

Search

Search

Type: Location

Folder

Filters

Group by

Save

X Clear

1-50 of 1159 items

Barcode	Name	Location	Modified	Schema
4C012	4C EE&SB fridge transient stor			4°C Fridge
4C002	4C Fridge 00271			4°C Fridge
4C009	4C Fridge 01223	DTU Buildi...	09/08/2018	4°C Fridge
4C008	4C Fridge 01233			4°C Fridge
4C014	4C Fridge 01871			4°C Fridge
4C015	4C Fridge Aarop			4°C Fridge
4C016	4C Fridge Adam	Bioinnovati...	15/04/2021	4°C Fridge
4C005	4C Fridge ANALYTICS			4°C Fridge
4C011	4C Fridge CFB00266			4°C Fridge
CFB01478	4C Fridge CFB01478			4°C Fridge
CFB01653	4C Fridge CFB01653	DTU Buildi...	19/11/2018	4°C Fridge
4C003	4C Fridge DSP1	DTU Buildi...	09/08/2018	4°C Fridge

# The basics of the Molecular Biology Tool

**Reach out when struggling with the platform:**

Biosustain Benchling support  
[lims\\_support@biosustain.dtu.dk](mailto:lims_support@biosustain.dtu.dk)



**Access Benchling:**

[biosustain.benchling.com](https://biosustain.benchling.com)

(login with DTU credentials)



**Additional resources:**

[LIMS Help guides](#)

[Benchling Help Center: Molecular Biology](#)



# Hands-on Benchling support

**Mondays 13:00 -14:00** (Room 222)

DTU  
Research Data  
Management Team

## DROP-IN HOURS

Get hands-on support for **Benchling** and  
other **data management** tasks.

**MONDAYS**  
13:00 – 14:00

2<sup>nd</sup> FLOOR

Mía López Portillo  
Ontiveros  
RDM/LIMS support

Image by Joamp on Freepik

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# 1. Introduction to the Molecular Biology Suite



# Functionalities and tools overview

## Sequence Alignment

- ✓ Alignment to template
- ✓ Consensus alignment
- ✓ Benchling BLAST

## Sequence Visualization

- ✓ Plasmid map
- ✓ Annotations and feature libraries creation (*Bulk auto-annotation*)
- ✓ Sequence search

## Construct Design

- ✓ RE-based cloning
- ✓ Golden Gate and Gibson assembly
- ✓ Bulk assembly
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- ✓ Worklists integration
- ✓ *In silico* PCR and digestions
- ✓ Customizable enzyme lists

## AA / Protein Analysis

- ✓ AA alignment
- ✓ Auto-fill, back and bulk translations
- ✓ Electrochemical properties overview

## CRISPR

- ✓ Guide RNA design
- ✓ On/Off-target scoring
- ✓ HR template design



# Functionalities and tools overview

Your sequence

Functionalities

The screenshot displays a web-based bioinformatics tool interface. At the top, there are navigation tabs: SEQUENCE MAP, LINEAR MAP, PLASMID, DESCRIPTION, METADATA, RELEVANT ITEMS, and RESULTS. A 'Share' button is visible on the right. Below the tabs, there are action buttons: 'Create', 'Analyze', 'Copy', and 'Create PDF'. The main area shows a DNA sequence with several restriction enzyme sites marked: BsrFI, EarI, HpaI, EarI, BseRI, and MscI. A pink bar labeled 'XI-3\UP' is highlighted across the sequence. A zoom slider is located at the top left of the sequence view. A vertical toolbar on the right side contains icons for bookmarking, copying, zooming, and other functions, which is highlighted by a yellow box and labeled 'Functionalities'. A yellow arrow points from the text 'Your sequence' to the sequence text area.

# Functionalities and tools overview

The screenshot displays a 'SEQUENCE MAP' interface with the following elements:

- Navigation tabs:** SEQUENCE MAP, LINEAR MAP, PLASMID, DESCRIPTION, METADATA, RELEVANT ITEMS, RESULTS.
- Tools:** Create, Analyze, Copy, Create PDF, Settings, Search.
- Sequence:**

```

aa c c g g c t g c t t t c a t g t g g a a c a g a a a g a a a t c g g g g c g c t c t c t t c t g t a t t c c t t t a g t t a a c g t t t t a t t c a g c c a t c t a a c c a t c a t a c c c
t t g g c g a c g a a a g t a c a c c t t g t c t t t c t t a g c c c c g c g a a g g a g a a g a c a t a a g g a a a t c a a t t g c a a a a a t a a g t c g g t a g a t t g g t a g t a t g g g

```
- Restriction Sites:** BsrFI, EarI, HpaI, EarI, BseRI, MscI.
- Annotations:** XI-3\UP (pink bar), primers (green arrows), CRISPR (red box around TCGCTAGCCAGO).
- Scale:** 1,610 to 1,700.

- Share
- Features (*annotations and translations*)
- Digests
- Primers
- History
- Alignments
- CRISPR
- Information (*topology, tags*)

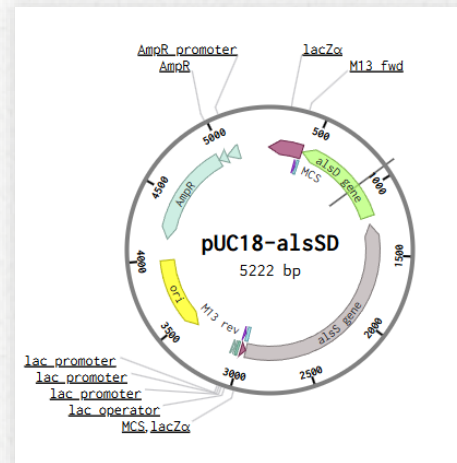
## 2. Training overview





# Training goals:

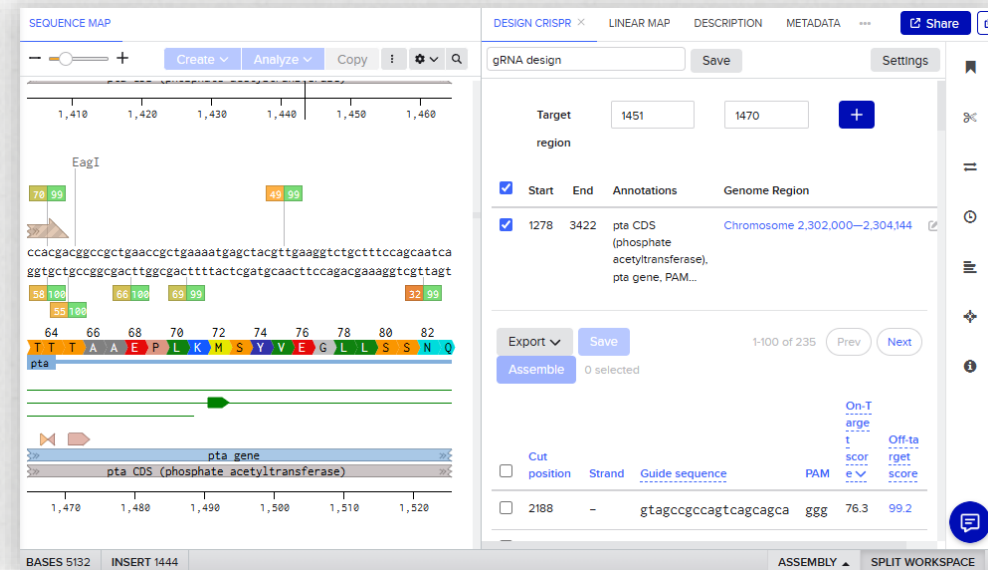
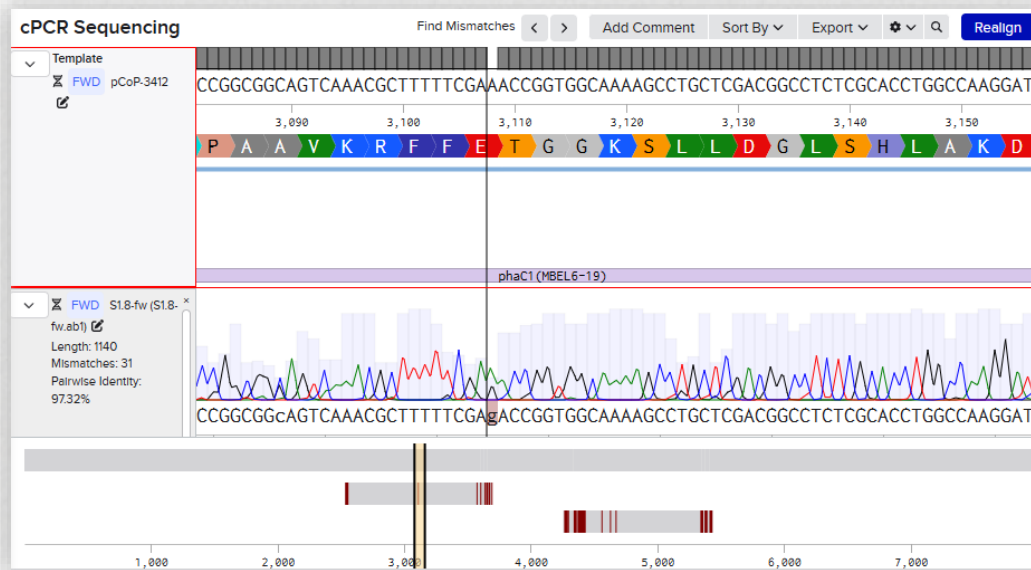
## The basics of...




- How to navigate the **sequence visualization** window and the workspace
- How to **assemble** simple constructs
- How to perform bulk assemblies with the **Combinatorial Assembly** tool

# Training goals:

## The basics of...



- How to **create a sequence alignment**
- How to use Benchling's **CRISPR** tools



# Today's work example:


Hypothetical scenario: **Production of acetoin in *E. coli***




*Scenario inspired by:*

Journal of the Taiwan Institute of Chemical Engineers 167 (2025) 105895

Contents lists available at [ScienceDirect](#)


 **Journal of the Taiwan Institute of Chemical Engineers**

 journal homepage: [www.journals.elsevier.com/journal-of-the-taiwan-institute-of-chemical-engineers](http://www.journals.elsevier.com/journal-of-the-taiwan-institute-of-chemical-engineers)

**Metabolic engineering of *Escherichia coli* for improved cofactor regeneration in lactate to acetoin via whole-cell conversion**

Chan-Hsiang Hsu, Sefli Sri Wahyu Effendi, Wan-Wen Ting, Yu-Hsiu Li, I-Son Ng\*

Department of Chemical Engineering, National Cheng Kung University, Tainan 70101, Taiwan



[Link to article](#)

# Today's work example:

Hypothetical scenario: **Production of acetoin in *E. coli***

1

Cloning vector  
design

Creation of  
expression vector  
library

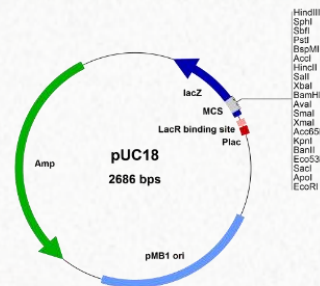
Gene knockout  
using  
CRISPR/Cas9

Sequence  
alignment

***alsS*** and ***alsD*** from *Bacillus subtilis*



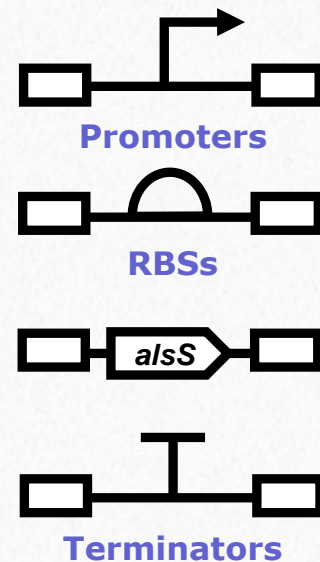
**pUC18** cloning vector



- ✓ Primer design
- ✓ *In silico* PCR
- ✓ Virtual digestion
- ✓ RE-based cloning

# Today's work example:

Hypothetical scenario: **Production of acetoin in *E. coli***



✓ Combinatorial cloning:  
**Golden Gate**

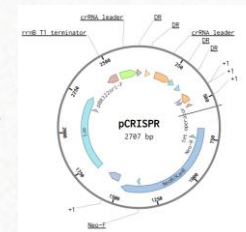
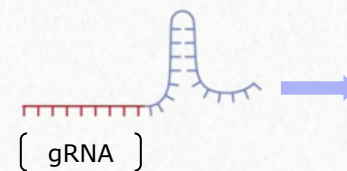
# Today's work example:

Hypothetical scenario: **Production of acetoin in *E. coli***



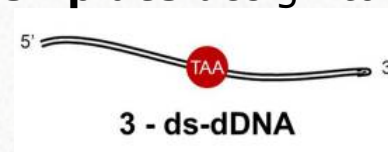
**Target:** *pta* in *E. coli*    **gRNA** design + assembly into pCRISPR

*pta*



- ✓ gRNA design
- ✓ HR template design

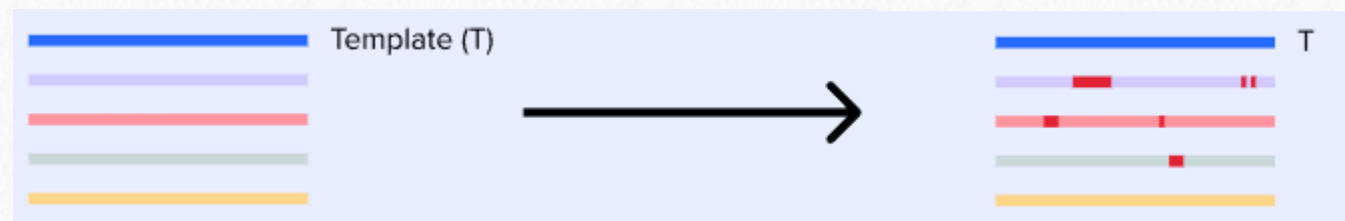
**HR template** design to KO *pta*



König, E., Zerbini, F., Zanella, I., Fraccascia, D., & Grandi, G. (2018). Multiple Stepwise Gene Knockout Using CRISPR/Cas9 in *Escherichia coli*. *Bio-protocol*, 8(2), e2688. <https://doi.org/10.21769/BioProtoc.2688>

# Today's work example:

Hypothetical scenario: **Production of acetoin in *E. coli***



✓ Multisequence alignment

# 3. The basics of sequences





This section will give you an overview of how to **import**, **visualize**, and **annotate** sequences. It also shows how to **optimize** a coding sequence's codons.



# 3. The basics of sequences

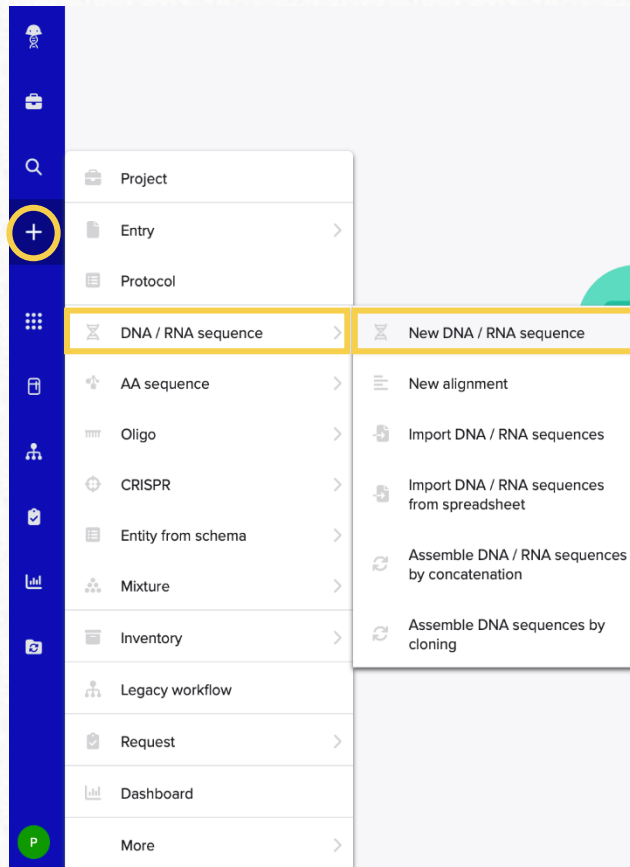
## 3.1 Sequence creation and import



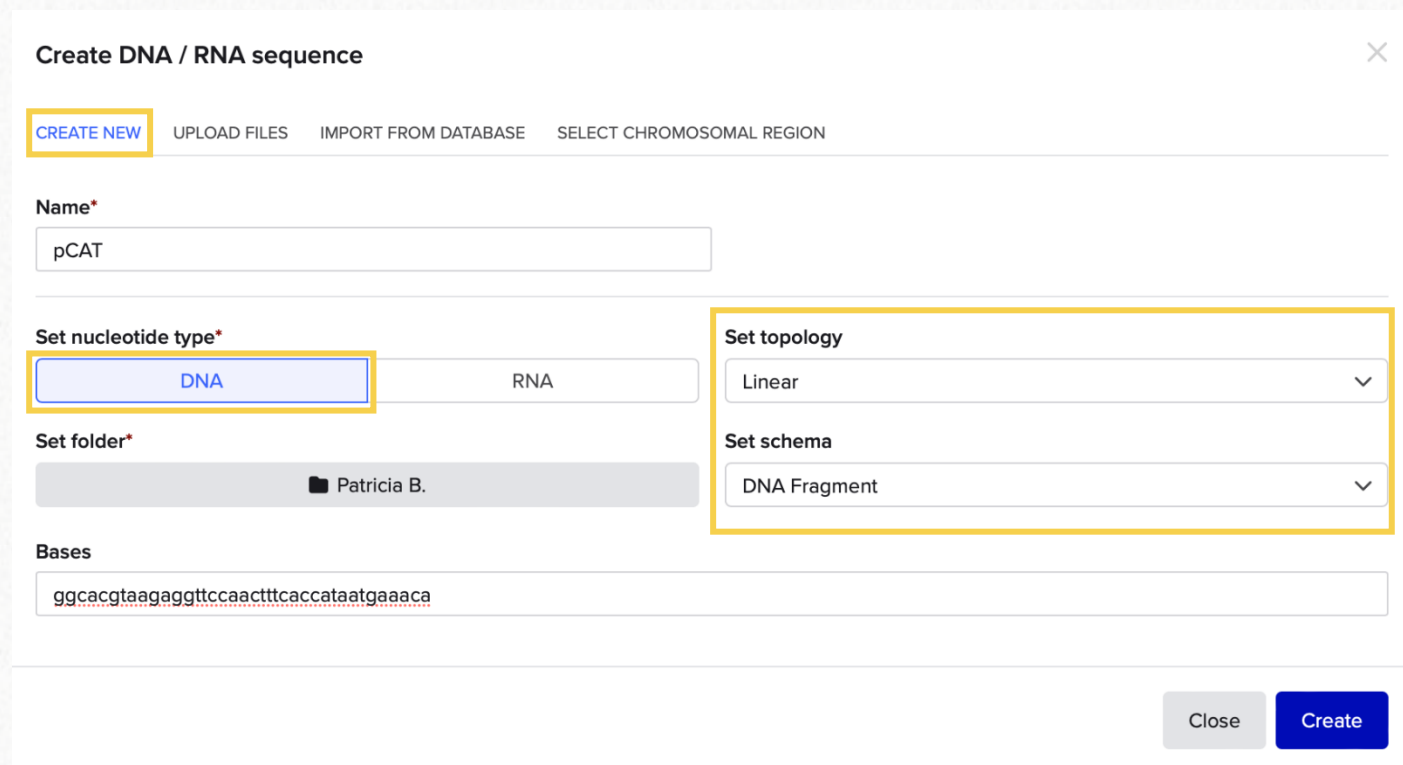
# Create and import a sequence

How to create a new entity from a nucleotide sequence

- 1 Create a new DNA sequence
- 2 You can paste or write down any nucleotide sequence of your interest, and you must assign the right topology and schema.



The screenshot shows the application's main menu on the left. The 'DNA / RNA sequence' option is highlighted in yellow, and its sub-menu is open, showing 'New DNA / RNA sequence' also highlighted in yellow. Other options in the sub-menu include 'New alignment', 'Import DNA / RNA sequences', 'Import DNA / RNA sequences from spreadsheet', 'Assemble DNA / RNA sequences by concatenation', and 'Assemble DNA sequences by cloning'.



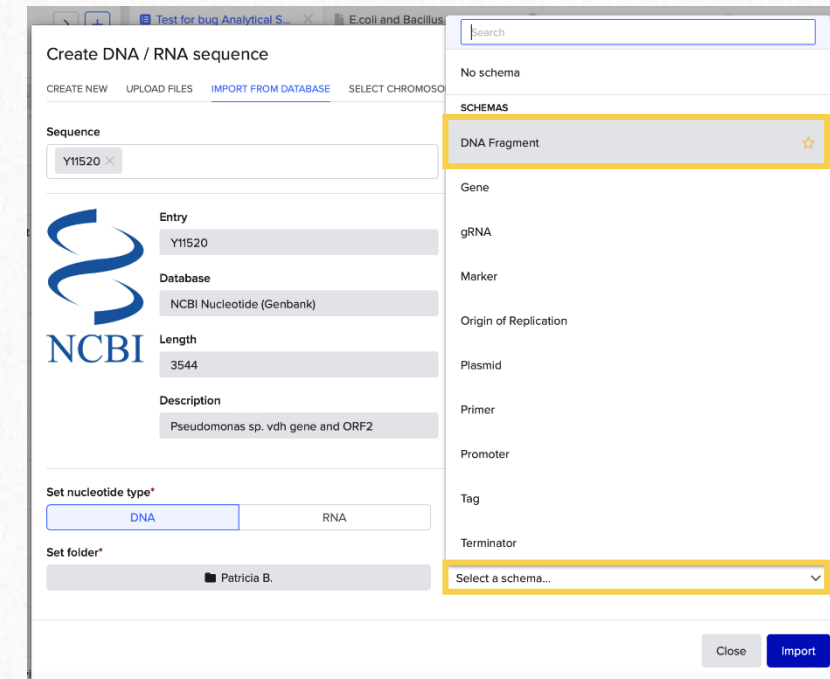
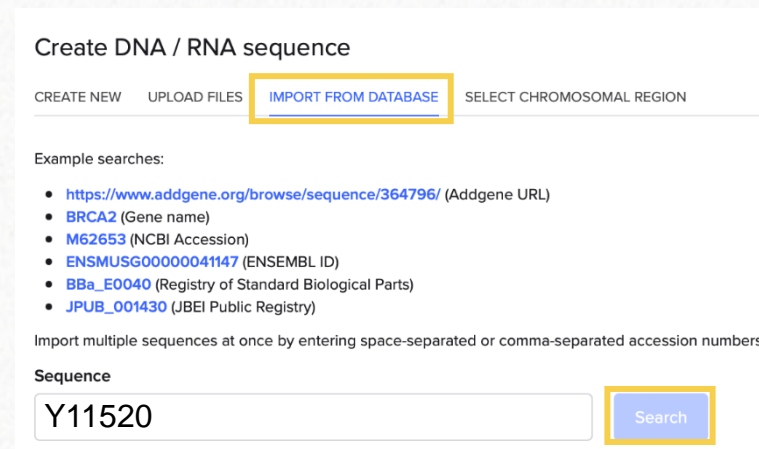
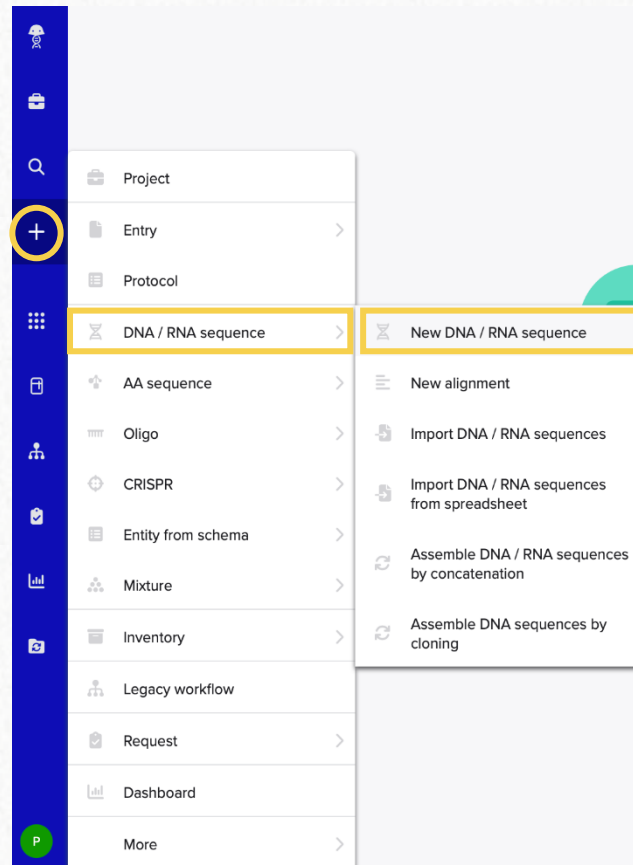
The screenshot shows the 'Create DNA / RNA sequence' dialog box. The 'CREATE NEW' button is highlighted in yellow. The 'Name' field contains 'pCAT'. The 'Set nucleotide type' dropdown is set to 'DNA'. The 'Set topology' dropdown is set to 'Linear'. The 'Set schema' dropdown is set to 'DNA Fragment'. The 'Bases' field contains the sequence 'ggcaccgtaagaggttccaacttcaccataatgaaaca'. The 'Set folder' field is set to 'Patricia B.'. The 'Close' and 'Create' buttons are visible at the bottom right.

