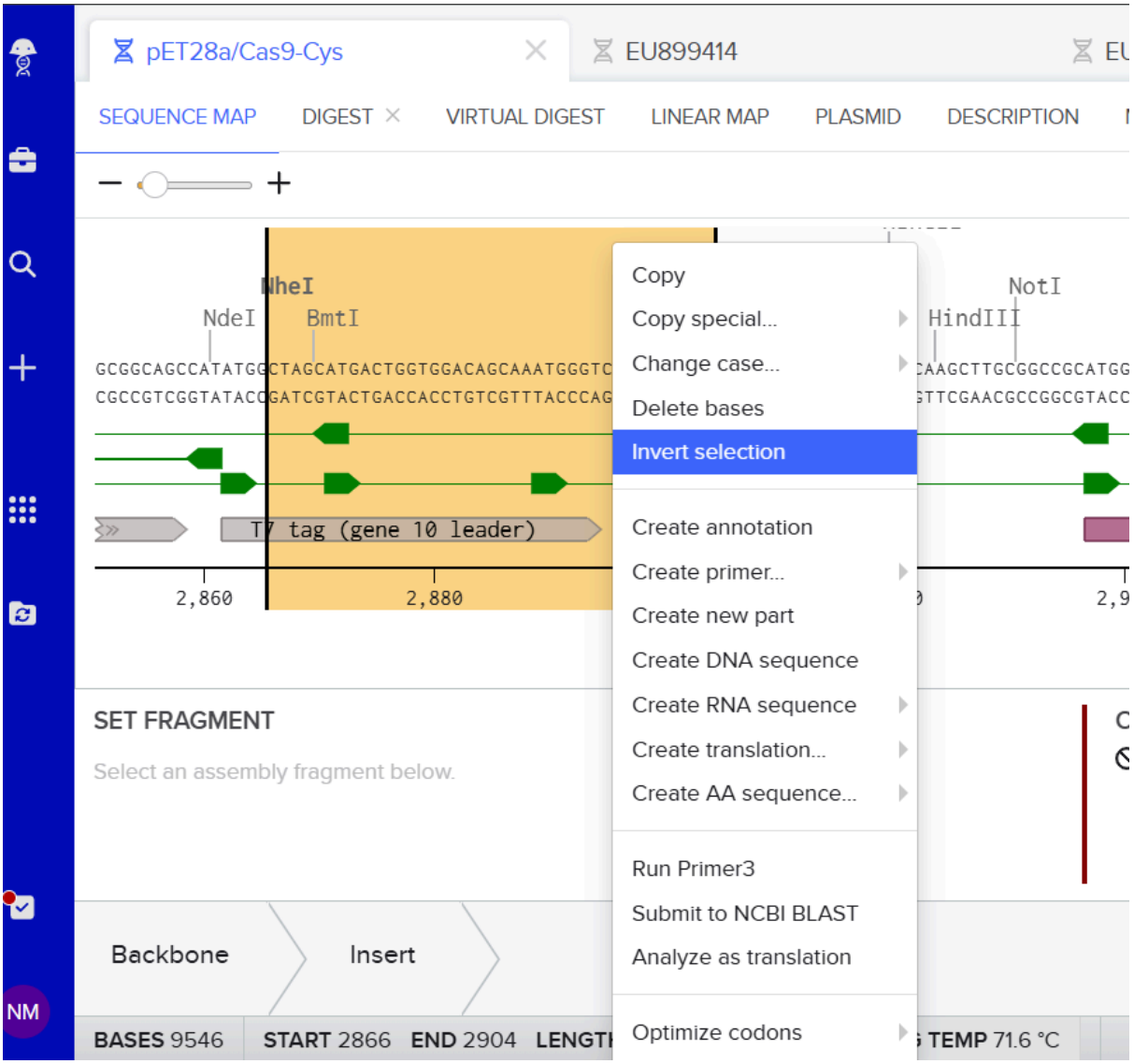


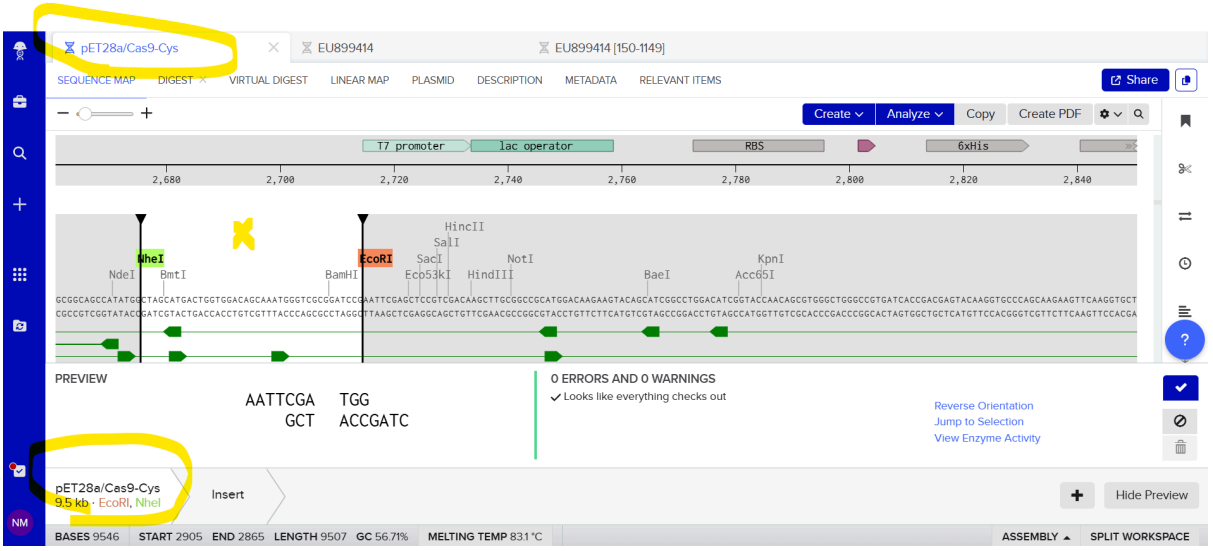
Enzymes	Cuts	Temp.	1.1	2.1	3.1	4/CS
EcoRI	1	37°C	25	100*	50	50*
NheI	1	—	Not available for this vendor			