- i** You can leave the **Bases** field **empty** and add your sequence later. This can be useful if you wish to copy and paste a sequence with its annotations.

# Create and import a sequence

## How to import of sequences from a database

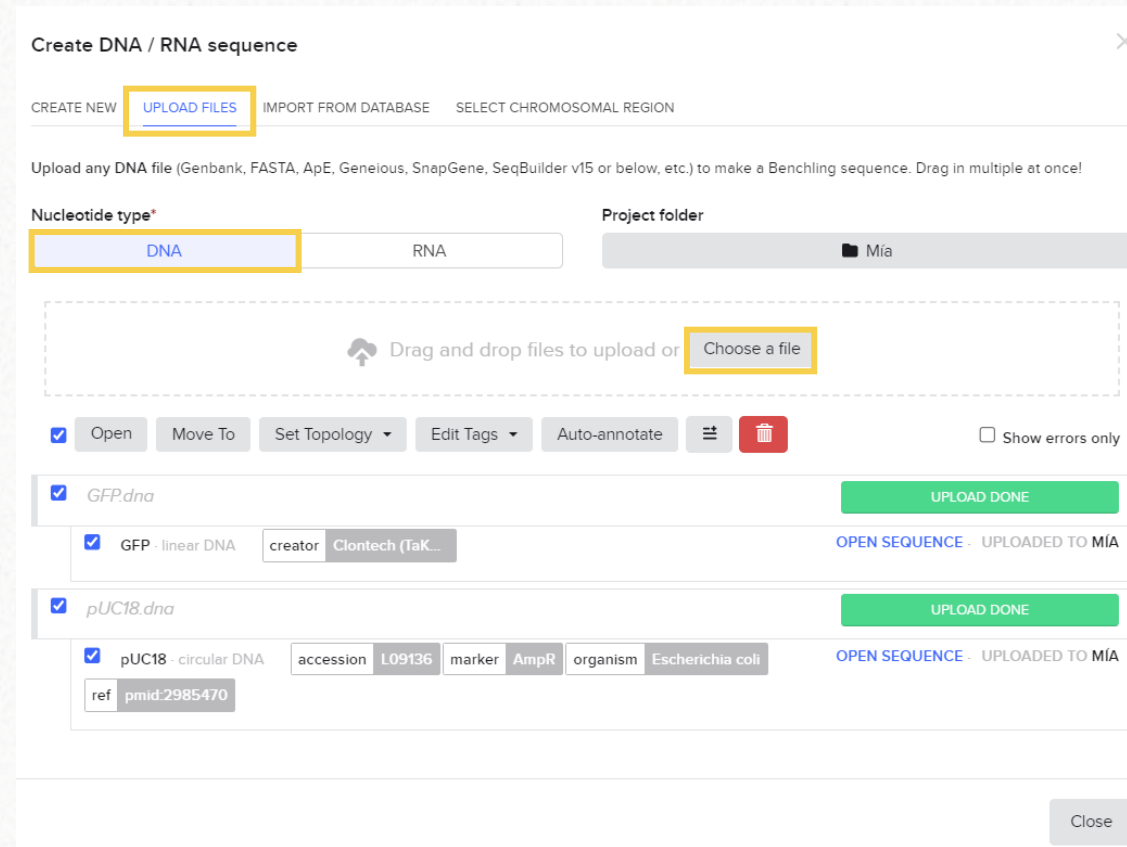
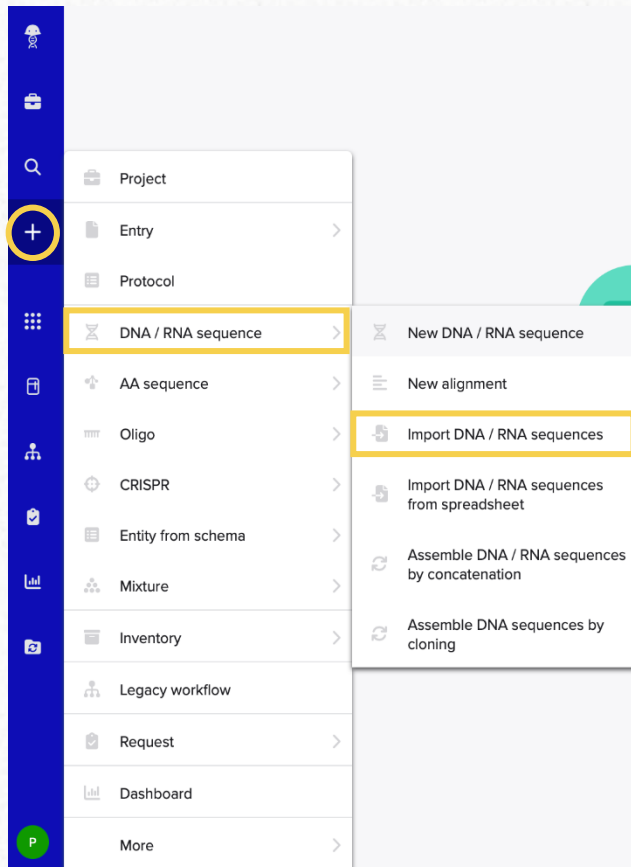
- 1 Create a new DNA sequence
- 2 You can write or paste a valid accession number from databases like GenBank, Addgene or the iGEM Registry
- 3 If the ID is valid, Benchling will show you the gene's description. You can set its schema and import it.



# Create and import a sequence

## How to import sequences from a file

- 1 Choose the **Import** sequences option
- 2 Choose the correct nucleotide type and select the sequence files. The sequences will be uploaded automatically to the folder you set.



**i** Remember to set the folder **before** uploading your files.

If you made a mistake, you can fix it by using the **Move to** option.

# Create and import a sequence

## How to import sequences from a file

The screenshot shows the 'Create DNA / RNA sequence' interface. At the top, there are tabs for 'CREATE NEW', 'UPLOAD FILES', 'IMPORT FROM DATABASE', and 'SELECT CHROMOSOMAL REGION'. Below this, a message says: 'Upload any DNA file (Genbank, FASTA, ApE, Geneious, SnapGene, SeqBuilder v15 or below, etc.) to make a Benchling sequence. Drag in multiple at once!'. There are two main sections: 'Nucleotide type\*' with 'DNA' and 'RNA' buttons, and 'Set folder' with a folder named 'Patricia'. A large dashed box contains the instruction 'Drag and drop files to upload' and a 'or choose a file' button. A yellow note box states: 'Note: GenBank sequences use the LOCUS for the sequence name. To use the filename instead, click here.' Below this, there are several buttons: 'Open', 'Move To', 'Set Topology' (highlighted with a yellow box), 'Edit Tags' (highlighted with a yellow box), 'Auto-annotate', and 'Show errors only'. The 'Set Topology' dropdown menu is open, showing 'Linear' and 'Circular' options. The 'Edit Tags' dropdown menu is also open, showing a list of tags: 'ACCESSION', 'DEFINITION', 'ORGANISM', and 'SOURCE', each with a checked checkbox. There is also a 'Create New Tag' button. At the bottom right, there is a 'Close' button. The interface also shows a file named 'Vector\_pBR322.gb' with a checked checkbox, and a green 'UPLOAD DONE' button. Below that, there is a button labeled 'OPEN SEQUENCE · UPLOADED TO PATRICIA'.

When uploading a sequence, it is possible to:

- i** **Change** its **topology** and **edit** the **tags** attached to your entity to make it easier to find.

# Create and import a sequence

## How to import sequences from a file

### Create DNA / RNA sequence

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

Upload any DNA file (Genbank, FASTA, ApE, Geneious, SnapGene, SeqBuilder v15 or below, etc.) to make a Benchling sequence. Drag in multiple at once!

Nucleotide type\* DNA RNA Set folder Patricia

Drag and drop files to upload or choose a file

Note: GenBank sequences use the *LOCUS* for the sequence name. To use the filename instead, [click here](#).

Open Move To Set Topology Edit Tags **Auto-annotate**  Show errors only

Vector\_pBR322.gb UPLOAD DONE

Exported - circular DNA ACCESSION J01749 DEFINITION Cloning vector... OPEN SEQUENCE · UPLOADED TO PATRICIA

ORGANISM synthetic DNA ... SOURCE synthetic DNA ...

Close

### Create DNA / RNA

CREATE NEW UPLOAD FILES

Upload any DNA file (Genbank, FASTA, ApE, Geneious, SnapGene, SeqBuilder v15 or below, etc.) to make a Benchling sequence. Drag in multiple at once!

Nucleotide type\* DNA RNA Set folder Patricia

Drag and drop files to upload or choose a file

Note: GenBank sequences use the *LOCUS* for the sequence name. To use the filename instead, [click here](#).

Open Move To Set Topology Edit Tags **Auto-annotate**  Show errors only

Vector\_pBR322.gb UPLOAD DONE

Exported - circular DNA ACCESSION J01749 DEFINITION Cloning vector... OPEN SEQUENCE · UPLOADED TO PATRICIA

ORGANISM synthetic DNA ... SOURCE synthetic DNA ...

Close

### Select Feature Libraries to use in auto-annotation

Select all / Clear selection

Select feature libraries

Affinity Tags

- annotations Eveline
- ART\_GEN feature library
- ART
- BI-Parts
- Biobricks
- Biobricks available
- CAL-DR
- Chenxi
- CLED features
- shared CLED features
- CM parts
- Parts for constructing MIA-CM strains
- CM parts.csv (imported 02/24/21 13:55:22)
- Common Plasmid Features
- Library of common plasmid features
- cPCR or PCR fragments
- CPE Plasmid Features
- Default Features

Show errors only

UPLOAD DONE

UPLOADED TO PATRICIA

Close

**i** You can **auto – annotate** the sequence from an existing list of features.

- This can also be done **in bulk** when using the expanded view of the selecting multiple entities at once

## Import of sequences from a file

### Create DNA / RNA sequence

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

Upload any DNA file (Genbank, FASTA, ApE, Geneious, SnapGene, SeqBuilder v15 or below, etc.) to make a Benchling sequence. Drag in multiple at once!

Nucleotide type\* DNA RNA Set folder Patricia

Drag and drop files to upload or choose a file

Note: GenBank sequences use the *LOCUS* for the sequence name. To use the filename instead, [click here](#).

Open Move To Set Topology Edit Tags Auto-annotate ☰ 🗑️  Show errors only

*Vector\_pBR322.gb* UPLOAD DONE

Exported · circular DNA ACCESSION J01749 DEFINITION Cloning vector... OPEN SEQUENCE · UPLOADED TO PATRICIA

ORGANISM synthetic DNA ... SOURCE synthetic DNA ...

Close

### Create DNA / RNA

CREATE NEW **LOAD**

Upload any DNA file (Genbank, FASTA, ApE, Geneious, SnapGene, SeqBuilder v15 or below, etc.) to make a Benchling sequence. Drag in multiple at once!

Nucleotide type\* DNA RNA

Note: GenBank sequences use the *LOCUS* for the sequence name. To use the filename instead, [click here](#).

Open Move To Set Topology Edit Tags Auto-annotate ☰ 🗑️  Show errors only

*Vector\_pBR322.gb* UPLOAD DONE

Exported · circular DNA ACCESSION J01749 DEFINITION Cloning vector... OPEN SEQUENCE · UPLOADED TO PATRICIA

ORGANISM synthetic DNA ... SOURCE synthetic DNA ...

Close

### Add items to entity worklist

New worklist Existing worklist

Worklist Name\*

Selected items

Exported

Add items to worklist

**i** You can also **create worklists or add to existing ones** to find your currently used entities faster.



# 3. The basics of sequences

## 3.2 Sequence visualization





# View, annotate and edit your sequences

Different viewing options:

**PRO TIP:**  
Click on **“split workspace”** to change the viewing mode to split screen/full screen

The screenshot shows a web-based sequence viewer for a pUC18 plasmid. The interface includes a search bar with 'pUC18' entered, a navigation menu with 'SEQUENCE MAP', 'PLASMID', 'LINEAR MAP', 'DESCRIPTION', 'METADATA', 'RELEVANT ITEMS', and 'RESULTS'. A 'Share' button is visible in the top right. The main area displays a DNA sequence with various annotations: restriction enzyme sites (NdeI, KsaI, HincII, AccI, BspMI, BfuAI, SbfI, PstI, XbaI, BamHI, SmaI, KpnI, SacI, XmaI, AvaI, TspMI, BsoBI, EcoRI, ApoI, BanII, Eco53kI, Acc65I), a 'lacZα' gene, 'M13 fwd' and 'M13 rev' primers, and 'lac operator' and 'lac promoter' elements. The sequence is shown in three segments with a scale from 180 to 640. At the bottom, there are tabs for 'BASES 2686', 'INSERT 1', and 'ASSEMBLY', with a 'SPLIT WORKSPACE' button highlighted in a yellow box.

# View, annotate and edit your sequences

## Sequence navigation:

## Functionalities

The screenshot displays the pUC18 plasmid sequence and its circular map. The linear map on the left shows the DNA sequence with various annotations including restriction sites (e.g., HindIII, SphI, PstI, XbaI, BamHI, SmaI, KpnI, SacI, BspMI, BfuAI, SalI, SbfI, XmaI, AvaI, Eco53kI, HincII, AccI, TspMI, BsoBI, ApoI, EcoRI), the lacZα gene, the Multiple Cloning Site (MCS), the lac operator, and the lac promoter. The circular map on the right shows the plasmid with features such as the AmpR gene, AmpR promoter, lacZα, MCS, ori, and various restriction sites. A sidebar on the right lists functionalities: Features (annotations and translations), Digests, Primers, History, Alignments, CRISPR, and Information (topology, tags).

# View, annotate and edit your sequences

## Sequence navigation:

- ✓ Click on any element or annotation in any of the views to select the corresponding sequence fragment
- ✓ See the **electrochemical properties** of the fragment on the bottom

**i PRO TIP:**  
Click on “*melting temperature*” to access the parameter settings. Different calculation algorithms are available.

# View, annotate and edit your sequences

## Sequence navigation:

The screenshot shows the 'SEQUENCE MAP' interface. At the top, there are buttons for 'Create' and 'Analyze', both highlighted with yellow boxes. Below these are 'Copy' and 'Create PDF' buttons. The main area displays a DNA sequence with a scale from 80 to 230. A red bar highlights a segment labeled 'lacZα'. A context menu is open over this segment, listing options such as 'Annotation', 'Primer', 'Translation', 'New AA sequence', 'New DNA', 'New RNA', 'New part', 'Run Primer3', 'Run Benchling BLAST', 'Submit to NCBI BLAST', 'Analyze as translation', and 'Optimize codons'.

This screenshot shows a different view of the sequence map. A red bar highlights a segment labeled 'lacZα'. A context menu is open over this segment, listing options such as 'Edit annotation', 'Delete annotation', 'Add to Feature Library', 'Copy', 'Copy special...', 'Change case...', 'Delete bases', 'Create new part', 'Create primer...', 'Create DNA sequence', 'Create RNA sequence', 'Create translation...', 'Create AA sequence...', 'Run Benchling BLAST', 'Submit to NCBI BLAST', and 'Analyze as translation'. The background shows a DNA sequence with a scale from 160 to 310. Other enzymes like BstAPI, NdeI, PluTI, SfoI, NarI, and KasI are indicated above the sequence.

- ✓ With a sequence fragment selected, see the "create" and "analyze" functions
- ✓ Right-click on a selection to unlock a new set of editing options

**i PRO TIP:** Click directly on any part of the sequence (not a fragment) and paste or write new bases directly.

# 3. The basics of sequences

## 3.3 Sequence annotation



# View, annotate and edit your sequences

## Sequence annotations

**2 Create an annotation**

**1 Select a sequence fragment**

BASES 2686    START 396    END 415    LENGTH 20    GC 60.00%    MELTING TEMP 61.3 °C

**3 Add the specifications**

Annotations list:

- lacZα 146-469
- M13 fwd 379-395
- MCS 399-455
- M13 rev 465-481
- lac operator 489-505

New annotation form:

Name:

Position:  -

Annotation type:

Color:

Strand:

Notes:

- ✓ Annotations are **automatically imported** with your sequences when uploading from databases and files



# View, annotate and edit your sequences

## Sequence annotations

The screenshot displays a bioinformatics tool interface for sequence annotation. On the left, a 'SEQUENCE MAP' view shows a linear DNA sequence with various features highlighted, including 'lacZα', 'MCS', and 'lac operator'. The sequence is shown in three segments with coordinates ranging from 320 to 540. On the right, a 'PLASMID' view shows a circular map of the plasmid with features like 'AmpR promoter', 'AmpR', 'ori', and 'lac promoter' labeled. A sidebar on the right lists 'ANNOTATIONS' with a 'Create new' button and a list of features like 'lacZα 146-469', 'M13 fwd 379-395', 'MCS 399-455', 'M13 rev 465-481', and 'lac operator 489-505'. At the bottom, there are buttons for 'Auto-annotate' and 'Edit feature libraries'.

**i** You can access the **“edit feature libraries”** and **“auto-annotate”** options at any time to create your own annotations list or use an existing one on your sequence

**!** Be aware that the **libraries are shared within the Center** so don't edit libraries that don't belong to you

# 3. The basics of sequences

## 3.4 Codon optimization



# View, annotate and edit your sequences

How to **codon optimize** a gene of interest for the host you want to express it in

- 1 Open the file with your gene of interest
- 2 Select the gene (for example, by clicking its annotation)

The screenshot shows two panels of a bioinformatics tool. The left panel, titled 'SEQUENCE MAP', displays DNA sequence with various annotations. A dropdown menu is open over the 'Create' button, with 'Forward' selected under the 'Translation' option. The right panel, titled 'LINEAR MAP', shows a genomic map with restriction enzyme sites and a 'TRANSLATIONS' window. In this window, a new translation is being created with the name 'vdh translation', position 1786-3242, and 485 amino acids. The bottom status bar shows sequence statistics: BASES 3544, START 1786, END 3242, LENGTH 1457, GC 58.48%, MELTING TEMP 83.5 °C.

✓ Before codon optimization, the DNA sequence must be translated

**i** If the sequence fragment selected is not a multiple of 3, the codon optimization will not be possible

- 4 Name your new translation

# View, annotate and edit your sequences

How to **codon optimize** a gene of interest for the host you want to express it in

5 Select the newly created translation and codon optimize it

The screenshot shows a web-based bioinformatics tool interface. On the left, a 'SEQUENCE MAP' view displays a DNA sequence with annotations for 'ORF2 gene', 'ORF2 CDS (enoyl-CoA hyd source)', and 'repeat\_region'. A dropdown menu is open over the 'Analyze' button, with 'Optimize codons' selected. On the right, the 'CODON OPTIMIZATION' panel is active, showing a 'Parameters' section with the following settings:

- Organism: Escherichia coli (K12)
- GC Content: Any (0 to 1)
- Uridine: mRNA Uridine Depletion (unchecked)
- Hairpin Parameters: Avoid Hairpins (checked), with values 20 and 200.

Below the parameters, there are sections for 'AVOIDED CUT SITES (0)', 'PRESERVED CUT SITES (0)', 'PROTECTED REGIONS (0)', and 'PATTERNS TO REDUCE (0)'. A table for 'Enzyme Name' and 'Cuts' is visible, with a note: 'For the specified enzymes, existing cut sites will be removed and no new cut sites will be created.' Buttons for '+ Add cut site to avoid' and 'Remove all cut sites' are present. At the bottom right, there is a 'Preview optimization' button.

- ✓ When codon optimizing, its possible to select the GC content and other details
- ✓ You can select cut sites to avoid or remove in your optimized sequence

# View, annotate and edit your sequences

How to **codon optimize** a gene of interest for the host you want to express it in

7 Take a look at the changes made and save the new optimized CDS sequence

The screenshot shows a web-based sequence optimization tool. The left panel displays a 'SEQUENCE MAP' with a DNA sequence and various annotations including restriction sites (XmnI, ApaI, PspOMI, BsaAI, PmlI), ORF2 gene, ORF2 CDS (enoyl-CoA hydratase), and a repeat region. The right panel shows an 'Optimization preview' table comparing metrics before and after optimization, and a list of specific codon changes.

Metric	Before	After
Rare codons	14	10
GC content	58%	58%
Uridine content	24%	22%
Hairpins	0	0

Location	Original	Optimized
1815	CTT → L (0.12)	TTA → L (0.15)
1824	GGT → G (0.29)	GGC → G (0.46)
1827	AAG → K (0.27)	AAA → K (0.73)
1830	CCT → P (0.17)	CCG → P (0.55)
1842	TCT → S (0.11)	AGC → S (0.33)
1848	GAG → E (0.3)	GAA → E (0.7)
1854	ACC → T (0.47)	ACT → T (0.16)
1860	GAG → E (0.3)	GAA → E (0.7)

Buttons: Back, Save, Save as new sequence

Footer: BASES 3544 START 1797 END 3242 LENGTH 1446 GC 58.44% MELTING TEMP 83.4 °C ASSEMBLY WIZARD SPLIT WORKSPACE

✓ You can keep the changes by saving the new sequence as a new entity or overwriting/editing your original sequence

# 4. Benchmarking access and folder setup



**LET'S MOVE TO BENCHLING TO START THE HANDS-ON!**

**Access Benchling:**

[biosustain.benchling.com](https://biosustain.benchling.com)

(login with DTU credentials)



# Create a training folder to work in

1. A blue sidebar on the left contains navigation icons, with a folder icon highlighted by a yellow circle and the number 1.

2. The breadcrumb path at the top reads "Projects / Biosustain Training / Molecular Biology Training", with the entire path highlighted by a yellow box and the number 2.

3. A dropdown menu is open, showing a search bar and a list of options: Folder, Entry, Protocol, DNA / RNA sequence, AA sequence, Oligo, Assembly, CRISPR, Entity from schema, Mixture, and More. The "Folder" option is highlighted by a yellow box and the number 3.

The main content area shows a list of folders with their last modified dates:

- Mia (Last modified 4 days ago)
- Agata (Last modified 21/03/2024)
- BS (Last modified 21/03/2024)
- Dushica (Last modified 18/06/2024)
- Ester (Last modified 20/03/2024)
- Ingrid (Last modified 21/03/2024)
- JY (Last modified 18/06/2024)
- Kostas test folder (Last modified 21/03/2024)
- Lilos (Last modified 21/03/2024)
- Max (Last modified 21/03/2024)

**Create folder**

Name\*

Location\*



Description








4. A yellow box highlights the "Create" button in the bottom right corner, with the number 4.


- ✓ Remember to select your own training folder when creating or importing sequences












# Copy the *Training Files* folder into your own


Projects / Biosustain Training /  
Molecular Biology Training  Saved Searches 

Search  Type: Folder, Entry, Dataset  1 filter  Save  × Clear    

< > 1-2 of 2 items, including items in subfolders  1 row selected


 Name 	Starred 	Owner	Modified 	Review Process
<input checked="" type="checkbox"/>  Training Files		DTU Biosustain	03/02/2025	
 Your Name		DTU Biosustain	03/02/2025	

Copy To... 


Item is currently in:  Molecular Biology Training

Projects

Filter...

- ★ Biosustain Training biosustain
  - Ester
  - Inventory
  - Joana
  - ▾ Molecular Biology Training
    - ✓ Your Name 

[Create new folder](#) (biosustain / Biosustain Training / Molecular Biology Training / Your Name)





Do not modify the *Training Files* folder! Make sure you are **copying** it, and **not moving** its contents.

# The *Training Files [Results]* folder

- i** You can find the **expected outputs** for each part of the hands-on in this folder, such as annealed primers, finalized assemblies and resulting constructs.

Projects / Biosustain Training / \*Molecular Biology Training /

\*Training Files [Results] Saved Searches

Search Type Filters

1-4 of 4 items

Name	Starred	Owner	Modified	Review Proces...	Description
1. Basic construct assembly		DTU Biosustain	10/02/2025		Includes: Primer design, i...
2. Combinatorial cloning		DTU Biosustain	10/02/2025		
3. CRISPR tools		DTU Biosustain	09/02/2025		Includes: gRNA design an...
4. Sequence alignments		DTU Biosustain	09/02/2025		

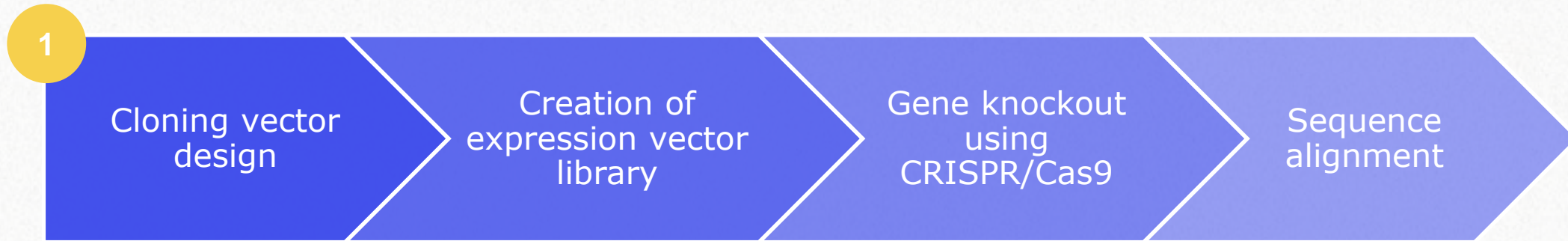


Do not modify the contents of this folder!

# 5. Basic construct assembly



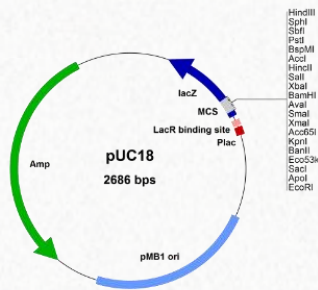
This is the first part of the *hands-on* example.



**alsS** and **alsD** from *Bacillus subtilis*



**pUC18** cloning vector



- ✓ Primer design
- ✓ *In silico* PCR
- ✓ Virtual digestion
- ✓ RE-based cloning

**Expected output:**

- alsSD fwd and rev primers
- alsSD PCR product
- Saved BamHI + Sall digestions for the alsSD PCR product and pUC18
- pUC18-alsSD plasmid

You will need the files in the **Basic construct assembly** subfolder.

The screenshot displays a bioinformatics software interface with the following components:

- Left Panel:** A file browser showing a folder named "1. Basic construct assembly". Inside, two files are listed: "alsSD source" (last modified 9 minutes ago) and "pUC18" (last modified 9 minutes ago). The "alsSD source" file is highlighted with a yellow border.
- Top Panel:** A tab titled "alsSD source" with sub-tabs for "SEQUENCE MAP" and "DESCRIPTION". The "SEQUENCE MAP" tab is active, showing a sequence viewer with a scale from 95 to 135 bp. The sequence is:
 

```

      tagtgaaacttatcacaagatattttaaattttacgtttaaaatgac
      atcactttgaatagtgttctataaattttaaaatgcaaattttacg
      
```
- Middle Panel:** A sequence viewer showing the "alsS CDS" (M T K A T K E Q K) and "alsS gene" (S L V K N R G A E L V V D C L) with their corresponding amino acid translations. The scale ranges from 140 to 180 bp.
- Right Panel:** A "LINEAR MAP" showing restriction enzyme sites (e.g., Tth111I, PflFI, StuI, MlyI, PleI, EagI, PciI, XbaI, KpnI, Acc65I, AhdI, BsrDI, StyI, NmeAIII, HpaI, BsmFI, BaeI, BsaAI, BtgI, Bsp1286I, BsiHKAI, BsrGI, BspHI, SphI, AgeI, SmlI, BtsXI, BssSI, BsrBI, BssSI, AlwNI, Bpu10I, BbvCI, PsiI, BtsI) and gene models for "alsS CDS", "alsD CDS", and "yw...S" (blue) and "alsS gene", "alsD gene", and "yw...e" (grey).
- Bottom Panel:** A status bar showing "BASES 3326" and "INSERT 154".

# 5. Basic construct assembly

## 5.1 Primer design

### 5.1.1 Manual primer design



## Manual primer creation

**Scenario:** Creating primers to add restriction sites to *alsSD*

- 1 Select ~ 22 bases at the start of *alsS*

The screenshot shows the Benchling interface for the *alsSD* source (3326 bp). On the left, the 'SEQUENCE MAP' view displays the DNA sequence with a 22-base region highlighted in yellow and red, corresponding to the amino acid sequence M T K A T K E Q K. On the right, the 'LINEAR MAP' view shows the gene structure with features like *alsS* CDS, *alsS* gene, *alsD* CDS, *alsD* gene, and *yw...S*. A primer tool window is open, showing options for 'Manual' and 'Wizard' primer creation, with a 'Create Primers' button highlighted. The bottom status bar indicates: BASES 3326, START 158, END 179, LENGTH 22, GC 31.82%, MELTING TEMP 50.8 °C.

**i** You can also attach **already existing** primers to your sequence if the entities are uploaded on Benchling

- 2 Access the primer tool and start to create a new primer manually

### 3 Select primer pair creation

The screenshot shows a software interface for primer design. On the left, a DNA sequence is displayed with a scale from 95 to 135. Below the sequence, a protein sequence is shown with amino acid letters (M, T, K, A, T, K, E, Q, K) and their corresponding codons (2, 4, 6, 8). The 'alsS CDS' and 'alsS gene' are indicated. On the right, the 'DESIGN PRIMER' panel is active, showing options for 'Primer Pair', 'Single Primer', and 'Primer Pair'. The 'Primer Pair' option is selected. The 'Forward' and 'Reverse' primer creation fields are visible, with a '3' Location' of 1 and 'Overhang' of 0 bp. A 'Cut Site' dropdown is set to 'AanI'. A 'Verify' section includes a 'Check Secondary Structure' button at 50 °C. At the bottom, a summary bar shows: BASES 3326, START 158, END 179, LENGTH 22, GC 31.82%, MELTING TEMP 50.8 °C, ASSEMBLY, and SPLIT WORKSPACE.

4 Set the 3' selected bases as forward (**start of alsS**)

5 Set the 5' selected bases as reverse (**end of alsD**)

✓ Make sure to select the **start of alsS** and the end of **alsD**



## Manual primer creation

- Look up **BamHI** restriction site in the *Cut site* dropdown menu
- Copy and paste the site at the beginning of the forward primer, and set the **overhang** to 6

Strand	Forward	Reverse
Bases	5' GGATCCatgacaaaagca acaaaagaac 3'	5' ttattcagggcttccttc agtt 3'
3' Location	179	2678
Overhang	6	0
Cut Site	BamHI	GGATCC

Use the dropdown above to look up restriction sites.

Strand	Forward	Reverse
Bases	5' GGATCCatgacaaaagca acaaaagaac 3'	5' GTCGACttattcagggct tccttcagtt 3'
3' Location	179	2678
Overhang	6	6
Cut Site	Sall	GTCGAC

Use the dropdown above to look up restriction sites.

- Repeat the process to add a **Sall** site at the beginning of the reverse primer

9 Name, select a location for your primers and save them

The screenshot shows a software interface for primer design. On the left, a 'SEQUENCE MAP' displays the 'alsD source' with a sequence map and a protein sequence: N L D N P D F A K D I E T T E G S P E \*. Below this, the 'alsD gene' and 'ywr0 gene' are shown. On the right, the 'DESIGN PRIMER' tab is active, showing a 'Primer Pair' selection. The 'Verify' section displays the following parameters:

$T_m$	50.8°C	53.4°C
GC Content	31.82%	40.91%
Length	28 bp	28 bp
Product Size	2554 bp	
$T_m$ Diff.	+2.57°C	

The 'Save' section shows the primer names 'alsSD-fwd' and 'alsSD-rev' and the 'Save To' location '1. Basic construct assembly'. A 'Save Primer Pair' button is highlighted. At the bottom, a status bar shows: BASES 3326, START 2678, END 2699, LENGTH 22, GC 40.91%, MELTING TEMP 53.4°C.

✓ Make sure to check that the melting temperatures of your primer pair are within an acceptable range

**PRO TIP:** You can adjust the default parameters for thermodynamic calculations

**PRO TIP:** Benchling offers the possibility to visualize **secondary structures** of your primers

Primer Pair: [Dropdown] Jump to Primer [Dropdown] Set from Selection [Dropdown]

Overhang: 0 bp 0 bp

Cut Site: AanI

Verify: **Check Secondary Structure** 37 °C

T <sub>m</sub>	56.1°C	69.8°C
GC Content	38.46%	73.91%
Length	26 bp	23 bp
Min ΔG Homodimer	-3.3 kcal All Structures	-13.8 kcal All Structures
Min ΔG Monomer	-0.1 kcal All Structures	-2.5 kcal All Structures
Product Size	1495 bp	
T <sub>m</sub> Diff.	+13.77°C	
Min ΔG Heterodimer	-6.3 kcal All Structures	

Name: fwd\_vdh rev\_vdh

Showing all heterodimer secondary structures for primers **ataatgacaataatgaggagtgccca** and **gcccgcggcgcccgaagatcgat** at 37°C.

ΔG (kcal)	Structure
-6.3	
-6	
-6.2	
-5.4	

Patricia

# 5. Basic construct assembly

## 5.1 Primer design

### 5.1.2 Primer wizard

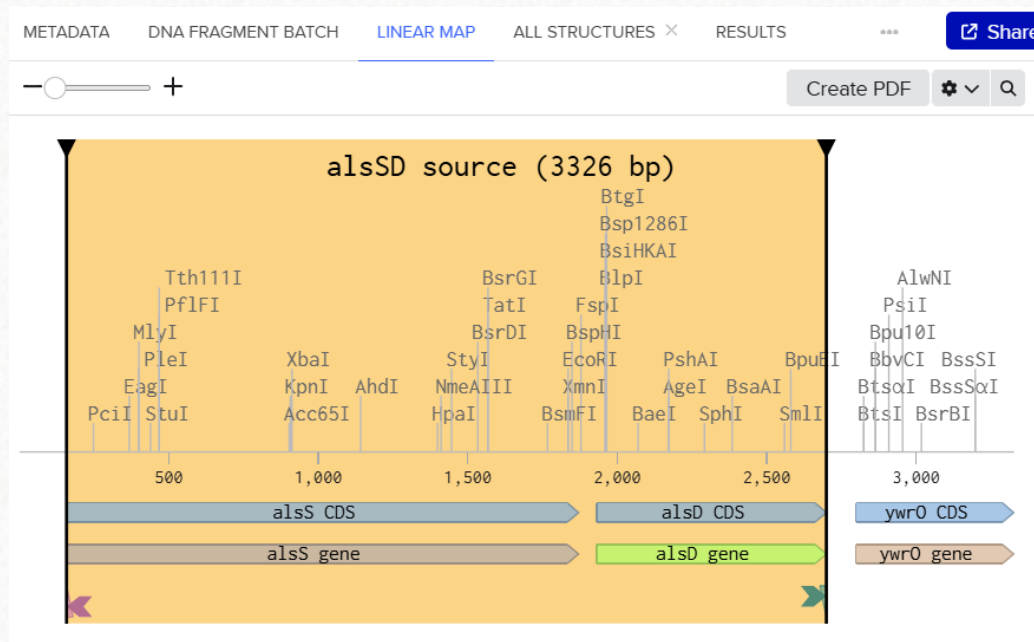


# Construct design

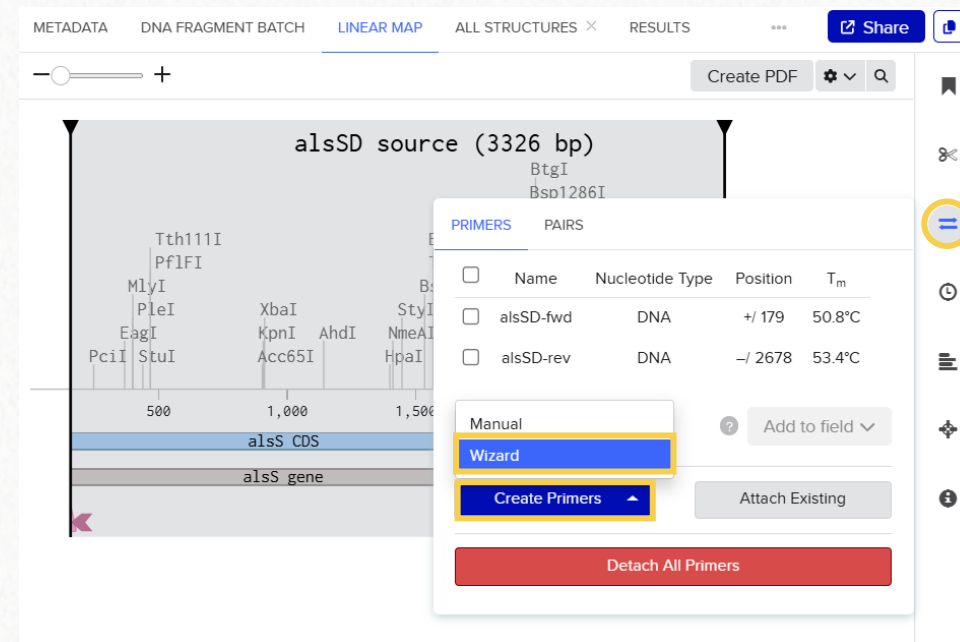
## Automatic primer creation – *Primer Wizard*

✓ Benchling has a tool for automatic primer creation called the **Primer Wizard**. To try it out, follow these steps:

1 Select the CDS of *alsS* and *alsD*



2 Access the primer tool and select *wizard*



**i** **PRO TIP:** Select both sequences by holding **Shift** while you click on the second one

### 3 Select PCR as sequencing task

The screenshot shows the Primer Wizard interface with the following components:

- SEQUENCE MAP:** Displays DNA sequence with annotations for `alsSD-fwd (DNA)`, `alsS CDS`, and `alsS gene`. A `PciI` restriction site is also indicated.
- PRIMER WIZARD:**
  - Task:** Set to `PCR`.
  - Region:** Target range from 158 to 2699. A `Use selection` button is highlighted.
  - Primer Parameters:**

	Min	Opt	Max
GC%	30	50	65
T <sub>m</sub>	45	62	65
Size	15	22	31
3' GC clamp	0		
- Bottom Bar:** Bases 3326, Start 158, End 2699, Length 2542, GC 44.89%, Melting Temp 78.1 °C.

### 4 Use your selected sequence

- ✓ Primer Wizard allows for different sequencing tasks
- ✓ Primer Wizard is powered by Primer3

**i** If you find any problem in the creation of the primers, choose a higher maximum amplicon size

## Automatic primer creation – Primer Wizard

6 Explore the primer options. You do not need to save them to continue with the next part of this tutorial.

The screenshot shows the 'PRIMER3 RESULTS' tab in a software interface. On the left, a 'SEQUENCE MAP' displays a DNA sequence with annotations for 'alsS CDS' and 'alsS gene'. A 'PciI' restriction site is also indicated. On the right, a table lists generated primers with columns for Penalty, Direction, % GC, T<sub>m</sub>°C, Location, Length, Product BP, and Primer. The first primer pair (ID 5.451) is selected, with its forward and reverse primers highlighted in blue. A blue arrow points from the 'Penalty' column header to an information icon in the text on the right.

Penalty	Direction	% GC	T <sub>m</sub> °C	Location	Length	Product BP	Primer
<input checked="" type="checkbox"/>	FWD	47.8%	57.6°	54-76	23	2909	5' aggcgaatcgatattggaggtc
<input checked="" type="checkbox"/>	REV	59.1%	62.0°	2941-2962	22	2909	5' tcgcacagctgctgttccttcg
<input type="checkbox"/>	FWD	50.0%	56.5°	55-76	22	2908	5' ggccaatcgatattggaggtc
<input type="checkbox"/>	REV	59.1%	62.0°	2941-2962	22	2908	5' tcgcacagctgctgttccttcg
<input type="checkbox"/>	FWD	47.8%	57.6°	54-76	23	2911	5' aggcgaatcgatattggaggtc
<input type="checkbox"/>	REV	59.1%	61.7°	2943-2964	22	2911	5' cctcgacagctgctgttccttc
<input type="checkbox"/>	FWD	47.8%	57.6°	54-76	23	2913	5' aggcgaatcgatattggaggtc
<input type="checkbox"/>	REV	59.1%	61.7°	2945-2966	22	2913	5' ttctcgacagctgctgttccttc
<input type="checkbox"/>	FWD	50.0%	56.2°	54-75	22	2909	5' aggcgaatcgatattggaggtc
<input type="checkbox"/>	REV	59.1%	62.0°	2941-2962	22	2909	5' tcgcacagctgctgttccttcg
<input type="checkbox"/>	FWD	50.0%	56.5°	55-76	22	2910	5' ggccaatcgatattggaggtc
<input type="checkbox"/>	REV	59.1%	61.7°	2943-2964	22	2910	5' cctcgacagctgctgttccttc
<input type="checkbox"/>	FWD	50.0%	56.5°	55-76	22	2912	5' ggccaatcgatattggaggtc
<input type="checkbox"/>	REV	59.1%	61.7°	2945-2966	22	2912	5' ttctcgacagctgctgttccttc
<input type="checkbox"/>	FWD	47.8%	57.6°	54-76	23	2912	5' aggcgaatcgatattggaggtc
<input type="checkbox"/>	REV	59.1%	62.5°	2944-2965	22	2912	5' tcctcgacagctgctgttccttc
<input type="checkbox"/>	FWD	47.8%	57.6°	54-76	23	2907	5' aggcgaatcgatattggaggtc
<input type="checkbox"/>	REV	59.1%	62.5°	2939-2960	22	2907	5' ccacaectctcttccttcgca

✓ It is possible to select primers independently of their pair, so you can mix and match as you need!

**i** By default, sorting is done based on Primer3 penalty score. The lower the penalty, the better the primer pair

# 5. Basic construct assembly

## 5.2 *In-silico* PCR





# Construct design

## In-silico PCR: Create a PCR product

- ✓ We will do an *in-silico* PCR using the primers created **manually**, to add the **BamHI** and **SaII** restriction sites.

alsSD source (3326 bp)

PRIMERS PAIRS

Primer	Position	Product Size
alsSD-fwd	+ / 179	2554
alsSD-rev	- / 2678	

Primer Pair Information

Link Primers

	Name	T <sub>m</sub>
Forward Primer	alsSD-fwd	50.8°C
Reverse Primer	alsSD-rev	53.4°C

Product Size: 2554 bp  
T<sub>m</sub> Difference: +2.6° C

Create PCR Product

Copy Selection to New DNA

Customize what gets copied over:

- Use primer bases instead of sequence (includes overhang if any)
- Annotations, translations, and primers
- Include annotations and translations not fully contained by selection
- Use reverse complement instead
- Preserve sequence indices
- Tags
- Description

Cancel Copy

- ✓ You can select what features to copy into the new DNA sequence that will be generated by the in-silico PCR
- ✓ The new entities will be saved by default in the folder that contains the original sequence

# Construct design

*In-silico PCR*: Create a PCR product

SEQUENCE MAP

—  +

Create ▾ Analyze ▾ Copy ⋮ ⚙ 🔍

BstYI  
BamHI

alsSD-fwd (DNA)

GGATCCatgacaaaagcaacaaaagaacaaaatcccttgtgaaaaacagagggcgagcttgt  
CCTAGGtactgttttcgttttctgttttttagggaacactttttgtctccccgcctcgaaca

2 4 6 8 10 12 14 16 18 20

M T K A T K E Q K S L V K N R G A E L V

alsS CDS

alsS gene

10 20 30 40 50 60

PciI

tgttgattgcttagtgagcaaggtgtcacacatgtattggcattccaggtgcaaaaattgatg  
acaactaacgaatcacctcgttccacagtgtgtacataaacgtaaggtccacgtttttaactac

22 24 26 28 30 32 34 36 38 40

V D C L V E Q G V T H V F G I P G A K I D

alsS CDS

alsS gene

BASES 2554

LINEAR MAP DESCRIPTION METADATA RESULTS ... [Share](#)

—  +

Create PDF ⚙ 🔍

alsSD source [158-2699] (2554 bp)

BtgI  
Bsp1286I  
BsiHKAI

Tth111I  
StuI  
EagI  
PciI  
PflFI  
MlyI  
BamHI

SspI  
XbaI  
KonI  
AhdI  
PleI  
BseYI  
Acc65I

StyI  
NmeAIII  
HpaI  
FokI  
NdeI  
BtsCI

BsrGI  
TatI  
BsrDI  
FspI  
BspHI  
EcoRI  
BsmFI  
XmnI  
PvuII  
BlpI

PshAI  
BpuEI  
SphI  
AccI  
AgeI  
BsaAI

SmI  
SalI

500 1,000 1,500 2,000 2,500

alsS CDS alsD CDS

alsS gene alsD gene

ASSEMBLY ▾ SPLIT WORKSPACE

✓ The new PCR product created contains the *alsSD* CDS and the desired restriction sites.

# 5. Basic construct assembly

## 5.3 Virtual digestion



## Virtual digestion

We will run two virtual digestions to create the **compatible sticky ends** for RE-based cloning in our gene of interest and the backbone (pUC18)

### Digestion of the backbone (open the pUC18 sequence)

The screenshot shows the software interface for virtual digestion. The main window displays the pUC18 sequence with various restriction enzyme sites. A 'NEW DIGEST' dialog box is open, showing a list of enzymes and their cut sites. The selected enzymes are BamHI and SalI. The 'Run digest' button is highlighted.

**1** Find and select the REs

Name	Cuts	Selected	Color
SacII	0	<input type="checkbox"/>	
BamHI	1	<input checked="" type="checkbox"/>	Red
SalI	1	<input checked="" type="checkbox"/>	Green
SapI	1	<input type="checkbox"/>	
Sau3AI	15	<input type="checkbox"/>	
Sau96I	6	<input type="checkbox"/>	

**2** Run the digestion

**3** Run the digestion

- ✓ The REs selected for this example are **BamHI** and **SalI**, which are single cutters in the MCS of pUC18.

Enzyme NcoI

CCATGG  
GGTACC

NEB

Use HF

Link: [NEB](#)

Inactivation: 80°C

Incubation: 37°C

Activity:

1.1	2.1	3.1	4/CS
100	100	100	100+

Isos.: None

Jump to Cut Site:

2192

NEW DIGEST | SAVED DIGESTS

Enzyme lists: [Manage enzyme lists](#)

Deduplicated commercial

Cut sites visible on maps: Single cutters

Find enzyme: [Clear selected](#)

nc

Name	Cuts	Selected	Color
HincII	4	NcoI	
NciI	10		
NcoI	1		

anywhere in the sequence

✓ in the current selection

only in the current selection

anywhere except the current selection

**PRO TIP:** The enzyme lists available can be managed, similarly to the features libraries and are shared within the Center.

**PRO TIP:** Click on any fragment of the sequence to select the enzyme list relevant to that fragment

## Digestion of the backbone

PLASMID DIGEST × VIRTUAL DIGEST METADATA ... [Share](#)

Digest  Save the digestion  Use HF ?

Enzymes	Cuts	Temp.	1.1	2.1	3.1	4/CS
BamHI	1	37°C	100	50	10	100
Sall	1	37°C	10	100	100	100

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
418	429	12	Sall	5'	BamHI	5'
430	417	2674	BamHI	5'	Sall	5'

- ✓ A saved digestion will allow you to easily find the fragments you need to work with for the assembly

### Digestion of the insert

1 Open the amplified *alsSD* sequence

2

3 Find and select the REs

4

Enzyme BamHI

GGATCC  
CCTAGG

NEB

Use HF ?

Link: NEB

Inactivation: N/A

Incubation: 37°C

Activity:

1:1	2:1	3:1	4:CS
100	50	10	100

Isos.: None

Jump to Cut Site: 2

Find enzyme

Clear selected

Name	Cuts	Selected	Color
BamHI	1	<input checked="" type="checkbox"/>	Green
SalI		<input type="checkbox"/>	Red

Show enzymes that cut

anywhere in the sequence

Highlight enzymes with compatible sticky ends

Run digest

The digest tab will open

LINEAR MAP DIGEST VIRTUAL DIGEST DESCRIPTION Share

Digest Save Save the digestion  Use HF ?

Enzymes	Cuts	Temp.	1:1	2:1	3:1	4:CS
BamHI	1	37°C	100	50	10	100
Sall	1	37°C	10	100	100	100

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
1	1	1	None	blunt	BamHI	5'
2	2549	2548	BamHI	5'	Sall	5'
2550	2554	5	Sall	5'	None	blunt

Run the digestion

### Gel visualization

- ✓ After running both digestions, you can easily visualize the resulting fragments in a simulated electrophoresis gel.
  - 1<sup>st</sup> lane: **Ladder**
  - 2<sup>nd</sup> lane: **Backbone**
  - 3<sup>rd</sup> lane: **Insert**
  
- ✓ If you click on the bands, you can easily select the DNA sequences that correspond to the digested fragments

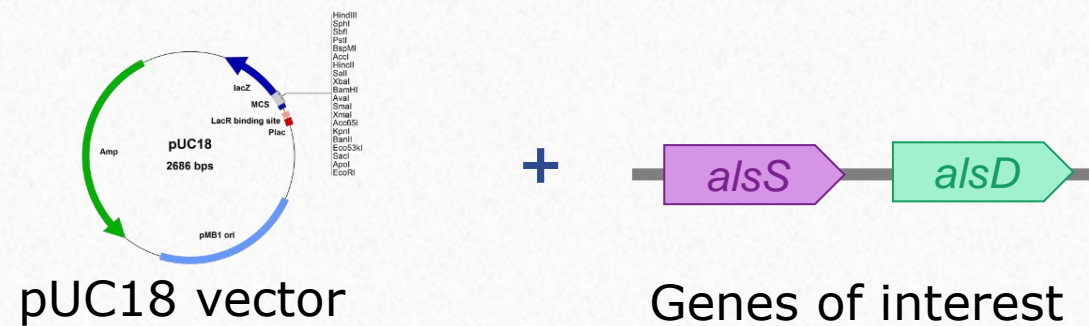


The screenshot shows a web-based interface for virtual digestion. The top navigation bar includes tabs for 'LINEAR MAP', 'DIGEST', 'VIRTUAL DIGEST' (selected), 'DESCRIPTION', 'METADATA', and 'RELEVANT ITEMS'. A 'Share' button is visible on the right. Below the navigation, a dropdown menu is open, listing various ladder options: 'Life 1 kb Plus' (selected), 'Life λ DNA-HindIII', 'Life 50 bp', 'NEB 2-Log', 'Bioline HyperLadder 1 kb Plus', 'GeneRuler 1 kb Plus', 'GeneRuler 1 kb', 'Generuler 1kb', 'GeneRuler\_1kb', and 'test'. The main area displays a gel image with a vertical scale on the left ranging from 12.0 kb to 200 bp. The gel shows a single band at approximately 2.0 kb. The bottom of the interface has buttons for 'ASSEMBLY WIZARD' and 'SPLIT WORKSPACE'.