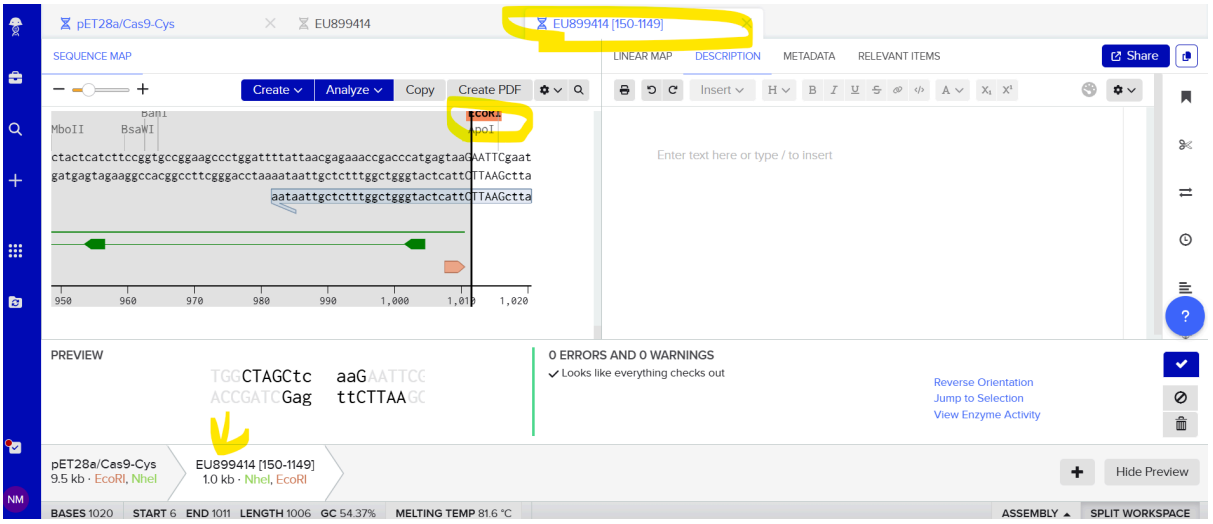
Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
2866	2904	39	NheI	5'	EcoRI	5'
2905	2865	9507	EcoRI	5'	NheI	5'

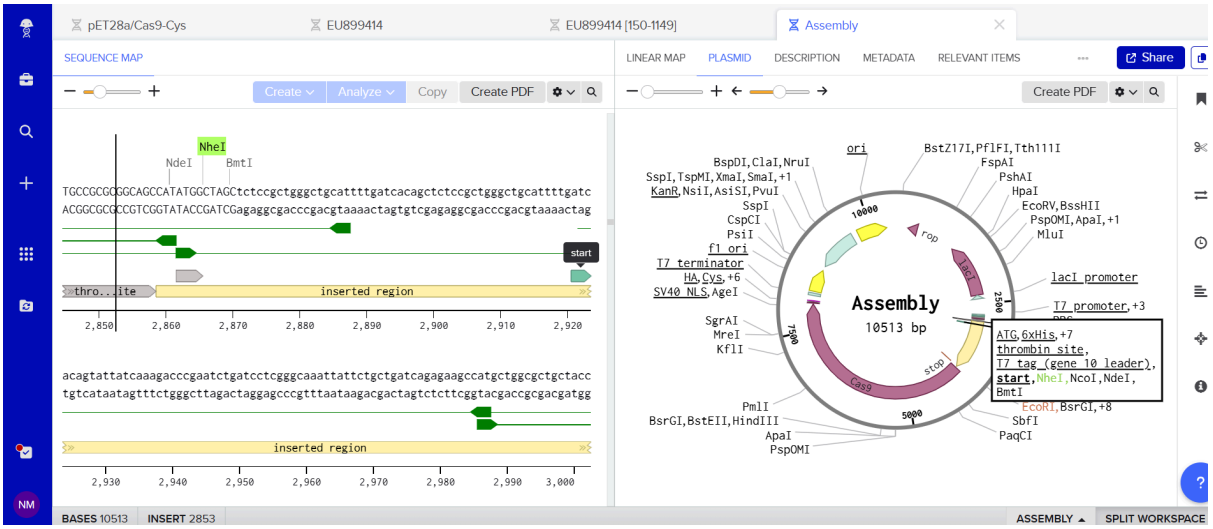


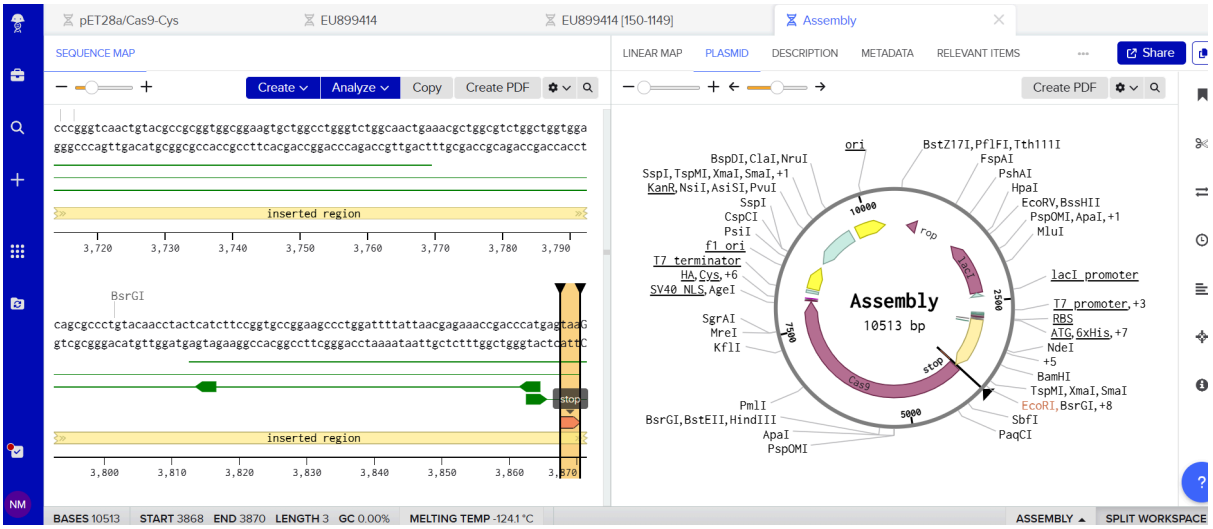

pET28a/Cas9-Cys
EU899414
EU899414 [150-1149]
SEQUENCE MAP
DIGEST
VIRTUAL DIGEST
Share
Analyze
Copy
Create PDF
Pick Assembly Strategy
Digest and Ligate
Simple restriction-enzyme based cloning.
Gibson
Homology-based, scarless, single-tube reaction assembly - see Gibson 2009.
Golden Gate
Quasi-scarless, single-tube reaction assembly - see Engler 2008.
Start
Cancel
Cas9
ApaI
PspOMI
BASES 9546
START 2865
END 2905
LENGTH 41
GC 58.54%
MELTING TEMP 73.0 °C
ASSEMBLY
SPLIT WORKSPACE


pET28a/Cas9-Cys
EU899414
SEQUENCE MAP
DIGEST
VIRTUAL DIGEST
LINEAR MAP
PLASMID
DESCRIPTION
NheI
NdeI
BmtI
NotI
HindIII
T7 tag (gene 10 leader)
2,860
2,880
Copy
Copy special...
Change case...
Delete bases
Invert selection
Create annotation
Create primer...
Create new part