**PRO TIP:** It's possible to choose between different ladders

# 5. Basic construct assembly

## 5.4 Assembly Wizard



## Assembly Wizard

The screenshot displays the Assembly Wizard interface. On the left, a 'SEQUENCE MAP' shows the 'alsS' gene with its CDS and various restriction sites like BstXI, BamHI, and PciI. The middle panel shows the 'alsSD source [158-2699] (2554 bp)' with a detailed map of restriction sites including Tth1111, PfuI, SspI, NheAIII, PvuII, BspI, BspHI, SmaI, BstXI, MlyI, XbaI, HpaI, NdeI, EcoRI, XmnI, BaeI, SphI, BpuEI, PciI, StuI, KpnI, FokI, BsrGI, BsmFI, BtgI, AgeI, BsaI, BstYI, PfiI, BseYI, Acc65I, AhdI, BtsCI, and TatI. On the right, another 'SEQUENCE MAP' shows the 'vdh' gene with sites like EcoRI, ApoI, BglI, and HaeII. A 'Pick Assembly Strategy' dialog box is overlaid on the right, with three options: 'Digest and Ligate' (selected), 'Gibson', and 'Golden Gate'. A yellow circle with the number '2' highlights the 'Start' button. A yellow circle with the number '1' highlights the 'ASSEMBLY' dropdown menu in the bottom navigation bar.

The Assembly Wizard allows you to use the following assembly strategies:

- ✓ Digest and Ligate (restriction enzyme-based cloning)
- ✓ Gibson assembly (no need for restriction enzymes)
- ✓ Golden Gate



## Digest and Ligate: Locate the Assembly Wizard work environment

The screenshot shows the Assembly Wizard interface with a circular plasmid map of pUC18 (2686 bp) on the left and a digestion table on the right. The plasmid map includes features like AmpR promoter, AmpR, M13 fwd, MCS, +3, lac promoter, lacZ, M13 rev, ori, and a 2600 bp region. The digestion table shows enzymes BamHI and Sall with their respective cut sites and overhangs.

Enzymes	Cuts	Temp.	1.1	2.1	3.1	4/CS
BamHI	1	37°C	100	50	10	100
Sall	1	37°C	10	100	100	100

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
418	429	12	Sall	5'	BamHI	5'
430	417	2674	BamHI	5'	Sall	5'

✓ This will remain open even if you go from one file to another

The screenshot shows the 'SET FRAGMENT' and 'OVERALL ASSEMBLY' panels. The 'SET FRAGMENT' panel has a 'Backbone' and 'Insert' section. The 'OVERALL ASSEMBLY' panel has a text input field containing 'pUC18-alsSD' and an 'Assemble' button. A yellow arrow points to the text input field with the label 'Name your construct'.

← Name your construct

## Digest and Ligate: Add the backbone

The screenshot shows the Assembly Wizard interface. On the left is a circular plasmid map of pUC18 (2686 bp) with various features labeled: AmpR promoter, AmpR, M13 fwd, MCS, +3 lac promoter, M13 rev, ori, and AmpR. On the right is a 'VIRTUAL DIGEST' table showing enzyme cuts and a 'RESULTS' table with a highlighted row for BamHI and Sall. Below the map is a 'PREVIEW' section with a 'Backbone' selection step (1) and an 'Insert' button. A yellow arrow points to the 'Backbone' selection. Below the preview is a 'PREVIEW' section showing the digested ends of the backbone: GATCCCC AGG GGG TCCAGCT. A yellow arrow points to the 'Set from Selection' button (3) and the 'Backbone' selection dropdown (4). A yellow arrow also points to the 'Backbone' selection dropdown (1).

Enzymes	Cuts	Temp.	11	21	31	4/CS
BamHI	1	37°C	100	50	10	100
Sall	1	37°C	10	100	100	100

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
418	429	12	Sall	5'	BamHI	5'
430	417	2674	BamHI	5'	Sall	5'

PREVIEW

Shift select two enzymes on the sequence map or run a digest and select a fragment.

1 Backbone Insert

BASES 2686 START 430 END 417 LENGTH 2674 GC 50.67% MELTING TEMP 80.5 °C ASSEMBLY SPLIT WORKSPACE

PREVIEW

GATCCCC AGG GGG TCCAGCT

0 ERRORS AND 0 WARNINGS  
 ✓ Looks like everything checks out

Reverse Orientation  
 Jump to Selection  
 View Enzyme Activity

2 Select the backbone

3 Set from Selection

4

✓ The Assembly Wizard shows the digested ends of the backbone

## Digest and Ligate: Add the insert

SEQUENCE MAP
LINEAR MAP

— +
Create PDF

alsSD source [158-2699] (2554 bp)

DIGEST 
VIRTUAL DIGEST
DESCRIPTION
METADATA
RESULTS
...
Share

Digest
Save
NEB
Use HF

Enzymes	Cuts	Temp.	11	2.1	3.1	4/CS
BamHI	1	37°C	100	50	10	100
Sall	1	37°C	10	100	100	100

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
1	1	1	None	blunt	BamHI	5'
2	2549	2548	BamHI	5'	Sall	5'
2550	2554	5	Sall	5'	None	blunt

Select the insert

PREVIEW

AGG      GATCCCC

TCCAGCT      GGG

Shift select two enzymes on the sequence map or run a digest and select a fragment.

pUC18  
2.7 kb · BamHI, Sall

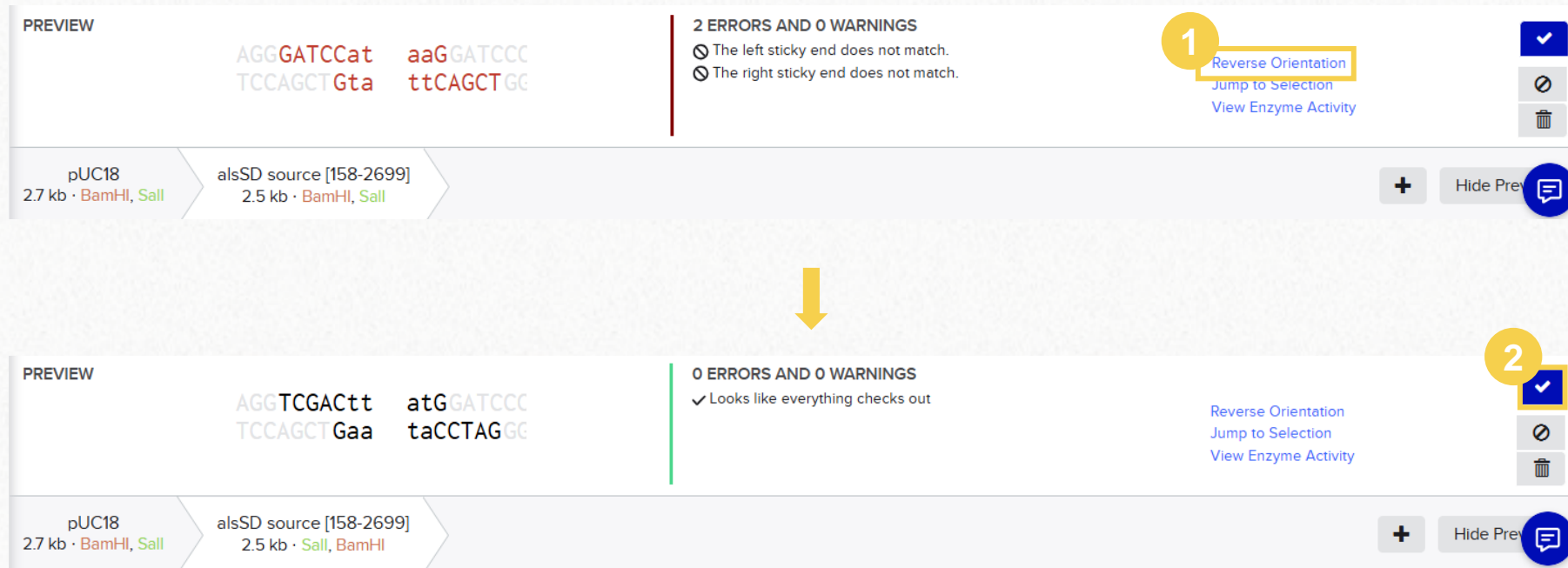
1
Insert

3
Set from Selection

+ Hide Pre

## Digest and Ligate: Check for compatibility

- ✓ The assembly wizard will check for compatibility between sticky ends.
- ✓ Depending on the orientation of your backbone and insert, you might need to make adjustments – such as in this case!



**PREVIEW**

AGGGATCCat    aaGGATCCC  
TCCAGCTGta    ttCAGCTGC

2 ERRORS AND 0 WARNINGS

- ⊗ The left sticky end does not match.
- ⊗ The right sticky end does not match.

1

Reverse Orientation  
Jump to Selection  
View Enzyme Activity

pUC18 2.7 kb · BamHI, SalI    alsSD source [158-2699] 2.5 kb · BamHI, SalI

+ Hide Pre

↓

**PREVIEW**

AGGTCGACTt    atGGATCCC  
TCCAGCTGaa    taCCTAGGC

0 ERRORS AND 0 WARNINGS

- ✓ Looks like everything checks out

2

Reverse Orientation  
Jump to Selection  
View Enzyme Activity

pUC18 2.7 kb · BamHI, SalI    alsSD source [158-2699] 2.5 kb · SalI, BamHI

+ Hide Pre

- ✓ In this scenario, it is necessary to click on "Reverse Orientation" so the ends match.

## Digest and Ligase: Assemble

**SET FRAGMENT**  
Select an assembly fragment below.

**OVERALL ASSEMBLY**  
✓ Looks like everything checks out

pUC18-alsSD **1**

Assemble

Hide Preview

pUC18  
2.7 kb · BamHI, Sall

alsSD source [158-2699]  
2.5 kb · Sall, BamHI

✓ You will be asked to choose a folder to save the construct in

**SEQUENCE MAP**

LINEAR MAP PLASMID DESCRIPTION METADATA RELEVANT ITEMS

Create Analyze Copy Create PDF

320 330 340 350 360 370 380 390

CCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTtattcagggtctcctcagttggttcgatatctttcgaataatca  
GGTCACGGTTCGAACGTACGGACGTCACGCTGaatagctcccgaaggagtcaacaaagctatagaagcgttttagt

AccI  
SbfI  
Sall

MCS  
lacZα  
alsD gene

400 410 420 430 440 450 460

gggttatccagattcgattaaagaaatccgctgttccggaagctgagattcatttttgagaatcgta  
cccaataggtctaagcgttaattcttttagcgacacaagccttcagacttaagtacaagtaaaaaactcttttagcat

alsD gene

470 480 490 500 510 520 530 540

accgtgcaatcctcaagcacatagtcaaaaacgtgctccgctgaattgcgtccttcgtaagtgacagtgatag  
tggcacgttagagttcgttatcagttttgacacagcggactaacgcaggaagcagttacttcacgtccactac

BASES 5222 INSERT 710

ASSEMBLY SPLIT WORKSPACE

**pUC18-alsSD**  
5222 bp

AmpR, AmpR\_promoter  
lacZα  
M13 fwd  
MCS  
alsD gene  
M13 rev  
lac promoter  
lac promoter  
lac promoter  
MCS, lacZα, +1

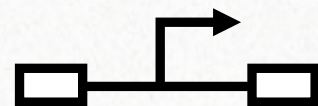
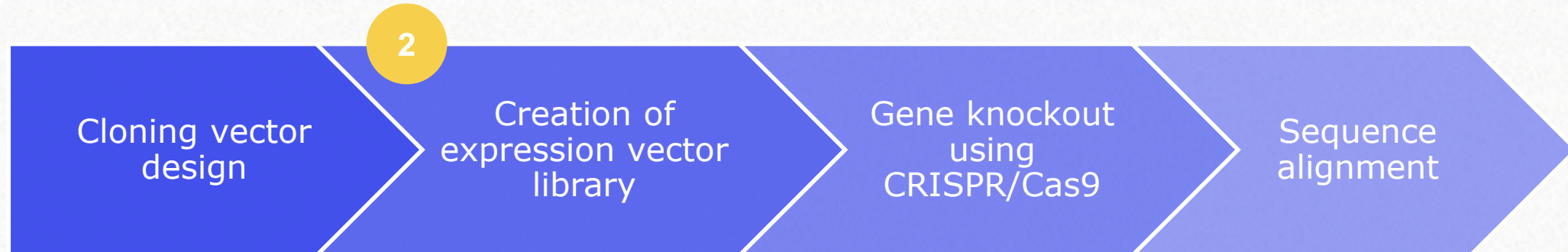
✓ The assembly is now done!



# 6. Combinatorial cloning: Golden Gate



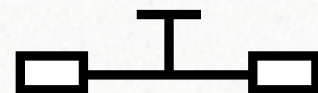
This is the second part of the *hands-on* example.



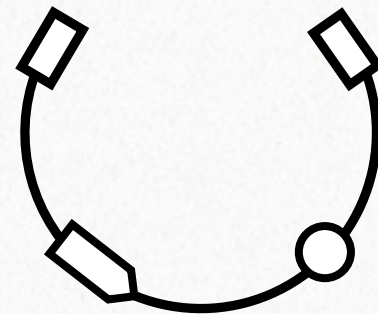
Promoters



RBSs



Terminators



Destination vector

✓ Combinatorial cloning:  
**Golden Gate**

#### Expected output:

- alsS expression vector library (combinatorial cloning assembly file)
- 27 resulting vector combinations

You will need the files in the **Combinatorial cloning** subfolder.

The screenshot displays a bioinformatics software interface for analyzing the *alsS* gene (1713 bp). The interface is divided into several sections:

- Left Panel (File Browser):** Shows a directory structure under "Your Name / Training Files / 2. Combinatorial cloning". It lists several files, including *alsS*, *pET-Ori-KanR*, and various promoters and RBS elements, all last modified 3 hours ago.
- Main Sequence Map:** Displays the DNA sequence of the *alsS* gene with restriction enzyme sites (BssS $\alpha$ I, BssSI, MboII, SacII, EciI, NspI, PstI) and the *alsS* CDS highlighted in a color-coded bar. The sequence is shown in two segments: positions 1-70 and 80-150.
- Right Panel (Linear Map):** Provides a detailed view of the *alsS* (1713 bp) gene structure, showing the *alsS* CDS and *alsS* gene. It lists numerous restriction enzymes (e.g., MmeI, MscI, SmaI, TspMI, XmaI, PstI, NspI, BsaHI, EciI, Bts $\alpha$ I, MboII, BtsI, SacII, PflMI, BssS $\alpha$ I, BstNI, BssSI, PspGI, AlwNI, BsmFI, BsrDI, BarI, BfaI, NaeI, SfiI, NgoMIV, FseI, BspMI, FokI, BspCNI, BspHI, XmnI, AgeI, AfeI, StyI, PpuMI, BsrGI, BtgZI, EcoRI, BfuAI, BtsCI, BspCNI, DdeI, PvuII, AhdI, BstXI) and their positions along the gene.
- Bottom Panel:** Shows the total length of the sequence (BASES 1713) and options for "ASSEMBLY" and "SPLIT WORKSPACE".



## Combinatorial Cloning Tool

- ✓ An alternative to the Assembly Wizard is the Combinatorial Cloning tool

It allows you to work with several cloning methods:

- ✓ **Golden Gate**
- ✓ **Gibson**
- ✓ **Homology**
- ✓ This tool is especially useful for **designing many constructs at once**

The screenshot displays the 'Golden Gate assembly' interface. It features a 'Bins & Spacers' section with three bins: BIN 1 (Backbone, 1 fragment), BIN 2 (Promoter, 3 fragments), and BIN 3 (Gene, 8 fragments). Below this is a 'Fragments' table with 12 rows, each representing a fragment with its sequence, bin, start/end coordinates, length, orientation, type, IIS enzyme, and a 'Frag' status.

Sequence	Bin	Start	End	Length	Orientation	Type	IIS enzyme	Frag
backbone	Backbone	2248	3314	1067	Forward	Bsal		Use
promoter001	Promoter	8	328	321	Forward	Bsal		Use
promoter002	Promoter	8	366	359	Forward	Bsal		Use
promoter003	Promoter	8	315	308	Forward	Bsal		Use
gene001	Gene	8	4007	4000	Forward	Bsal		Use
gene002	Gene	8	4191	4184	Forward	Bsal		Use
gene003	Gene	8	4188	4181	Forward	Bsal		Use
gene004	Gene	8	4004	3997	Forward	Bsal		Use
gene005	Gene	8	4188	4181	Forward	Bsal		Use
gene006	Gene	8	4004	3997	Forward	Bsal		Use
gene007	Gene	8	4001	3994	Forward	Bsal		Use
gene008	Gene	8	4185	4178	Forward	Bsal		Use

At the bottom, there is a 'Constructs' table with 4 rows, showing the assembly of backbone, overhangs, promoters, and genes into specific constructs.

Name	Backbone	Overhang	Promoter	Overhang	Gene
backbone-promoter001-gene001	backbone	AACA	promoter001	CGAT	gene001
backbone-promoter001-gene002	backbone	AACA	promoter001	CGAT	gene002
backbone-promoter001-gene003	backbone	AACA	promoter001	CGAT	gene003
backbone-promoter001-gene004	backbone	AACA	promoter001	CGAT	gene004

The right side of the interface shows a grid of 9 circular construct diagrams, each representing a different combination of backbone, promoter, and gene. Each diagram is labeled with its name and includes a 'No associated primers' note.

## Combinatorial Cloning Tool: How to access it

The screenshot displays the interface of the Combinatorial Cloning Tool. On the left, a sidebar lists training files, with '2. Combinatorial cloning' selected. A search bar and filter options are visible. The main workspace shows a 'SEQUENCE MAP' for the 'alsS' gene. A context menu is open over the 'Assembly' option, with 'Assemble DNA sequences by cloning' selected. The sequence map shows the DNA sequence with restriction enzyme sites (BssSxI, MboII, EciI, etc.) and a color-coded amino acid sequence (K S L V K N R G A E L V V D C L V E). The right panel shows a 'LINEAR MAP' of the 'alsS (1713 bp)' gene, with various restriction enzyme sites and the 'alsS CDS' highlighted. The bottom status bar indicates 'BASES 1713' and 'ASSEMBLY' mode.

## Combinatorial Cloning Tool: Configuration

The screenshot shows the 'Assemble DNA' configuration window in a software interface. The window is titled 'Assemble DNA' and contains several fields and options. Five yellow circles with numbers 1 through 5 are overlaid on the interface to highlight specific elements:

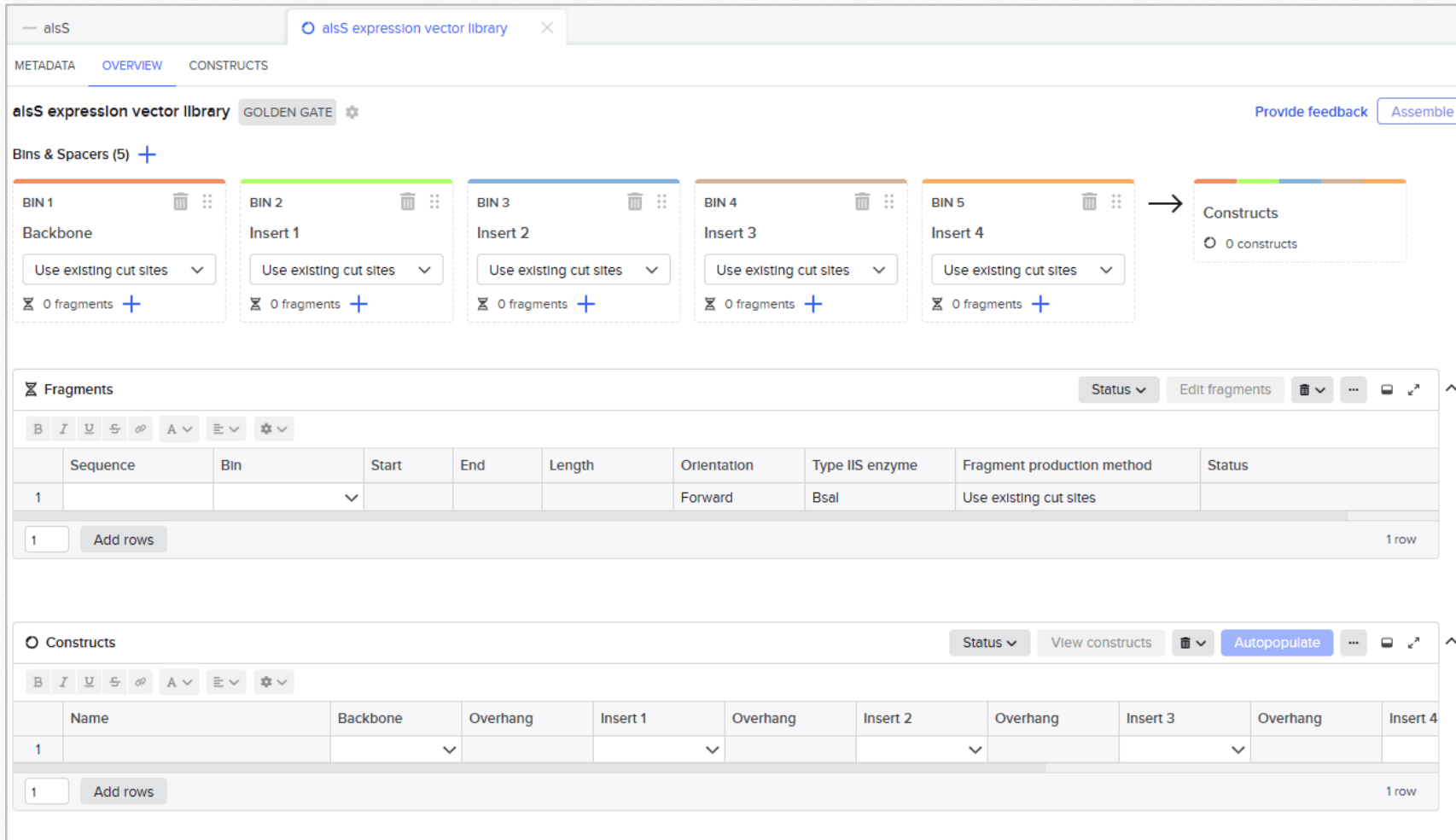
- 1: The 'Name\*' field, which contains the text 'alsS expression vector library'.
- 2: The 'Number of fragment bins\*' field, which is a numeric input set to '5'.
- 3: The 'Cloning method' section, where 'Golden Gate' is selected among other options like 'Gibson' and 'Homology'.
- 4: The 'Type IIS Restriction Enzyme' dropdown menu, which is set to 'Bsal'.
- 5: The 'Save' button at the bottom right of the window.

Other visible fields include 'Project folder\*' (set to '1. Basic construct assembly'), 'Topology of construct' (set to 'Circular'), and 'Fragment production method' (set to 'Use existing cut sites'). A blue information box is visible below the cloning method options, stating: 'Join up to 15 DNA fragments into a single piece using Type IIS restriction enzymes and T4 DNA ligase. Show details'. At the bottom of the window, there are 'Cancel' and 'Save' buttons.

**i** You can modify these parameters later (before finalizing the assembly)

**i** The only thing you will not be able to modify later is the **cloning method**

## Combinatorial Cloning Tool: Full view



The screenshot shows the 'alsS expression vector library' interface. It features five bins (BIN 1 to BIN 5) with 'Backbone' and 'Insert' fields. Each bin has a 'Use existing cut sites' dropdown and a '0 fragments' indicator. A 'Constructs' panel on the right shows '0 constructs'. Below the bins is a 'Fragments' table with one row:

	Sequence	Bin	Start	End	Length	Orientation	Type IIS enzyme	Fragment production method	Status
1						Forward	Bsal	Use existing cut sites	

At the bottom, there is a 'Constructs' table with one row:

	Name	Backbone	Overhang	Insert 1	Overhang	Insert 2	Overhang	Insert 3	Overhang	Insert 4
1										

**i** You can add multiple fragments to each bin to create several combinations

**i** All added fragments will show up here  
(You can change some configurations)

**i** When you're done adding your fragments, you can autopopulate this table with all possible combinations!



## Combinatorial Cloning Tool: Bins and spacers

**i** You can **rename** the bins for better organization.

Bins & Spacers (5) +

- BIN 1**: Backbone (highlighted), Use existing cut sites, 0 fragments +
- BIN 2**: Promoter, Use existing cut sites, 0 fragments +
- BIN 3**: RBS, Use existing cut sites, 0 fragments +
- BIN 4**: CDS, Use existing cut sites, 0 fragments +
- BIN 5**: Terminator, Use existing cut sites, 0 fragments +

Constructs: 0 constructs

✓ For our example, **rename** your bins according to this picture.

**i** You can choose whether to use **existing cut sites** or a **primer pair** in each bin

**BIN 2**

Promoter

Use a primer pair (selected)

Use existing cut sites  
Digest fragments at Type IIS enzyme cut sites to expose overhangs.

Use a primer pair (highlighted)  
Use a primer pair to introduce Type IIS enzyme cut sites and overhangs.

✓ For our example, set all bins except for the **Backbone** to use a **primer pair**.

**i** It is possible to add **spacers** (max. 20 nt) between bins, which will be incorporated in the primer design. At least one of the bins next to the spacer must be set to use a primer pair.

Bins & Spacers (5) +

Bin 1: Backbone

Options: Add new bin, Add new spacer (highlighted)

Resulting configuration:

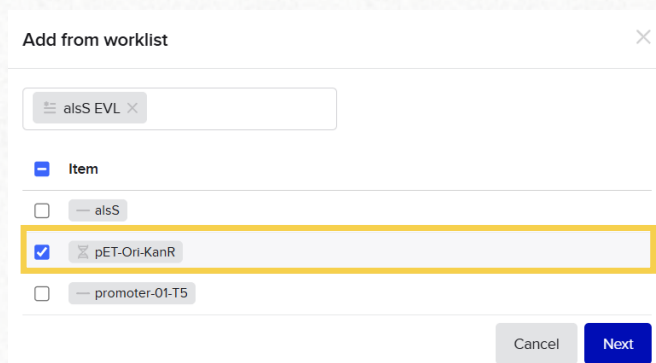
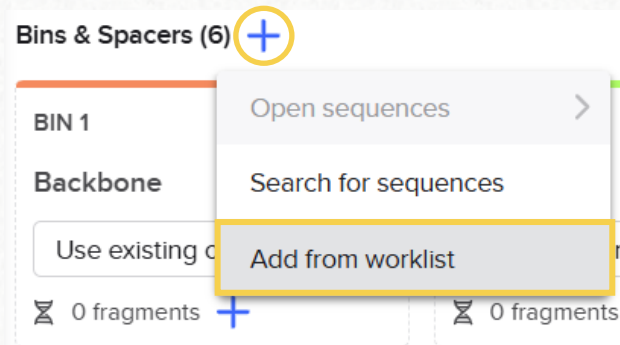
- BIN 4**: CDS, Use a primer pair, 0 fragments +
- SPACER 1**: Spacer, AATTCGAT (highlighted)
- BIN 5**: Terminator, Use existing cut sites, 0 fragments +

✓ Spacers will not be used in our example.

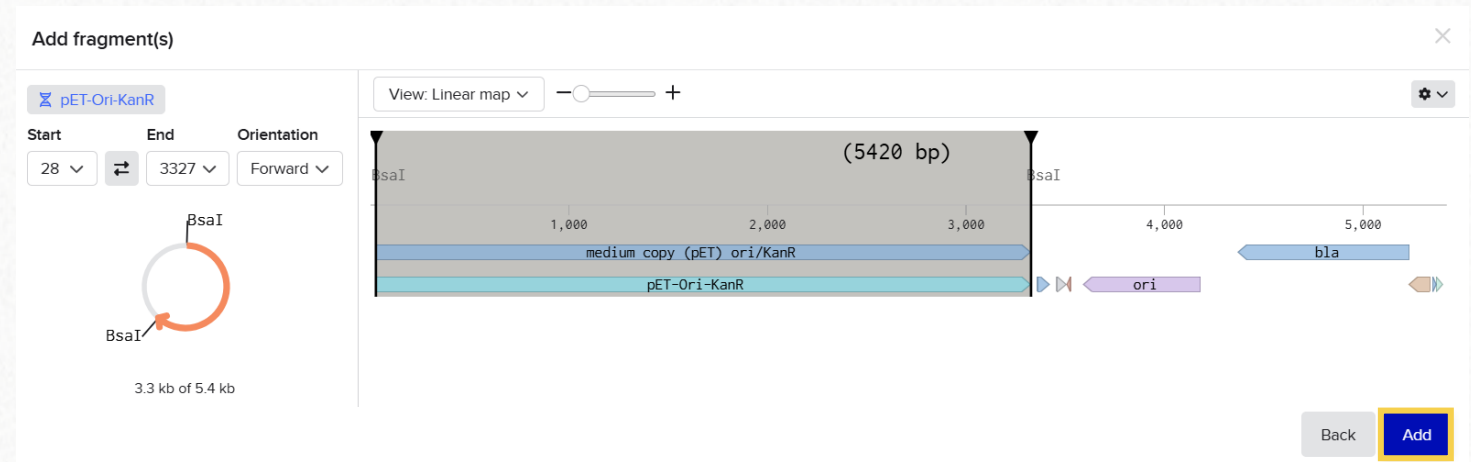
## Golden Gate: Set fragments in corresponding bins

### 1. Backbone

- 1 Find and select the **backbone** file (pET-Ori-KanR)



- 2 Verify the selection is correct and click "Add"

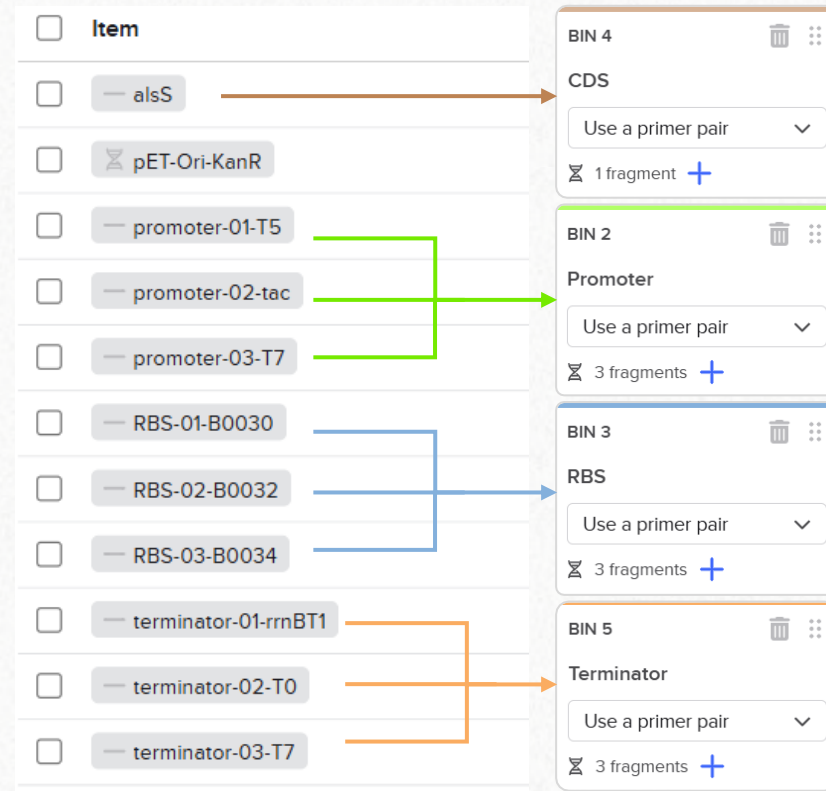


- ✓ Since this bin was configured to use **existing cut sites**, Benchling has detected the **BsaI** sites in the sequence and automatically selected the region between them.
- ✓ If you choose the option to **create a primer pair** for a sequence, you will be able to freely select the region you'd like to use.

## Golden Gate: Set fragments in corresponding bins

### 2. Inserts

- 3 Repeat the process for each bin following each category.  
Keep the entire sequences.



- ✓ Primers with appropriate overhangs will be designed for the assembly of these fragments following the position of the bins.

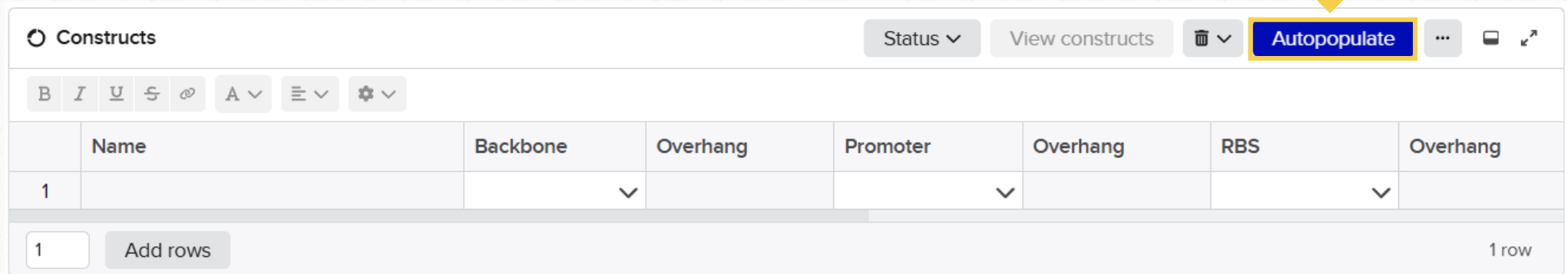
## Golden Gate: Verify the fragments

- ✓ You should obtain a table like this one.
- ✓ By clicking on a specific row, you will be able to edit the fragments if you need to do so. You can also change the bin a sequence corresponds to, and even remove sequences.

Fragments											
											Status
											Edit fragments
											Close
B I U S @ A [ ] [ ] [ ]											
	Sequence	Bin	Start	End	Length	Orientation	Type IIS enzyme	Fragment production method	Preferred 5' primer	Preferred 3' primer	Status
1	pET-Ori-KanR	Backbone	28	3327	3300	Forward	Bsal	Use existing cut sites			Looks good
2	promoter-01-T5	Promoter	1	45	45	Forward	Bsal	Use a primer pair			Looks good
3	promoter-02-tac	Promoter	1	46	46	Forward	Bsal	Use a primer pair			Looks good
4	promoter-03-T7	Promoter	1	36	36	Forward	Bsal	Use a primer pair			Looks good
5	RBS-01-B0030	RBS	1	52	52	Forward	Bsal	Use a primer pair			Looks good
6	RBS-02-B0032	RBS	1	50	50	Forward	Bsal	Use a primer pair			Looks good
7	RBS-03-B0034	RBS	1	49	49	Forward	Bsal	Use a primer pair			Looks good
8	alsS	CDS	1	1713	1713	Forward	Bsal	Use a primer pair			Looks good
9	terminator-01-rrnBT1	Terminator	1	110	110	Forward	Bsal	Use a primer pair			Looks good
10	terminator-02-T0	Terminator	1	126	126	Forward	Bsal	Use a primer pair			Looks good
11	terminator-03-T7	Terminator	1	71	71	Forward	Bsal	Use a primer pair			Looks good

## Golden Gate: Populate the “constructs” table

- 4 Click the “**Autopopulate**” button to fill the **Constructs** table with all possible combinations of your fragments.



The screenshot shows the 'Constructs' table in the software interface. The table has columns for Name, Backbone, Overhang, Promoter, Overhang, RBS, and Overhang. The 'Autopopulate' button is highlighted with a yellow arrow, indicating the action to be taken.

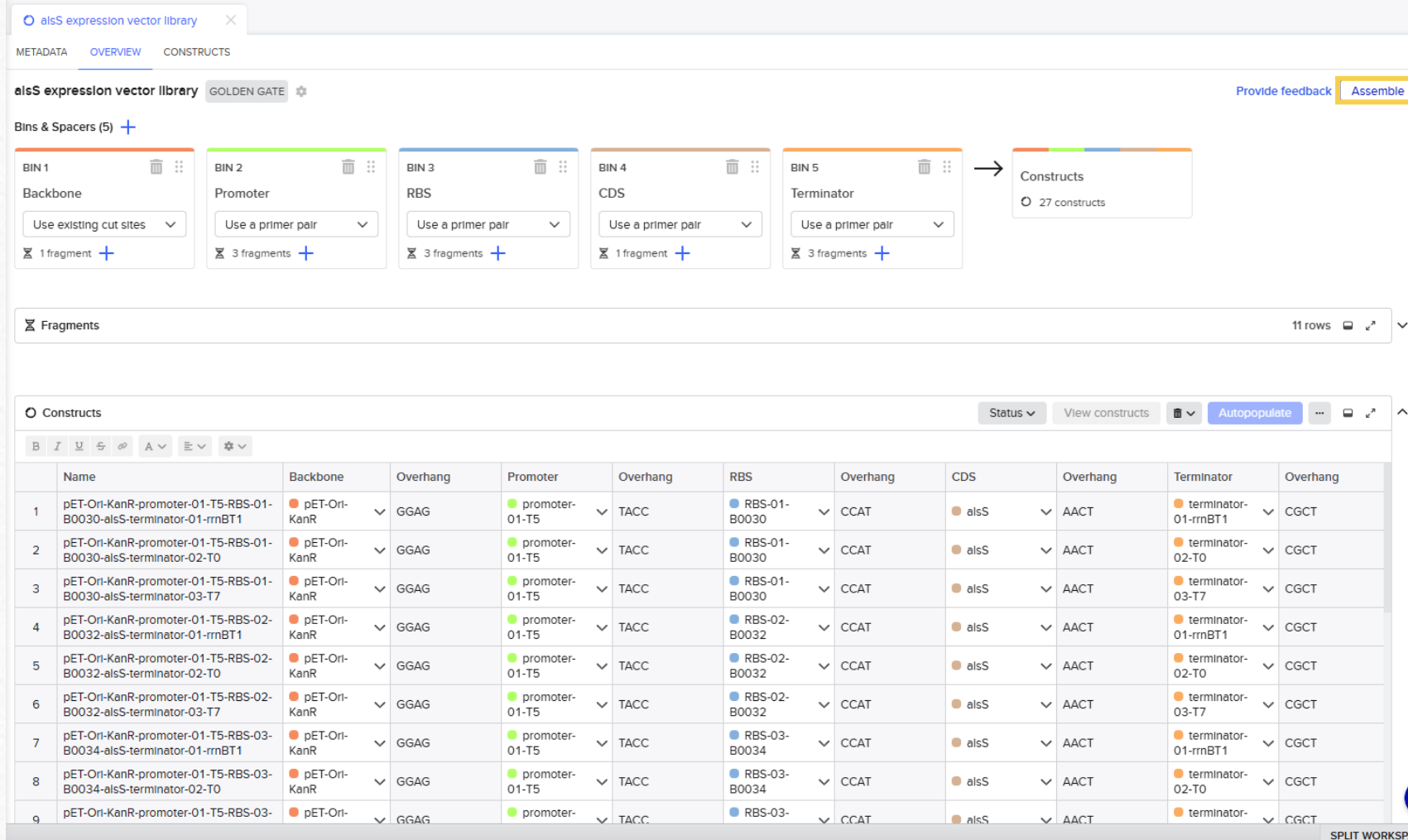
	Name	Backbone	Overhang	Promoter	Overhang	RBS	Overhang
1		▼		▼		▼	

1 Add rows 1 row

- ✓ You can also create combinations **manually**, with the option of **skipping** bins if you wish to do so
- ✓ It's also possible to **remove** rows that you are not interested in.

## Golden Gate: Finalize the assembly

- 5 Click the “**Assemble**” button to create **primer** (optional), **fragment** (optional) and **plasmid** files for all of your constructs.



The screenshot shows the 'alsS expression vector library' interface. At the top right, the 'Assemble' button is highlighted with a yellow box and a yellow arrow pointing to it. Below the 'Assemble' button, there are five bins (BIN 1 to BIN 5) for selecting components: Backbone, Promoter, RBS, CDS, and Terminator. Each bin has a dropdown menu for 'Use a primer pair' and a plus sign to add fragments. A 'Constructs' box on the right shows 27 constructs. Below the bins, there is a 'Fragments' section with 11 rows. At the bottom, there is a table of constructs.

	Name	Backbone	Overhang	Promoter	Overhang	RBS	Overhang	CDS	Overhang	Terminator	Overhang
1	pET-Ori-KanR-promoter-01-T5-RBS-01-B0030-alsS-terminator-01-rmBT1	pET-Ori-KanR	GGAG	promoter-01-T5	TACC	RBS-01-B0030	CCAT	alsS	AACT	terminator-01-rmBT1	CGCT
2	pET-Ori-KanR-promoter-01-T5-RBS-01-B0030-alsS-terminator-02-T0	pET-Ori-KanR	GGAG	promoter-01-T5	TACC	RBS-01-B0030	CCAT	alsS	AACT	terminator-02-T0	CGCT
3	pET-Ori-KanR-promoter-01-T5-RBS-01-B0030-alsS-terminator-03-T7	pET-Ori-KanR	GGAG	promoter-01-T5	TACC	RBS-01-B0030	CCAT	alsS	AACT	terminator-03-T7	CGCT
4	pET-Ori-KanR-promoter-01-T5-RBS-02-B0032-alsS-terminator-01-rmBT1	pET-Ori-KanR	GGAG	promoter-01-T5	TACC	RBS-02-B0032	CCAT	alsS	AACT	terminator-01-rmBT1	CGCT
5	pET-Ori-KanR-promoter-01-T5-RBS-02-B0032-alsS-terminator-02-T0	pET-Ori-KanR	GGAG	promoter-01-T5	TACC	RBS-02-B0032	CCAT	alsS	AACT	terminator-02-T0	CGCT
6	pET-Ori-KanR-promoter-01-T5-RBS-02-B0032-alsS-terminator-03-T7	pET-Ori-KanR	GGAG	promoter-01-T5	TACC	RBS-02-B0032	CCAT	alsS	AACT	terminator-03-T7	CGCT
7	pET-Ori-KanR-promoter-01-T5-RBS-03-B0034-alsS-terminator-01-rmBT1	pET-Ori-KanR	GGAG	promoter-01-T5	TACC	RBS-03-B0034	CCAT	alsS	AACT	terminator-01-rmBT1	CGCT
8	pET-Ori-KanR-promoter-01-T5-RBS-03-B0034-alsS-terminator-02-T0	pET-Ori-KanR	GGAG	promoter-01-T5	TACC	RBS-03-B0034	CCAT	alsS	AACT	terminator-02-T0	CGCT
9	pET-Ori-KanR-promoter-01-T5-RBS-03-	pET-Ori-	GGAG	promoter-	TACC	RBS-03-	CCAT	alsS	AACT	terminator-	CGCT

**i** After assembling the construct(s), this Combinatorial Cloning file cannot be edited anymore.

## Golden Gate: Save the constructs and related files

The image shows three sequential screenshots of the 'Assemble DNA' dialog box, illustrating the workflow for Golden Gate cloning. Each window has a close button (X) in the top right corner.

- Window 1:** Shows the initial state with three steps: 1. Save constructs, 2. Save fragments, and 3. Save primers. Below the steps, it says 'Add constructs to a folder and optionally set a schema'. There are two dropdown menus: 'Set location\*' (set to 'Mia') and 'Set schema' (set to 'Plasmid'). A checkbox 'Add constructs to a worklist' is present. At the bottom, there are 'Cancel' and 'Next' buttons. A yellow arrow points from the 'Next' button to the next window.
- Window 2:** Shows the workflow progressing. Step 1 'Save constructs' is now completed with a green checkmark. Step 2 'Save fragments' is active. A blue information bar states 'Saving fragments is optional.' Below it is a checkbox 'Create DNA Sequences to represent amplified fragments' with an information icon. At the bottom, there are 'Back' and 'Next' buttons. A yellow arrow points from the 'Next' button to the next window.
- Window 3:** Shows the workflow nearly complete. Step 1 'Save constructs' and Step 2 'Save fragments' are both completed with green checkmarks. Step 3 'Save primers' is active. A blue information bar states 'Saving primers is optional.' Below it is a checkbox 'Create DNA Oligos to represent newly designed primers' with an information icon. At the bottom, there are 'Back' and 'Assemble' buttons.

**i** You can choose whether to create files for every primer and related amplicon.

**i** If you choose not to create the primer files, you will still be able to find them later.

## Golden Gate: Results

- ✓ After you finalize the assembly, you can move over to the "Constructs" tab to see the resulting constructs.
- ✓ You can view the primer information summarized in a table.

1

Clicking here will take you to the sequence file of the construct

2

## Primer view

View constructs

SEQUENCE PRIMERS

Fragment	Orientation	Action	Primer	Bases	T <sub>m</sub> whole (°C)
1	promoter-01-T5	5' primer	promoter-01-T5_forward	TTTCATGG... 44 bp	62.22
2	promoter-01-T5	3' primer	promoter-01-T5_reverse	TTTCATGG... 40 bp	62.08
3	RBS-01-B0030	5' primer	RBS-01-B0030_forward	TTTCATGG... 45 bp	59.16
4	RBS-01-B0030	3' primer	RBS-01-B0030_reverse	TTTCATGG... 44 bp	59.93
5	alsS	5' primer	alsS_forward	TTTCATGG... 34 bp	65.81
6	alsS	3' primer	alsS_reverse	TTTCATGG... 34 bp	63.86
7	terminator-01-rmBT1	5' primer	terminator-01-rmBT1_forward	TTTCATGG... 33 bp	69.64
8	terminator-01-rmBT1	3' primer	terminator-01-rmBT1_reverse	TTTCATGG... 40 bp	68.77

**i** You can copy this table or download it as a CSV file.

## Sequence view

View constructs

SEQUENCE PRIMERS

View: Plasmid map

alsS\_forward  
RBS-01-B0030\_reverse  
RBS-01-B0030\_forward  
promoter-01-T5\_reverse  
promoter-01-T5\_forward

5224 bp





# 7. CRISPR tools

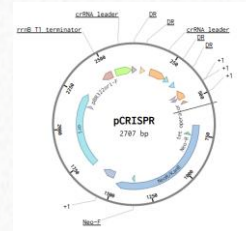
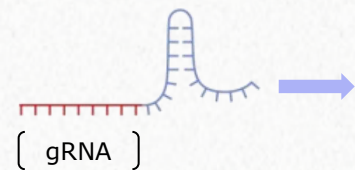


This is the third part of the *hands-on* example.

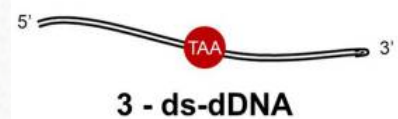


**Target:** *pta* in *E. coli*    **gRNA** design + assembly into pCRISPR

*pta*



**HR template** design to KO *pta*



- ✓ gRNA design
- ✓ HR template design

**Expected output:**

- Selected gRNA for the *pta* gene
- Forward and reverse primers to clone the gRNA into pCRISPR via *Bsa*I
- pCRISPR-*pta*-gRNA construct
- Modified *pta* sequence for KO
- HR template for KO

König, E., Zerbini, F., Zanella, I., Fraccascia, D., & Grandi, G. (2018). Multiple Stepwise Gene Knockout Using CRISPR/Cas9 in *Escherichia coli*. *Bio-protocol*, 8(2), e2688. <https://doi.org/10.21769/BioProtoc.2688>

You will need the files in the **CRISPR tools** subfolder.

The screenshot displays a web-based bioinformatics tool interface. On the left, a sidebar shows a file browser with the path `... / Your Name / Training Files / 3. CRISPR tools`. Below the search bar, two files are listed: `pCRISPR` (last modified 6 days ago) and `pta source` (last modified 6 days ago). The main workspace is titled `pta source` and is split into two panels.

The left panel, labeled `SEQUENCE MAP`, shows the raw DNA sequence in three segments:
 

```

    ttccacaccgccagctcagctggcggtgctgttttgaaccgccaatcgggcg
    aaagtgtggcggtcgagtcgaccgccacgacaaaacattggcggttagccgc
    taacgaaaggataaacctgtcccgtattattatgctgatccctaccggaacc
    attgctttctcctatttggcacagggcataataatcactagggatggccttgg
    agcgtcggctctgaccagcgtcagccttggcggtgatccgtgcaatggaacgcaaag
    tcgagccagactggctcagtcggaaccgcactaggcacgttaccttgcgtttc
    
```

 Below the sequence, three horizontal bars represent gene models: a green bar for the full sequence, a blue bar for `pta gene`, and a grey bar for `pta CDS (phosph...yltransferase)`. A green arrow indicates a restriction enzyme site labeled `PshAI` at the start of the second segment. The x-axis at the bottom shows genomic coordinates from 2,301,000 to 2,302,030.
 