Create DNA sequence
Create RNA sequence
Create translation...
Create AA sequence...
Run Primer3
Submit to NCBI BLAST
Analyze as translation
Optimize codons
SET FRAGMENT
Select an assembly fragment below.
Backbone
Insert
BASES 9546
START 2866
END 2904


pET28a/Cas9-Cys
EU899414
EU899414 [150-1149]
SEQUENCE MAP
DIGEST
VIRTUAL DIGEST
LINEAR MAP
PLASMID
DESCRIPTION
METADATA
RELEVANT ITEMS
Share
Create
Analyze
Copy
Create PDF
T7 promoter
lac operator
RBS
6xHis
NheI
EcoRI
NdeI
BmtI
BamHI
SacI
Eco53kI
HindIII
HincII
SalI
NotI
BaeI
KpnI
Acc65I
PREVIEW
AATTCGA TGG
GCT ACCGATC
0 ERRORS AND 0 WARNINGS
Looks like everything checks out
Reverse Orientation
Jump to Selection
View Enzyme Activity
pET28a/Cas9-Cys
9.5 kb · EcoRI, NheI
Insert
Hide Preview
BASES 9546
START 2905
END 2865
LENGTH 9507
GC 56.71%
MELTING TEMP 83.1 °C
ASSEMBLY
SPLIT WORKSPACE