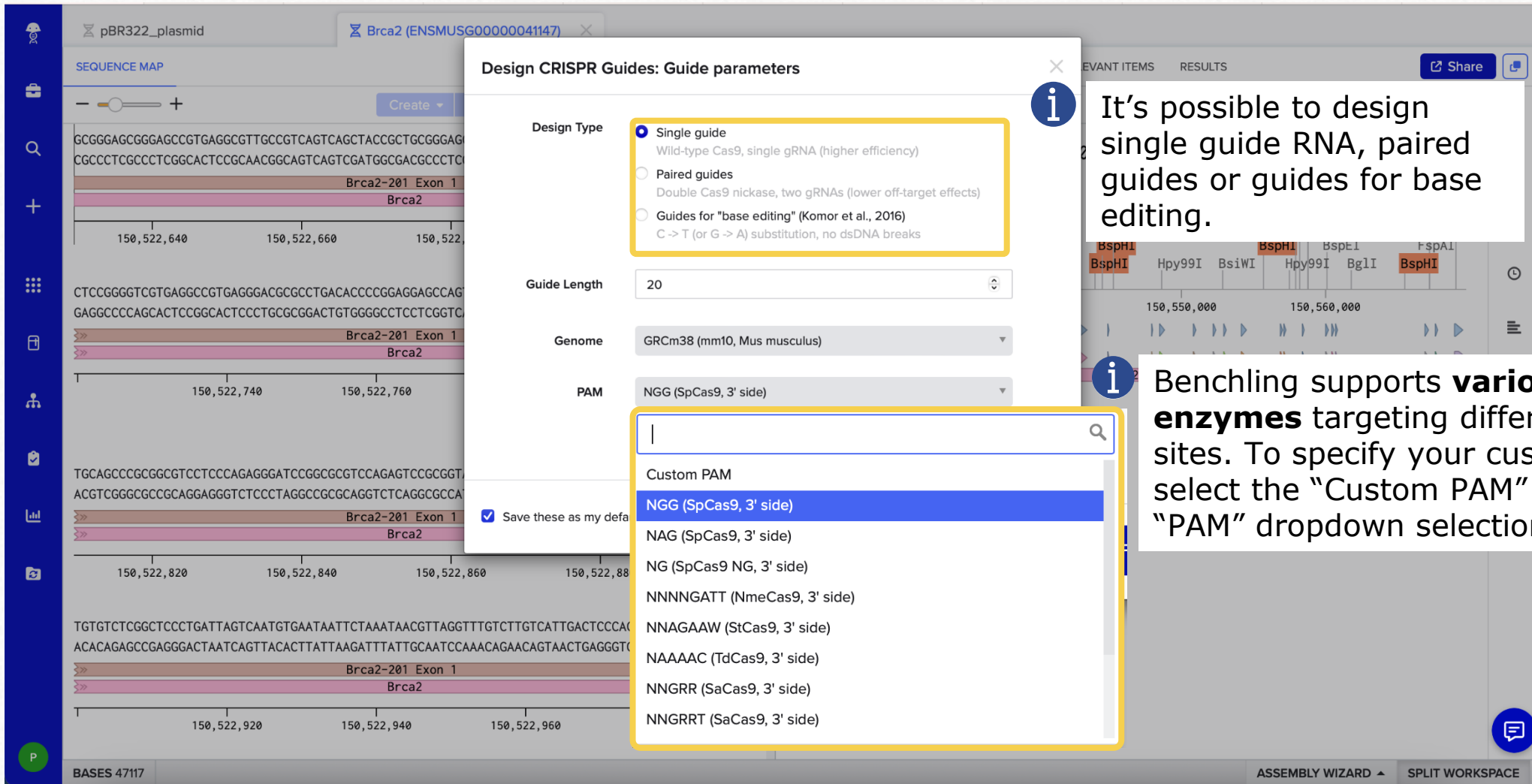
The right panel, labeled `LINEAR MAP`, shows a zoomed-in view of the `pta source (3929 bp)` region. It features a scale from 2,303,000 to 2,305,000 bp. Two gene models are shown: a blue bar for `pta gene` and an orange bar for `yfcC gene`. Below them, their respective CDS regions are shown: `pta CDS (pho...ransferase)` and `yfcC CDS...rotein)`. The interface includes various controls like zoom sliders, search bars, and a 'Share' button.

## Tool overview

**i** It is possible to create guide RNA sequences and Homologous recombination templates using the CRISPR tool. There are 2 ways to access it:

The screenshot shows the CRISPR tool interface. On the left, a sidebar is open to the 'CRISPR' menu, with 'CRISPR guides' and 'HR template' highlighted. A yellow box highlights the 'CRISPR' menu item in the sidebar, and another yellow box highlights the 'CRISPR' sub-menu in the main interface. The main interface displays a 'SEQUENCE MAP' for 'pBR322\_plasmid' and a 'PLASMID' map. The plasmid map shows various features like 'bla\_sig\_peptide', 'AmpR\_promoter', 'tet\_promoter', and 'ROP protein'. A yellow box highlights the 'CRISPR' sub-menu in the main interface, which contains options like 'Design and analyze guides', 'Saved Guide Analyses', and 'Design HR Template (ssODN)'. A blue arrow points from an information icon to the text on the right.

By default, Benchling will use the open sequence as to design the gRNA on



**Design CRISPR Guides: Guide parameters**

**Design Type**

- Single guide  
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**

**Genome** GRCm38 (mm10, Mus musculus)

**PAM** NGG (SpCas9, 3' side)

Save these as my default

**PAM Dropdown List:**

- Custom PAM
- NGG (SpCas9, 3' side)**
- NAG (SpCas9, 3' side)
- NG (SpCas9 NG, 3' side)
- NNNGATT (NmeCas9, 3' side)
- NNAGAAW (StCas9, 3' side)
- NAAAAC (TdCas9, 3' side)
- NNGRR (SaCas9, 3' side)
- NNGRRT (SaCas9, 3' side)

**Informational Callouts:**

- It's possible to design single guide RNA, paired guides or guides for base editing.
- Benchling supports **various Cas enzymes** targeting different PAM sites. To specify your custom PAM, select the "Custom PAM" option in the "PAM" dropdown selection.

# 7. CRISPR tools

## 7.1 gRNA design







- 3 Change the genome to ***E. coli* BL21(DE3)**. 4 Click **Finish** and continue.

### Design CRISPR guides: Guide parameters

**Design type**

Single guide  
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**

**Genome**

**PAM**

[Show advanced settings](#)

Save these as my default CRISPR settings

**Finish**

**i** Setting the genome is important for **off-target** calculations.

- 5 Give a name to your CRISPR design tab and save it so you can come back to it if you need to.

- 6 Select the **pta CDS** annotation. The target region will be set automatically.



The screenshot displays the CRISPR design tool interface. The left panel, titled 'SEQUENCE MAP', shows a DNA sequence with a 'pta gene' and 'pta CDS (phosph...yltransferase)' annotation. A yellow arrow points to the 'pta CDS' annotation. The right panel, titled 'DESIGN CRISPR', shows a 'pta gRNA' input field with a 'Save' button. Below the input field, a 'Target region' section displays coordinates '2302000' and '2304144' with a blue '+' button. A blue arrow points from the '+' button in the 'Target region' section to the '+' button in the 'DESIGN CRISPR' section.

SEQUENCE MAP DESCRIPTION

METADATA DNA FRAGMENT BATCH DESIGN CRISPR × ...

pta gRNA Save Settings

To get started, create a **target region** by selecting it on the sequence map and pressing +.

Target region 2302000 2304144 +

7 Click the + button to generate gRNA candidates.

8 Set the genome region as shown to obtain accurate **off-target** scores for the gRNA candidates.

SEQUENCE MAP DESCRIPTION

tttcacaccgccagctcagctggcggtgctgttttgaaccgcccaaatcggcgg  
aaagtgtggcggtcgagtcgaccgccacgacaaaacattgggcggttagccgcc

2,301,93501,94001,94501,95001,95501,96001,96501,97001,975

taacgaaaggataaacctgtcccgtattattatgctgatccctaccggaacc  
attgctttctcctatttggcacagggcataataatcagactagggatggccttgg

57 49  
56 50  
35 49  
56 50  
51 49  
38 49

pta gene  
pta CDS (phosph...yltransferase)

2,301,99001,99502,00002,00502,01002,01502,02002,02502,030

PshAI

62 50  
52 50  
56 49  
59 50

METADATA DNA FRAGMENT BATCH DESIGN CRISPR x ...

pta gRNA Save Settings

Target 2302000 2304144 +

region

<input checked="" type="checkbox"/>	Start	End	Annotations	Genome Region
<input checked="" type="checkbox"/>	2302000	2304144	pta CDS (phosphate acetyltransferase), pta gene	No region set

You don't have a genome region set above, so scores may not match scores from other sites. Benchling uses the genome region to locate your target region and to ignore potential off-target sites in that part of the genome.

The Doench, Fusi et al. (2016) paper publishes two models for scoring guides - one that includes the position of the cut within the translated gene and a simpler model that looks only at the guide sequence.

Genome region

Setting a genome region will remove it from off-target analysis.

None

Chromosome 2302000 2304144

Find genome matches ⚠

Set genome region Cancel

## gRNA design

**i** Benchmarking will show you a list of potential gRNAs to choose from. You can sort them by **on-target** or **off-target** score, or browse your sequence and select the best one for your needs based on its location.

The screenshot shows a CRISPR design tool interface. On the left, a 'SEQUENCE MAP' displays a DNA sequence with a 'pta gene' and 'pta CDS (phosphate acetyltransferase)' highlighted. A vertical line indicates the 'EagI' restriction site. On the right, the 'DESIGN CRISPR' tab shows a list of potential gRNAs. The table below is a representation of the data shown in the screenshot:

position	Strand	Guide sequence	PAM	On-Target score	Off-target score
2302910	-	gtagccgccagtcagcagca	ggg	76.3	99.2
2302761	-	agaaagtgcaggatttaacg	cgg	73.0	100.0
2303021	-	gaagctctgcaggctcagag	agg	72.0	100.0
2303477	+	cgaacagctggaagacaacg	tgg	71.8	99.4
2302391	+	aatcgctaaaacgctgaatg	cgg	71.6	100.0
<input checked="" type="checkbox"/> 2302190	+	tgcgaaactttccaccacga	cgg	70.6	99.4
2302210	-	tcaacgtagctcattttcag	cgg	69.7	99.8
2303170	-	agctgataacggaacgcagg	cgg	69.0	100.0
2303277	+	gccgctatctgtgctgaacg	tgg	68.8	99.8
2302436	-	acgctctttcagctgttccg	ggg	68.5	99.6
2302508	-	cagtttgttaacgataacgc	cgg	68.3	99.9

**9** Sort by **on-target** score.

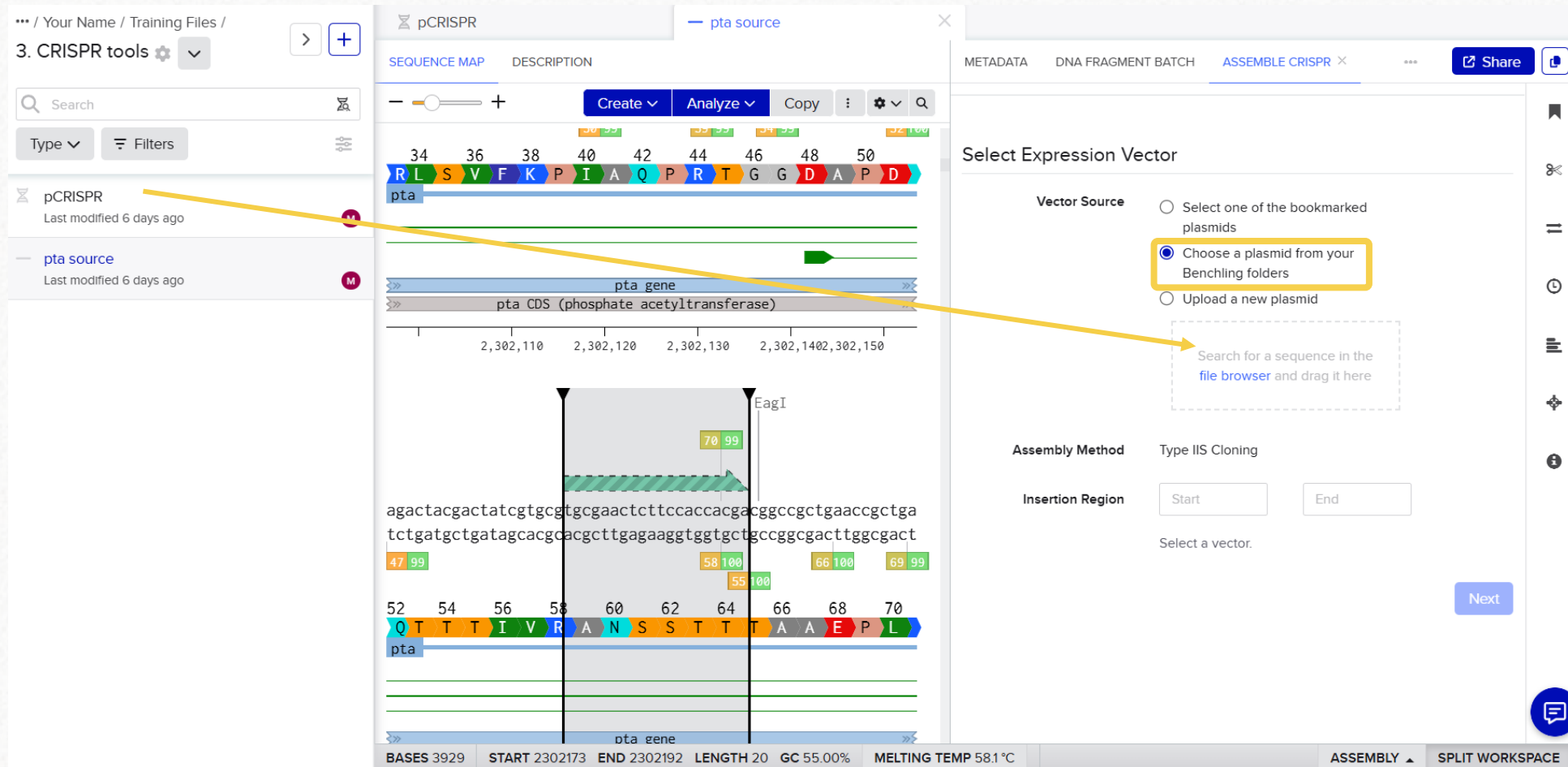
**10** Select the gRNA as shown.

**i** By clicking the blue **Save** button, you can create a file with your selected gRNA(s). You should do it for this example.

**11** Click **Assemble**.

**i** This option will allow you to place the chosen gRNA into a plasmid with Type IIS restriction sites.

12 Select the **Choose a plasmid from your Benchling folders** option and drag the **pCRISPR** file into the box.



3. CRISPR tools

Search

Type Filters

pCRISPR  
Last modified 6 days ago

pta source  
Last modified 6 days ago

pCRISPR

pta source

SEQUENCE MAP DESCRIPTION

CREATE ANALYZE COPY

34 36 38 40 42 44 46 48 50

R L S V F K P I A Q P R T G G D A P D

pta

pta gene

pta CDS (phosphate acetyltransferase)

2,302,110 2,302,120 2,302,130 2,302,140 2,302,150

EagI

70 99

agactacgactatcgtgcgtgcgaactctccaccacgacggccgctgaaccgctga

tctgatgctgatagcagcgcgcttgagaaggtggtgctgccggcgacttggcgact

47 99 58 100 66 100 69 99

52 54 56 58 60 62 64 66 68 70

Q T T T I V R A N S S T T T A A E P L

pta

pta gene

BASES 3929 START 2302173 END 2302192 LENGTH 20 GC 55.00% MELTING TEMP 58.1 °C

ASSEMBLY SPLIT WORKSPACE

METADATA DNA FRAGMENT BATCH ASSEMBLE CRISPR

Share

Select Expression Vector

Vector Source

- Select one of the bookmarked plasmids
- Choose a plasmid from your Benchling folders**
- Upload a new plasmid

Search for a sequence in the file browser and drag it here

Assembly Method Type IIS Cloning

Insertion Region Start End

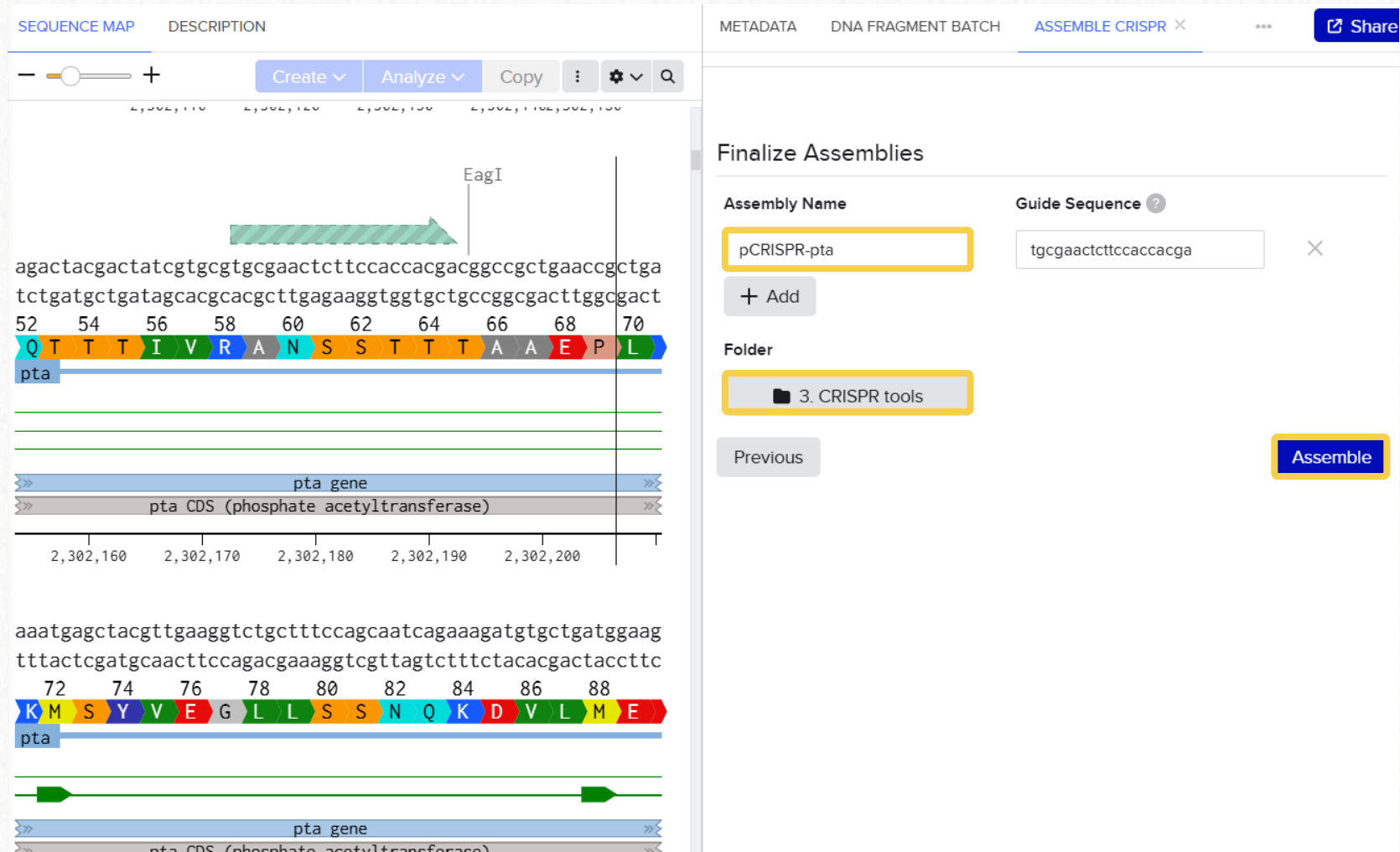
Select a vector.

Next

i The drag-and-drop option does not work in Safari.



- 15 Name your assembly, choose a location to save it and click **Assemble**.



The screenshot displays the CRISPR tools interface, divided into two main panels: the left panel for sequence analysis and the right panel for assembly finalization.

**Left Panel (SEQUENCE MAP):**

- Shows a genomic map with a green arrow indicating the location of the *EagI* restriction site.
- Displays the DNA sequence:
 

```

      agactacgactatcgtgctgcaactcttcaccacgacggcgcgtgaaccgctga
      tctgatgctgatagcagcagcgttgagaaggtggctgcccgcgacttggcgact
      52 54 56 58 60 62 64 66 68 70
      Q T T T I V R A N S S T T T A A E P L
      pta
      
```
- Below the sequence, a protein sequence is shown:
 

```

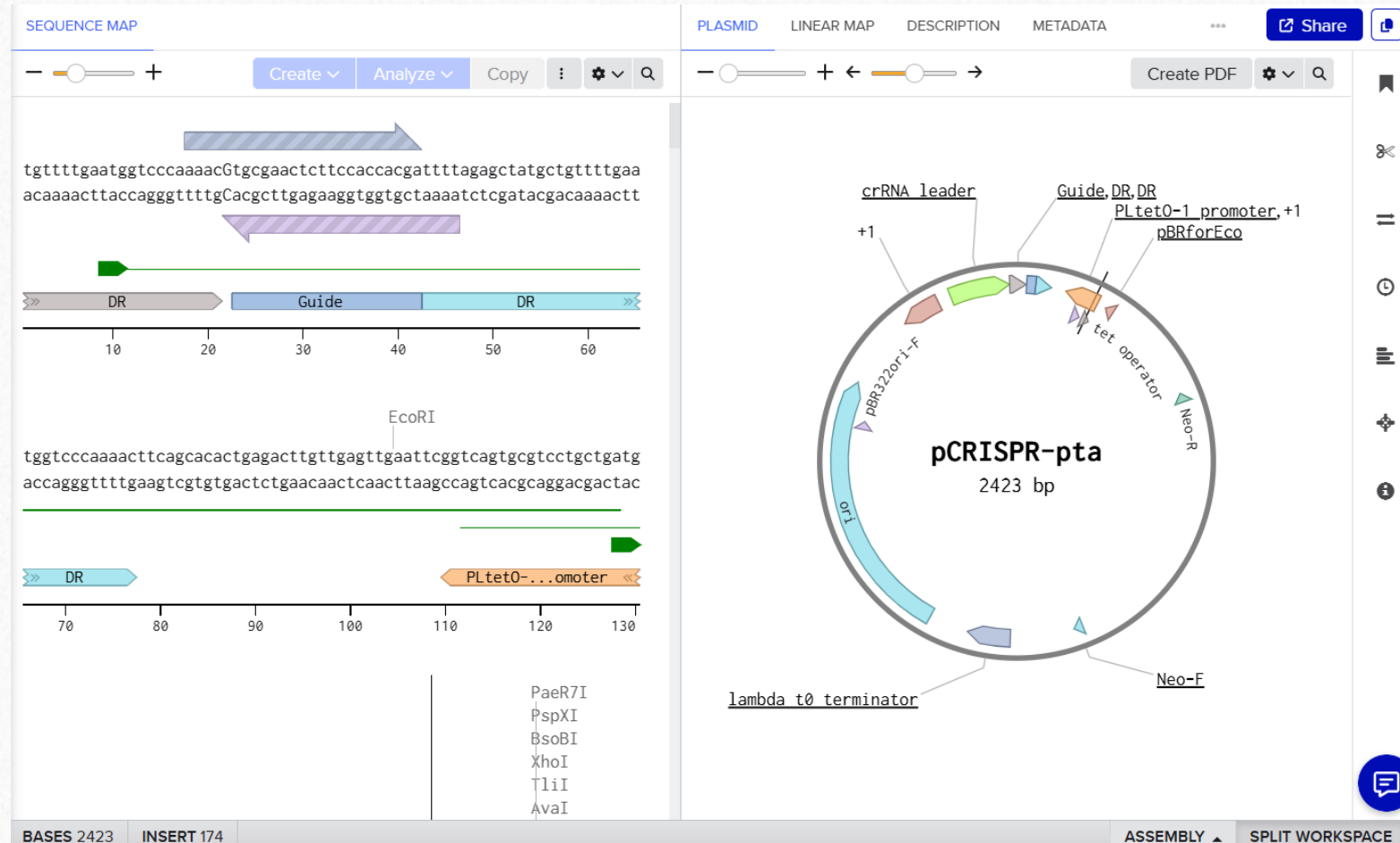
      K M S Y V E G L L S S N Q K D V L M E
      pta
      
```
- Gene annotations include "pta gene" and "pta CDS (phosphate acetyltransferase)".

**Right Panel (Finalize Assemblies):**

- Assembly Name:
- Guide Sequence:
- Folder:
- Buttons: "Previous" and "Assemble" (highlighted in yellow).

## gRNA design

- ✓ The result will be an expression vector with your chosen gRNA and a primer pair that can be annealed and ligated into the plasmid after digestion with BsaI.
- ✓ This can also be done with multiple gRNAs at a time.





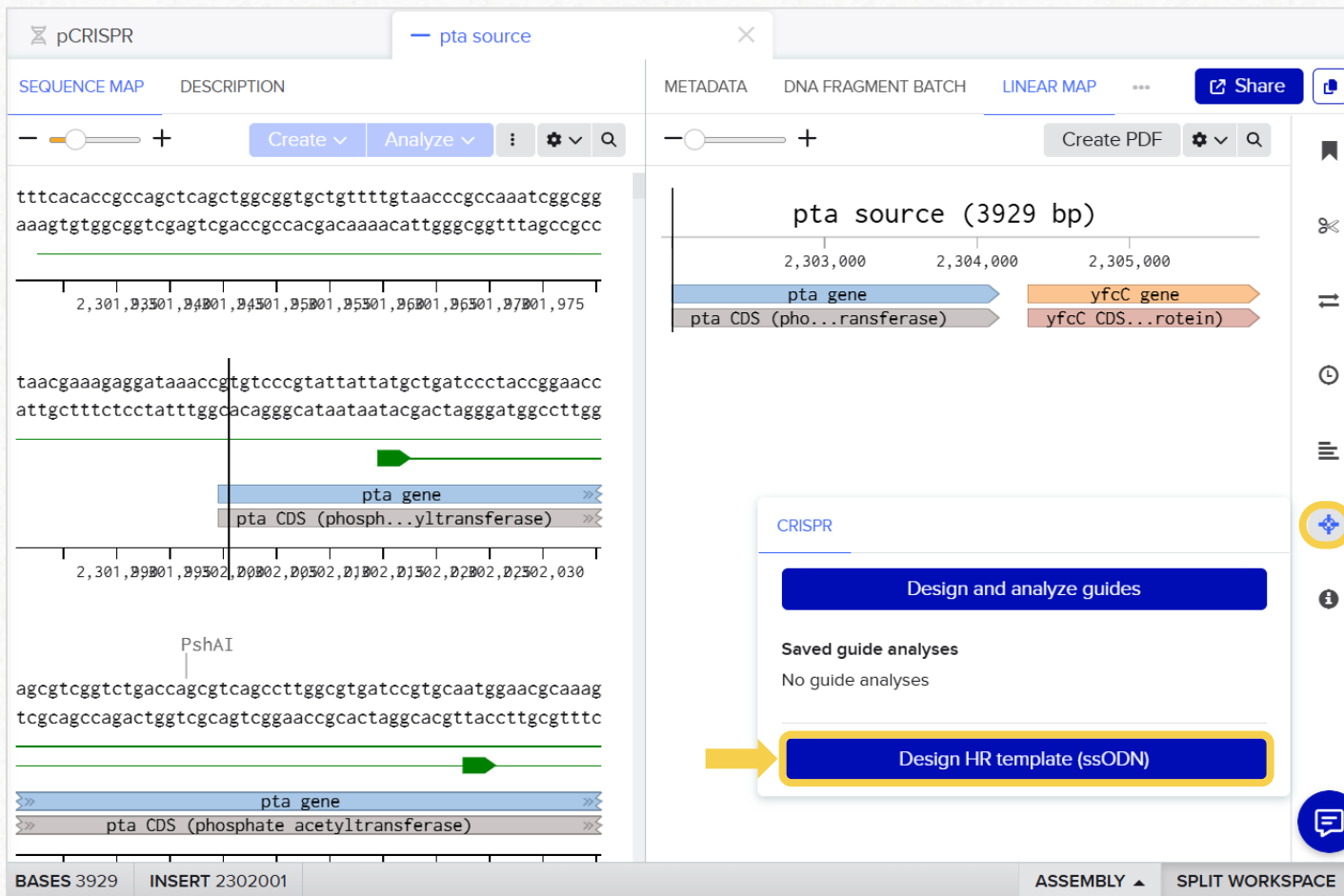
# 7. CRISPR tools

## 7.2 HR template design



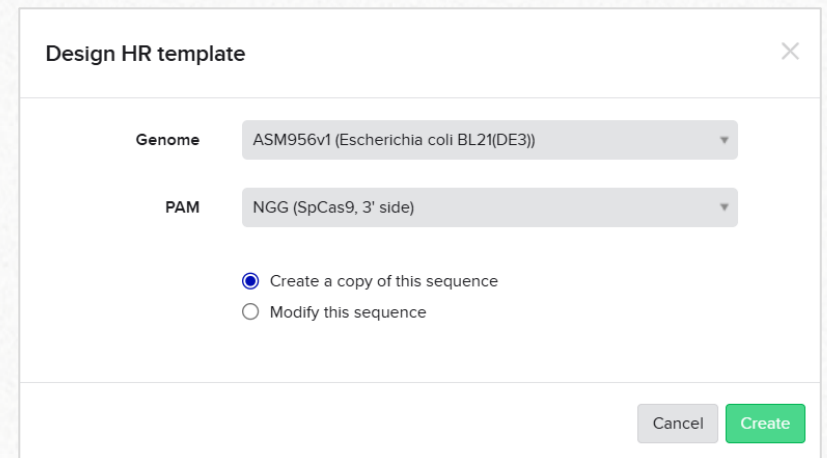
## HR template design

- 1 Open the **pta source** file.
- 2 Access the **HR template design** menu.



The screenshot displays the pCRISPR web interface. The main window shows the 'pta source' file (3929 bp) with a linear map and sequence. The 'pta gene' and 'pta CDS (phosphatyltransferase)' are highlighted. A 'PshAI' site is also indicated. The 'Design HR template' dialog box is open, showing the 'Genome' as 'ASM956v1 (Escherichia coli BL21(DE3))' and the 'PAM' as 'NGG (SpCas9, 3' side)'. The 'Create a copy of this sequence' option is selected. The 'Design HR template (ssODN)' button is highlighted with a yellow arrow.

- 3 Select the option to create a copy of the sequence.



The 'Design HR template' dialog box is shown, with the following settings:

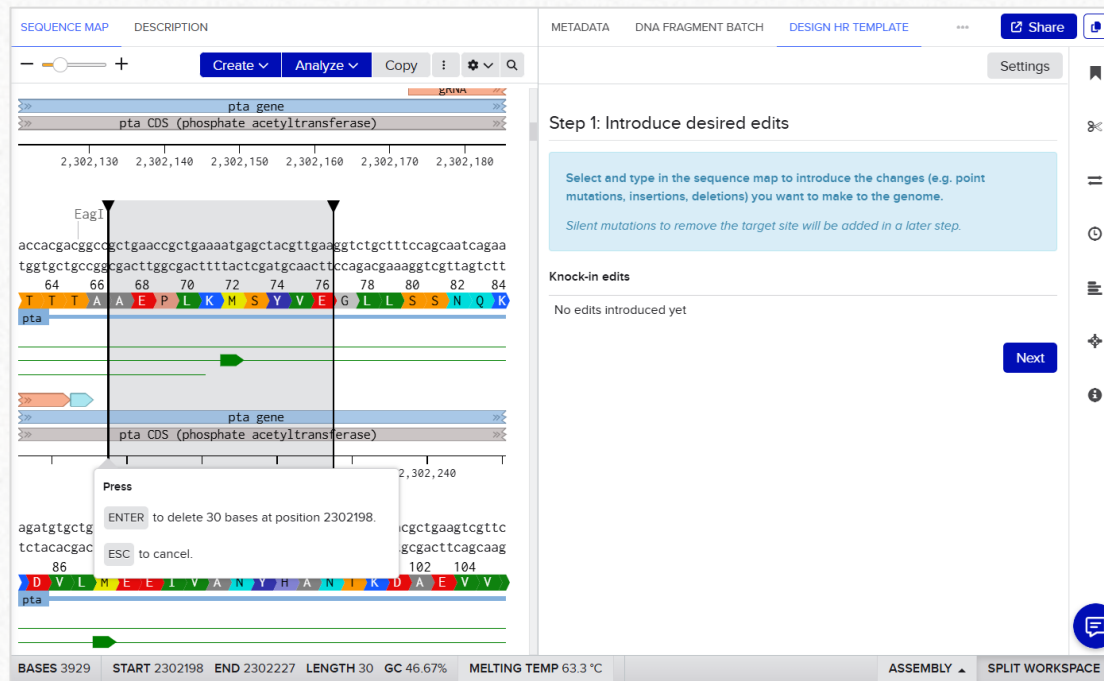
- Genome: ASM956v1 (Escherichia coli BL21(DE3))
- PAM: NGG (SpCas9, 3' side)
- Create a copy of this sequence
- Modify this sequence

Buttons: Cancel, Create

## HR template design

**i** You can introduce the desired modifications to the sequence, but do not remove the gRNA region nor its PAM. Benchling will look for **both** of them. The **PAM removal** will be done by the tool at a later stage.

**4** Delete 30 nt as shown.



SEQUENCE MAP DESCRIPTION METADATA DNA FRAGMENT BATCH DESIGN HR TEMPLATE ... Share

ptgRNA  
pta gene  
pta CDS (phosphate acetyltransferase)

2,302,130 2,302,140 2,302,150 2,302,160 2,302,170 2,302,180

EagI

accacgacgcccctgaaccgctgaaatgagctacgttgaagctctgtttccagcaatcagaa  
 tggctgcccggcgacttggcacttttactcgtcaactccagacgaaagctgtagtctt  
 64 66 68 70 72 74 76 78 80 82 84  
 T T T A A E P L K M S Y V E G L L S S N Q K  
 pta

pta gene  
pta CDS (phosphate acetyltransferase)

2,302,240

Press  
 ENTER to delete 30 bases at position 2302198.  
 ESC to cancel.

cgctgaagtcgttc  
 gcgacttcagcaag  
 102 104

D V L M E E I V A N Y H A N S K D A E V V  
 pta

BASES 3929 START 2302198 END 2302227 LENGTH 30 GC 46.67% MELTING TEMP 63.3 °C ASSEMBLY SPLIT WORKSPACE

Step 1: Introduce desired edits

Select and type in the sequence map to introduce the changes (e.g. point mutations, insertions, deletions) you want to make to the genome.

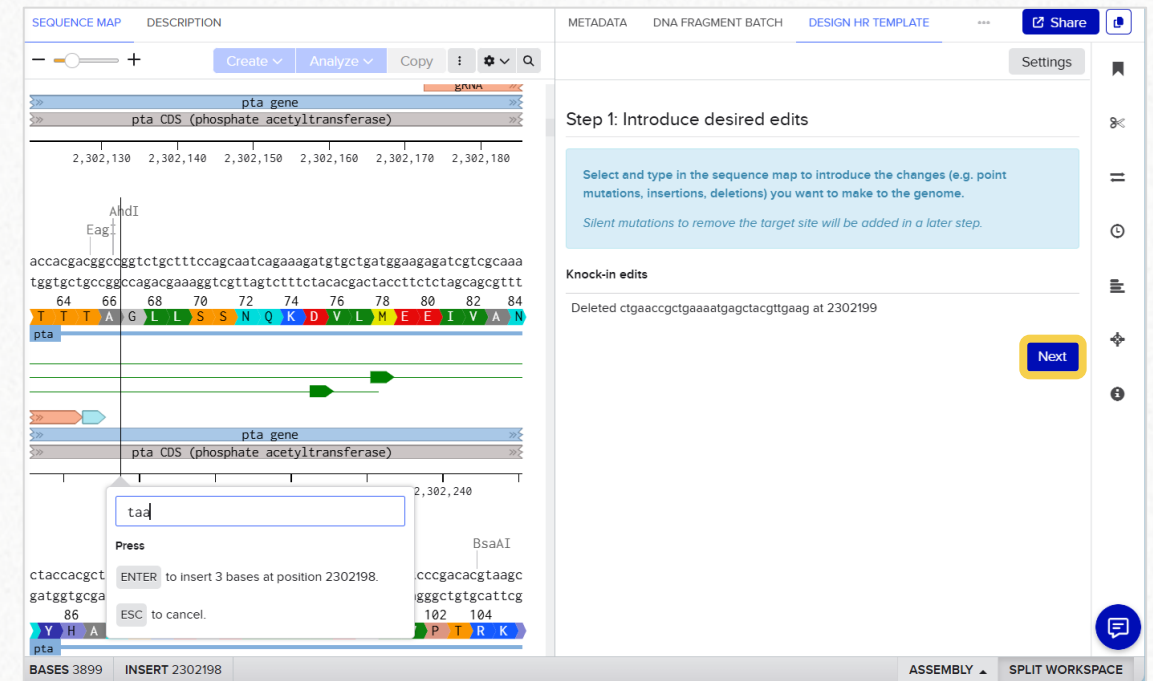
Silent mutations to remove the target site will be added in a later step.

Knock-in edits

No edits introduced yet

Next

**5** Insert a stop codon **in-frame** of the *pta* CDS.



SEQUENCE MAP DESCRIPTION METADATA DNA FRAGMENT BATCH DESIGN HR TEMPLATE ... Share

ptgRNA  
pta gene  
pta CDS (phosphate acetyltransferase)

2,302,130 2,302,140 2,302,150 2,302,160 2,302,170 2,302,180

EagI

accacgacgcccgctgctttccagcaatcagaaagatgctgtaggaagatcgtcgcgaaa  
 tggctgcccggcgacttggcacttttactcgtcaactccagacgaaagctgtagtctt  
 64 66 68 70 72 74 76 78 80 82 84  
 T T T A A G L L S S N Q K D V L M E E I V A N  
 pta

pta gene  
pta CDS (phosphate acetyltransferase)

2,302,240

Press  
 ENTER to insert 3 bases at position 2302198.  
 ESC to cancel.

cccgcacgtaagc  
 gggctgtgcattcg  
 102 104

Y H A P T R K  
 pta

BASES 3899 INSERT 2302198 ASSEMBLY SPLIT WORKSPACE

Step 1: Introduce desired edits

Select and type in the sequence map to introduce the changes (e.g. point mutations, insertions, deletions) you want to make to the genome.

Silent mutations to remove the target site will be added in a later step.

Knock-in edits

Deleted ctgaaccgctgaaatgagctacgttgaag at 2302199

Next

**6** Click **Next**.

**i** Benchling will select the region needed to create the HR template. You can adjust the length of the selection.

**SEQUENCE MAP** DESCRIPTION

Editing disabled because... Copy Create PDF Settings

20 22 24 26 28 30 32 34 36 38 40  
 S L G V I R A M E R K G V R L S V F K P I

pta

pta gene  
 pta CDS (phosphate acetyltransferase)

2,302,070 2,302,080 2,302,090 2,302,100 2,302,110

42 44 46 48 50 52 54 56 58 60 62  
 A Q P R T G G D A P D Q T T T I V R A N S S

pta

pta gene  
 pta CDS (phosphate acetyltransferase)

2,302,130 2,302,140 2,302,150 2,302,160 2,302,170 2,302,180

accacgacggcctaaggctctgctttccagcaatcagaaagatgtgctgatggaagatcgtcgc

METADATA DNA FRAGMENT BATCH **DESIGN HR TEMPLATE** Share

Step 2: Adjust HR arms

Adjust the region to use as the HR template by clicking and dragging the ends of the selection on the sequence map.

A 200 bp region around your mutations has already been selected. At least 50 bp on each side flanking the mutations is recommended.

Template region

2302098 - 2302297 Reset to default

Template Length: 200 bp  
 Left arm length: 100 bp  
 Right arm length: 99 bp

Knock-in edits

Deleted gctgaaccgctgaaaatgagctactg at 2302198

Deleted g at 2302199

Back Next

**7** Click **Next**.



### 8 Paste the gRNA sequence: tgcgactcttccaccacga

SEQUENCE MAP DESCRIPTION METADATA DNA FRAGMENT BATCH DESIGN HR TEMPLATE LINEAR MAP Share

Editing disabled because the sequenc... Copy Create PDF Settings

Step 3: Remove target site

Confirm the mutated bases and click Next to continue. Modify the mutations by clicking on the synonymous codons below.

Our default suggestion is to mutate the PAM, unless it occurs in a translation with no possible silent mutations. In that case, we try to introduce 2 mutations in the guide sequence, selecting codons to keep the [CAI](#) close to the original value.

tgcgactcttccaccacga

	R	A	N	S	S	T	T	T	A
Wildtype	cgt	gcg	aac	tct	tcc	acc	acg	acg	gcc
Mutations	AGA	GCA	AAC	AGC	AGC	ACA	ACA	ACA	GCA
	AGG	GCC	AAT	AGT	AGT	ACC	ACC	ACC	GCC
	CGA	GCG		TCA	TCA	ACG	ACG	ACG	GCG
	CGC	GCT		TCC	TCC	ACT	ACT	ACT	GCT
	CGG			TCG	TCG				
	CGT			TCT	TCT				
Final	cgt	gcg	aac	tct	tcc	acc	acg	ACC	gcc

Bases included to complete triplets are colored grey and the PAM site is colored blue.

Back Next

**i** The PAM will be removed from the HR template to prevent the degradation of the ssODN. You can choose from several alternatives, as shown in the table.

**9** Click **Next**.

## Step 4: Summary

## Knock-in edits

Deleted gctgaaccgctgaaaatgagctacgt at 2302026

Deleted g at 2302027

<b>Template Range</b>	2302098 to 2302297
<b>Guide</b>	tgcgaaactcttccaccacga
<b>Original Target Site</b>	... cgt gcg aac tct tcc acc acg <b>acg</b> gCC ...
<b>After Site Removal</b>	... cgt gcg aac tct tcc acc acg <b>ACC</b> gCC ...

Copy the [template](#) or its [reverse complement](#) to your clipboard.

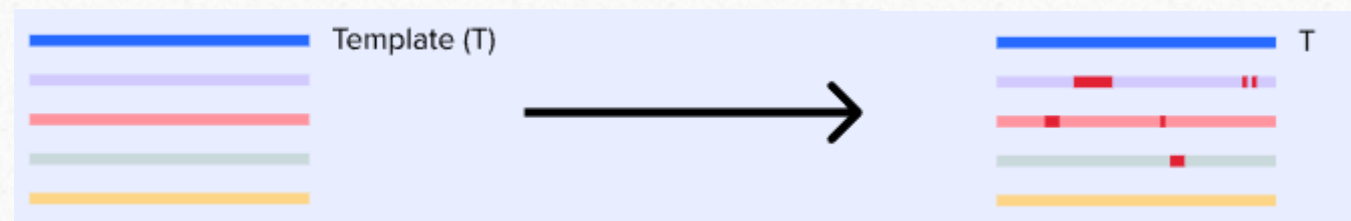
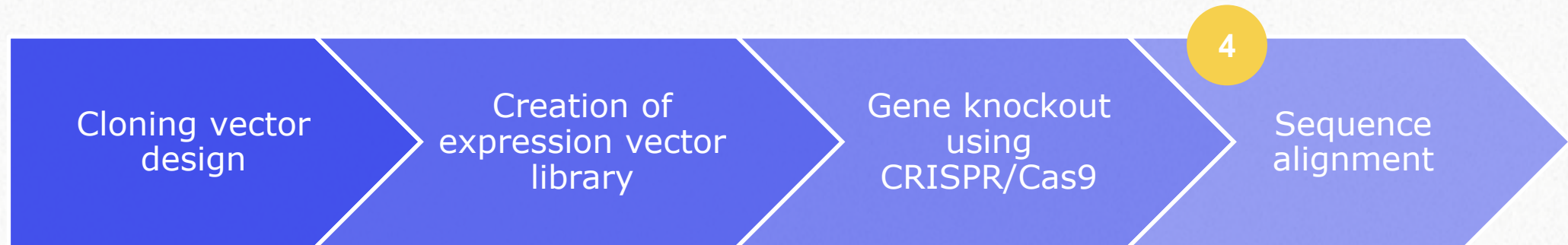
To design a template for the same knock-in edits but with a different guide, [click here](#).

- ✓ After the design process, you can copy the resulting HR template and paste it onto a new DNA sequence file to save it.

# 8. Sequence alignments



This is the fourth part of the *hands-on* example.



✓ Multisequence alignment

**Bonus:** How to do consensus alignments

**Expected output:**

- Alignments using pSEVA6311-phaC-pct540 as template
  - Sanger sequencing alignments
  - Plasmid sequencing alignment





You will need the files in the **Sequence alignments** subfolder.

... / Your Name / Training Files /

### 4. Sequence alignments

Search

Type ▾ Filters

- FW-seq-1  
Last modified 6 days ago
- MID-seq-1  
Last modified 6 days ago
- plasmid-seq  
Last modified 6 days ago
- pSEVA6311-phaC-pct540**  
Last modified 6 days ago

pSEVA6311-phaC-pct540

PLASMID DESCRIPTION

**pSEVA6311-phaC-pct540**  
7955 bp

Propionate-CoA transferase  
phaC1 (MBEL6-19)  
chnR-PchnB-GA-R  
PS1-1 (mismatches: 0)  
Preo -10  
chnR  
NdeI, +3  
oriT  
rep gene  
EcoRV  
EcoRV

METADATA PLASMID BATCH SEQUENCE MAP

Share

Create ▾ Analyze ▾

```

tgtatctcagggtgcattgtgtcattgttccgtgatatagcttctcataagcca
acatagagtcaccacgtaacacagtaaacaggcactatatcgaagagtattcgg
106 108 110 112 114 116 118 120 122
V S Q G A L C H L F R D I A S H K P
7642-1261

```

Propionate-CoA transferase

5 10 15 20 25 30 35 40 45 50 55

```

ggcgtatttacaaggtaggtatcggtagctttcattgacccagaaatggcggcg
ccgcataaatgtttccatccatagccatgaaagtaactggggtctttaccgccc
124 126 128 130 132 134 136 138 140
G V F T K V G I G T F I D P R N G G
7642-1261

```

Propionate-CoA transferase

60 65 70 75 80 85 90 95 100 105 110

```

gtaaagtaatgatattaccaagaagatattgtgaattgtagagattaaggg
catttcattactataatggttcttctataacaacttaaccatctctaattccc

```

BASES 7955 INSERT 1180 ASSEMBLY SPLIT WORKSPACE

# 8. Sequence alignments

## 8.1 Alignment tool



## Alignment tool overview

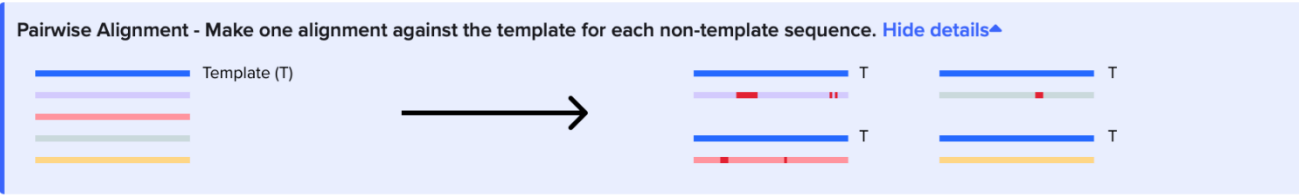
- i** In a real-life scenario, the construct sequences could be sent to sequencing. The results could then be analyzed using the **alignment tool** in Benchling.
- ✓ There are **three alignment options** and several alignment programs available:

### Create DNA / RNA alignment

1 Choose input 2 Define parameters

Pairwise Multisequence Consensus

Pairwise Alignment - Make one alignment against the template for each non-template sequence. [Hide details](#)



Template(s) + Non-template sequence(s)

Choose an alignment program.

Auto (MAFFT) Show parameters

Alignments performed via [MAFFT v7 \(Katoh, Standley 2013\)](#).

- 1 Pairwise alignment:**  
Sequences are compared against a template sequence, creating individual alignment files for each non-template seq.

## Alignment tool overview

- i** In a real-live scenario, the construct sequence could be sent to sequencing. The results could then be analysed using the **alignment tool** in Benchling.
- ✓ There are **three alignment options** and several alignment programs available:

Create DNA / RNA alignment

1 Choose input 2 Define parameters

Pairwise **Multisequence** Consensus

**Multisequence Alignment** - The results will be attached as a single alignment on the template sequence. [Hide details](#)

Template (T) → T

If you'd like to perform a contig alignment (shown below), we recommend selecting the MAFFT "local pairwise" algorithm.

Template (T) → T

Template(s) + Non-template sequence(s)

Choose an alignment program.