pET28a/Cas9-Cys
EU899414
EU899414 [150-1149]
SEQUENCE MAP
LINEAR MAP
DESCRIPTION
METADATA
RELEVANT ITEMS
Share
Create
Analyze
Copy
Create PDF
Insert
Enter text here or type / to insert
MboII
BsaWI
EcoRI
NpoI
PREVIEW
TGGCTAGCtc aaG
ACCGATCGag ttCTTAA
0 ERRORS AND 0 WARNINGS
Looks like everything checks out
Reverse Orientation
Jump to Selection
View Enzyme Activity
pET28a/Cas9-Cys
9.5 kb · EcoRI, NheI
EU899414 [150-1149]
1.0 kb · NheI, EcoRI
Hide Preview
BASES 1020
START 6
END 1011
LENGTH 1006
GC 54.37%
MELTING TEMP 81.6 °C
ASSEMBLY
SPLIT WORKSPACE


pET28a/Cas9-Cys
EU899414
EU899414 [150-1149]
Assembly
SEQUENCE MAP
LINEAR MAP
PLASMID
DESCRIPTION
METADATA
RELEVANT ITEMS
Share
Create
Analyze
Copy
Create PDF
NheI
NdeI
BmtI
start
thro...ite
inserted region
Assembly
10513 bp
ori
rop
lacI
T7 promoter
start
cas9
ATG, 6xHis, +7
thrombin site,
T7 tag (gene 10 leader),
start, NheI, NcoI, NdeI,
BmtI
EcoRI, BsrGI, +8
BstZ17I, PflFI, Tth111I
FspAI
PshAI
HpaI
EcoRV, BssHII
PspOMI, ApaI, +1
MluI
lacI promoter
T7 promoter, +3
SbfI
PaqCI
PmlI
BsrGI, BstEII, HindIII
ApaI
PspOMI
SgrAI
MreI
KflI
f1 ori
T7 terminator
HA, Cys, +6
SV40 NLS, AgeI
CspCI
PsiI
SspI
BspDI, ClaI, NruI
SspI, TspMI, XmaI, SmaI, +1
KanR, NsiI, AsiSI, PvuI
BASES 10513
INSERT 2853
ASSEMBLY
SPLIT WORKSPACE


pET28a/Cas9-Cys
EU899414
EU899414 [150-1149]
Assembly
SEQUENCE MAP
LINEAR MAP
PLASMID
DESCRIPTION
METADATA
RELEVANT ITEMS
Share
Create
Analyze
Copy
Create PDF
cccgggtcaactgtacgccgcggtggcggaagtgctggcctgggtctggcaactgaaacgctggcgtctggctggtgga
gggcccagttgacatgcggcgccaccgccttcacgaccggacccagaccgttgactttgcgaccgcagaccgaccacct
inserted region
3,720
3,730
3,740
3,750
3,760
3,770
3,780
3,790
BsrGI
cagcgccctgtacaacctactcatcttccggtgccggaagccctggattttattaacgagaaaccgacccatgagtaaG
gtcgcgggacatgttggatgagtagaaggccacggccttcgggacctaaaataattgctctttggctgggtactcattC
stop
inserted region
3,800
3,810
3,820
3,830
3,840
3,850
3,860
3,870
Create PDF
Assembly
10513 bp
ori
BstZ17I,PflFI,Tth111I
FspAI
PshAI
HpaI
EcoRV,BssHII
PspOMI,ApaI,+1
MluI
lacI promoter
T7 promoter,+3
RBS
ATG,6xHis,+7
NdeI
+5
BamHI
TspMI,XmaI,SmaI
EcoRI,BsrGI,+8
SbfI
PaqCI
PspOMI
ApaI
BsrGI,BstEII,HindIII
PmlI
MreI
KflI
SgrAI
SV40 NLS,AgeI
HA,Cys,+6
T7 terminator
f1 ori
PsiI
CspCI
SspI
KanR,NsiI,AsiSI,PvuI
SspI,TspMI,XmaI,SmaI,+1
BspDI,ClaI,NruI
10000
rop
lacI
2500
stop
Cas9
5000
7500
BASES 10513
START 3868
END 3870
LENGTH 3
GC 0.00%
MELTING TEMP -124.1 °C
ASSEMBLY
SPLIT WORKSPACE