Auto (MAFFT) Show parameters

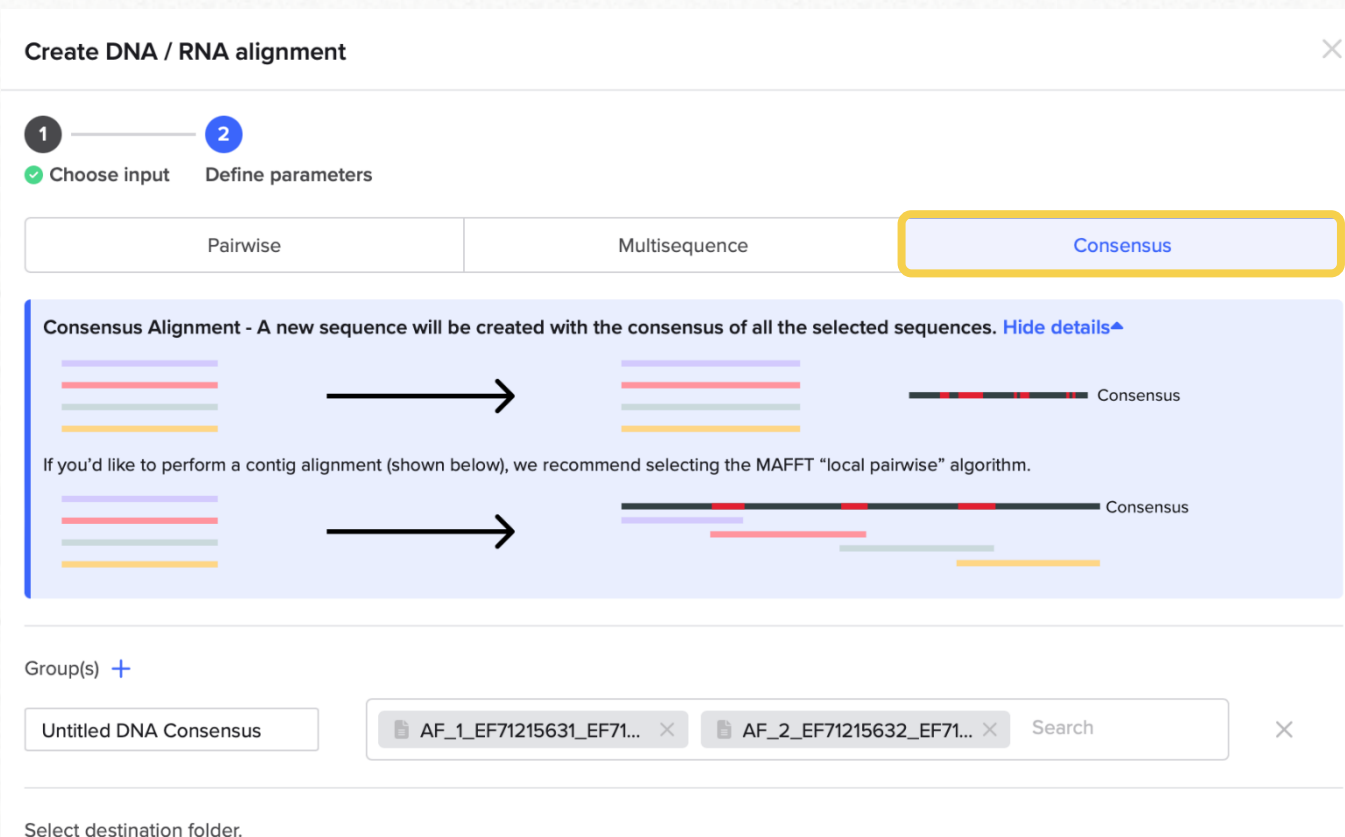
2

### Multisequence alignment:

Multiple sequences are compared against a template sequence, creating a unique alignment file for all the non-template sequences

## Alignment tool overview

- i** In a real-live scenario, the construct sequence could be sent to sequencing. The results could then be analysed using the **alignment tool** in Benchling.
- ✓ There are **three alignment options** and several alignment programs available:



Create DNA / RNA alignment

1 Choose input 2 Define parameters

Pairwise Multisequence **Consensus**

Consensus Alignment - A new sequence will be created with the consensus of all the selected sequences. [Hide details](#)

If you'd like to perform a contig alignment (shown below), we recommend selecting the MAFFT "local pairwise" algorithm.

Group(s) +

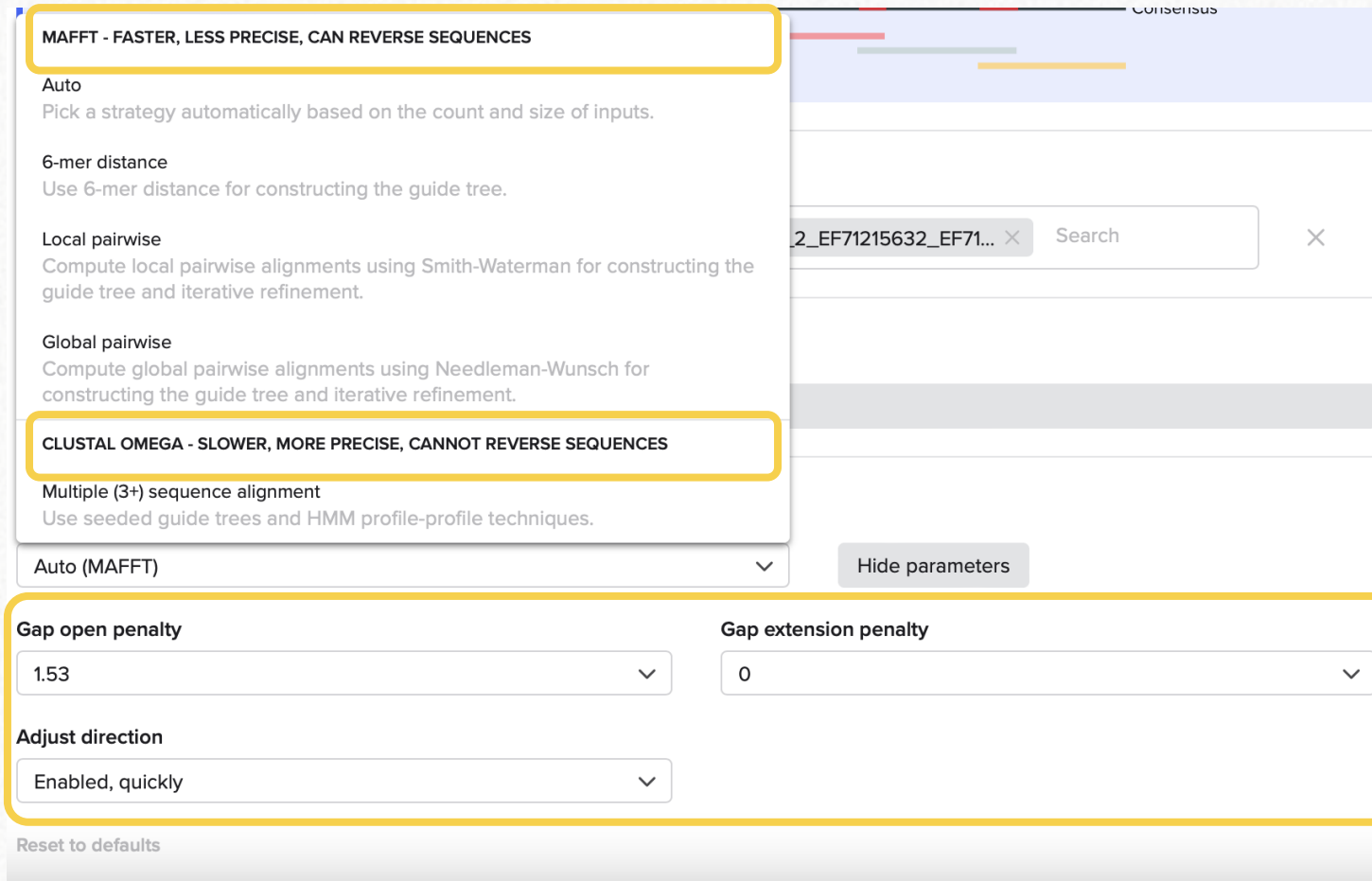
Untitled DNA Consensus AF\_1\_EF71215631\_EF71... AF\_2\_EF71215632\_EF71... Search

Select destination folder.

**3**

### Consensus alignment:

Multiple sequences are compared against each other, creating a new sequence from the consensus region of all the sequences.



**MAFFT - FASTER, LESS PRECISE, CAN REVERSE SEQUENCES**

Auto  
Pick a strategy automatically based on the count and size of inputs.

6-mer distance  
Use 6-mer distance for constructing the guide tree.

Local pairwise  
Compute local pairwise alignments using Smith-Waterman for constructing the guide tree and iterative refinement.

Global pairwise  
Compute global pairwise alignments using Needleman-Wunsch for constructing the guide tree and iterative refinement.

**CLUSTAL OMEGA - SLOWER, MORE PRECISE, CANNOT REVERSE SEQUENCES**

Multiple (3+) sequence alignment  
Use seeded guide trees and HMM profile-profile techniques.

Auto (MAFFT) ▼ Hide parameters

**Gap open penalty** 1.53 ▼ **Gap extension penalty** 0 ▼

**Adjust direction** Enabled, quickly ▼

[Reset to defaults](#)

- ✓ It's possible to choose between multiple types of **MAFFT** algorithms and **Crustal Omega** multisequence algorithm to power the alignment.
- ✓ Some of the key parameters of these can be changed as needed.

# 8. Sequence alignments

## 8.2 Multisequence alignment



# Alignment creation

## Multisequence alignment

1 Open the **Sequence alignments** folder.

2 Select all the files in the folder. From the **Analyze** menu, select **Create DNA/RNA Alignment**.

The screenshot shows a file management interface with a table of sequence files. The 'Name' column is selected, and the 'Analyze' menu is open with 'Create DNA / RNA Alignment' highlighted. The table contains the following data:

Name	Inventory	ID	Modified	Authors	Descr
FW-seq-1			11/02/2025		
MID-seq-1			11/02/2025		
plasmid-seq	No inventory availa...		11/02/2025		
pSEVA6311-phaC-pct540	No inventory availa...		11/02/2025		

**i** This way of starting alignments can be helpful if you have multiple sequences to work with.



### Create DNA / RNA alignment

1 Choose input — 2 Define parameters

Upload sequence and trace files (.ab1, .ftv, .fasta, .gb, and .geneious). RNA uploads are not currently supported.

Drag and drop to upload or

Search for a DNA / RNA sequence.

---

Create a DNA / RNA sequence from scratch.

**Nucleotide type\***

DNA  RNA

Sequences

FW-seq-1 ×  MID-seq-1 ×  plasmid-seq ×  pSEVA6311-phaC-pct540 ×

3 Click **Next**.

# Alignment creation

## Multisequence alignment




- 4 Configure the alignments to create two separate ones, as shown, both using **pSEVA6311-phaC-pct540** as template.



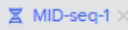
### Create DNA / RNA alignment



1 Choose input    2 Define parameters

Pairwise    **Multisequence**    Consensus

Multisequence Alignment - The results will be attached as a single alignment on the template sequence. [Show details](#)

Template(s)   Non-template sequence(s) 

   Search

  Search

Choose an alignment program.

**MAFFT**  
recommended for nucleotide alignments  
Faster, less precise, can reverse sequences

Clustal Omega  
recommended for amino acid alignments  
Slower, more precise, cannot reverse sequences

Auto (MAFFT)    Show parameters

Alignments performed via [MAFFT v7 \(Katoh, Standley 2013\)](#).

Back    **Create Alignment**

- 5 Create the alignments.

6 Go to the file you used as template and open the **Alignments** menu. You will find both alignments here.

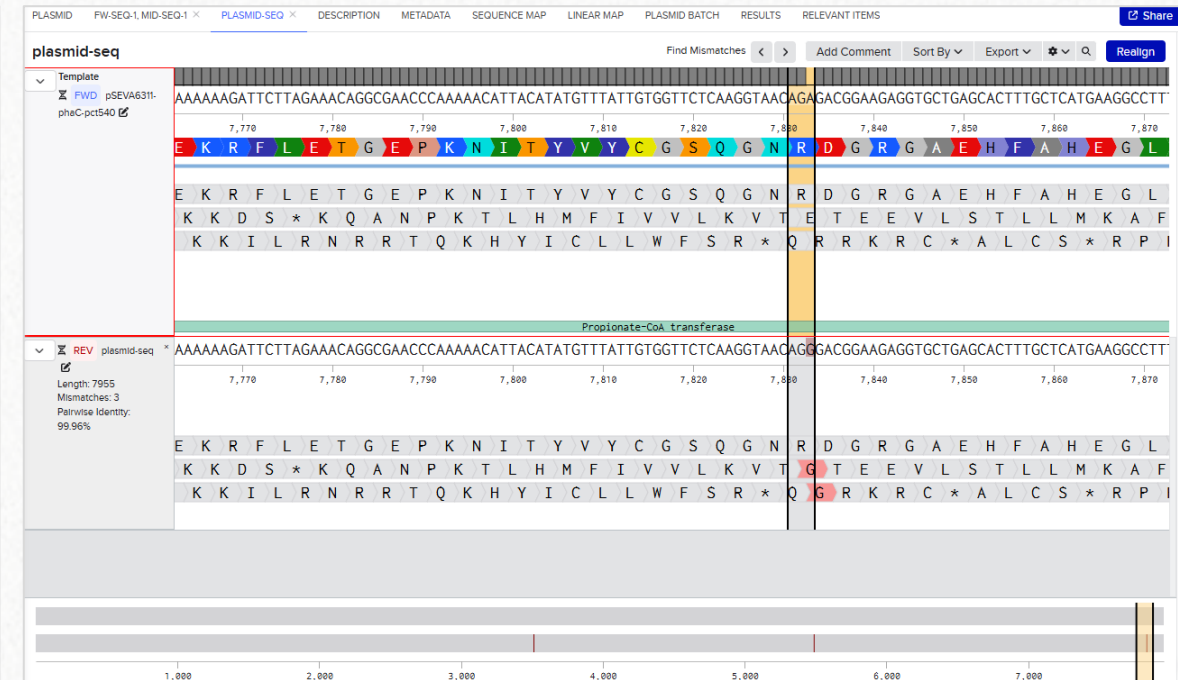
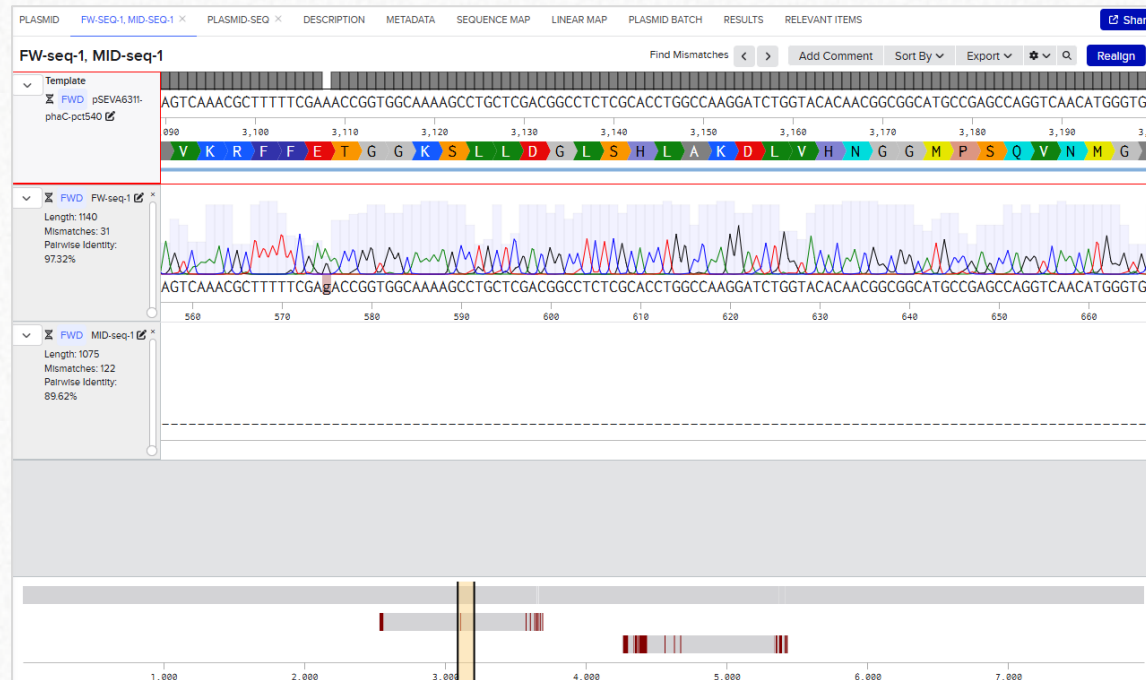
The screenshot displays the Benchling interface for the plasmid **pSEVA6311-phaC-pct540** (7955 bp). On the left, a circular plasmid map shows features including *Propionate-CoA transferase*, *phaC1* (MBEL6-19), *chnR*, *acc3* CDS, *oriT*, *rep gene*, *PSI-1* (mismatches: 0), and *chnR*. On the right, the **SEQUENCE MAP** view shows a protein sequence with a highlighted region (residues 106-122) corresponding to the sequence **V S Q G A L C H L F R D I A S H K P**. A **SEQUENCE ALIGNMENTS** panel is open, showing a **Create New Alignment** button and a **Saved Alignments** section with a yellow arrow pointing to it. The saved alignments list includes **FW-seq-1, MID-seq-1** and **plasmid-seq**, both dated 17/02/2025 18:55.

7 Open the alignments.

# Alignment creation

## Multisequence alignment

- ✓ You can now see and browse your resulting alignments.
- ✓ You may notice the first one includes trace files, which can help you assess the quality of the sequencing and assess whether the result can be considered accurate.
- ✓ The second one is a sequencing file for the whole plasmid. You can assess mismatches and toggle certain view options to check, for example, for amino acid changes in your CDS.



# 8. Sequence alignments

## 8.3 Consensus alignment



SEQUENCE MAP DNA CONSENSUS × LINEAR MAP DESCRIPTION METADATA RELEVANT ITEMS RESULTS Share

**DNA Consensus** Realign In Sync Find Mismatches < > Add Comment Sort By Export Settings Search

**Consensus**  
 DNA Consensus

**REV**  
 AF\_2\_EF71215632\_EF71  
 215632  
 (AF\_2\_EF71215632\_EF71215632.ab1)

**FWD**  
 AF\_1\_EF71215631\_EF712  
 15631  
 (AF\_1\_EF71215631\_EF71215631.ab1)

**Overview navigation panel**

BASES 1387 ASSEMBLY WIZARD SPLIT WORKSPACE

Consensus region fragment

Analysed sequences

# Alignment creation

## Consensus alignment navigation

You can jump from one mismatch to the other easily

**Find Mismatches** < >

Mismatches from the consensus sequence are marked in red.

You can edit what elements to visualize

Translation and reading frames have some additional settings that can be customized

The screenshot displays the DNA Conser interface with the following elements:

- Consensus:** DNA Consensus track showing the sequence: `tgcaagtctgtggagttccatgtataagggtatacatattagaaaagctttacgattatataactaacatcatgtacaaaacaat`
- REV:** Reverse strand track showing the sequence: `TGCAAGTTCGTGGAGTTCATGTATAAGGTATACATATTAGAAAAGCTTTACGATTATATACTAACATCATGTACAAAACAAT`
- FWD:** Forward strand track showing the sequence: `GGTCCCCCAATTACAGCTTTACGATTATATACTAACATCATGTACAAAACAAT`
- Annotations:** Amino acid indices track showing: `L Q V L W S S M Y K V Y I L E K L Y D Y I L T S C T K Q`
- Translations:** Amino acid indices track showing: `C K F C G V P C I R Y T Y * K S F T I I Y * H H V Q N N`
- Trace Quality:** A chromatogram showing signal intensity for each base.
- Mismatches:** A yellow box highlights mismatches in the amino acid sequence: `S P Q L E` (top) and `G P P N * S` (bottom).
- Settings Menu:** A list of visualization options:
  - Annotations
  - Translations
  - Amino Acid Indices
  - Reading Frames
  - Primers
  - Alignment Axis
  - Sequence Axis
  - DNA
  - Trace
  - Trace Quality
  - Quality-based capitalization (20)
  - Votes
  - Row Statistics
  - Comments
  - Expanded Mini-Map
- Navigation:** A 'Find Mismatches' button with left and right arrows.
- Footer:** Bases 1387, Start 278, End 281, Length 4, GC 0.00%, Melting Temp -83.7 °C, Assembly Wizard, Split Workspace.

# 9. Tips and tricks





# Tips and tricks

## Overview:

- You can work in bulk using the expanded view of the workspace
- Re-indexing of sequences when creating alignments.
- Benchmarking [trouble-shooting articles](#) and [Help center](#) offers many resources, frequently asked questions and articles that can help you
  - Biosustain learning material: [Brilliant Basics: The Molecular Biology Suite - LIMS Help Guides](#)



# Tips and tricks

## Work in bulk using the expanded view

You can use the **expanded view** of the workspace to:

- ✓ Edit, move, archive... entities in bulk
- ✓ Create Multi-sequence alignments, attach and detach primers, autofill annotations and transcriptions, auto annotate...

**Pro TIP:** if you access the expanded view from the search, you will have access to all your entities, not only the ones contained in a particular project folder. Also, more filters will be available

Projects / RDM\_Support

Search

Type Filters

MolBio_training_DNA Consensus	Last modified 16 hours ago	P
Alignment file 1_circular	Last modified 7 hours ago	P
Alignment file 2_circular	Last modified 16 hours ago	P
Alignment file1	Last modified 16 hours ago	P
Alignment file 2	Last modified 16 hours ago	P
pBR322_linear	Last modified 18 hours ago	P
P (Cat) promoter	Last modified 18 hours ago	P

# Tips and tricks

## Work in bulk using the expanded view

You can use the **expanded view** of the workspace to:

- ✓ Register, edit, move, archive... entities in bulk

The screenshot displays a workspace interface with a table of items. The table has columns for Name, Inventory, ID, Modified, Entry Dates, Authors, and Review Stat. Four items are listed, each with a checked checkbox in the Name column. Above the table is a toolbar with various icons. Blue callout boxes with arrows point to specific icons: 'Register' points to the expanded view icon (a 3x3 grid), 'Move to' points to the move icon (a folder with an arrow), 'Copy to' points to the copy icon (two overlapping documents), 'Archive' points to the archive icon (a document with a checkmark), 'Create request' points to the request icon (a document with a plus sign), 'Add to worklist' points to the worklist icon (a document with a plus sign), and 'Export' points to the export icon (a document with a plus sign). The interface also shows a search bar, a 'Type' dropdown, and 'Filters'.

<input type="checkbox"/>	Name	Inventory	ID	Modified	Entry Dates	Authors	Review Stat
<input checked="" type="checkbox"/>	MolBio_training_DNA Consensus			31/10/2023		Patricia Brito Diaz	
<input checked="" type="checkbox"/>	Alignment file 1_circular			01/11/2023		Patricia Brito Diaz	
<input checked="" type="checkbox"/>	Alignment file 2_circular			31/10/2023		Patricia Brito Diaz	
<input checked="" type="checkbox"/>	Alignment file1			31/10/2023		Patricia Brito Diaz	

# Tips and tricks

The screenshot displays a software interface with a table of items and a context menu. The table has columns for 'Name' and 'Inventory'. The context menu is open over the table, showing various actions. The 'Analyze' option is highlighted with a yellow box, and a yellow arrow points from it to a 'More' dropdown button in the table's right-hand side.

Projects / RDM\_Support Saved Searches

Search Type Filters

< > 1-24 of 24 items

Name	Inventory
<input checked="" type="checkbox"/> MolBio_training_DNA Consensus	
<input checked="" type="checkbox"/> Alignment file 1_circular	
<input checked="" type="checkbox"/> Alignment file 2_circular	
<input checked="" type="checkbox"/> Alignment file1	

Context Menu:

- Create DNA / RNA Alignment
- Create AA Alignment
- Auto-Annotate
- Attach Primers
- Auto-fill part fields
- Auto-fill translations
- Auto-fill transcriptions
- Set topology
- Codon optimize
- Remove annotations
- Detach primers
- Unlink parts
- Remove translations
- Back translate

Table Actions:

- Open
- Analyze
- Bulk edit
- Refresh
- More

# Tips and tricks

## Autoindexing when creating alignments

When creating an alignment of circular sequences, Benchling by default performs an **auto indexing** of these sequences.

To change this, after creating the alignment, you will have to realign the file and unmark the “automatically reindex” box.

The screenshot shows the Benchling interface for sequence alignment. A yellow circle with the number '1' highlights the 'Realign' button in the top toolbar. The interface displays a sequence alignment between a template and a sample. The template sequence is GGTCCCCCAATTAGAGCTTTACGATTATATACTAACATCATGTACAAAACAATTTAATAATGATCTGTATTGCTGGCTCAATCCACGTAAATTAATGCCTCAGCACTAGTCCTGCAGGGTAAC. The sample sequence is G P P N \* S F T I I Y \* H H V Q N N L I M I C I A G S I H V N \* C L S T S P A G V T V P P I R A L R L Y T N I M Y K T I \* \* \* S V L L A Q S T \* I N A S A L V L Q G \* F. Below the sequences is a chromatogram showing the signal for each base. The 'Realign' button is highlighted in blue, and the 'Out of Sync' button is highlighted in orange. The 'Find Mismatches' button is also visible. The interface includes a sidebar with a 'Template' section and a 'Sample' section. The 'Sample' section shows the sequence EF71215631\_EF71215631 with a length of 1088, 0 mismatches, and 100% pairwise identity. The 'Realign' button is highlighted with a yellow circle and the number 1.

# Tips and tricks

## Autoindexing when creating alignments

**2** Realign DNA / RNA

1 Choose Input 2 Define parameters

Upload sequence and trace files (.ab1, .ftv, .fasta, .gb, and .genelous). RNA uploads are not currently supported.

Drag and drop to upload or

Search for a DNA / RNA sequence.

Search by name

Create a DNA / RNA sequence from scratch.

Nucleotide type\*

DNA  RNA

Name  Bases

Current sequences

Sequence	Use Latest Version
<input checked="" type="checkbox"/> Alignment file 2_circular	<input checked="" type="checkbox"/>
<input checked="" type="checkbox"/> EF71215631_EF71215631	<input type="checkbox"/>

New sequences

**3** Realign DNA / RNA

1 Choose Input 2 Define parameters

Pairwise  Multisequence  Consensus

Your realignment must be the same type as your original alignment.

Multisequence Alignment - The results will be attached as a single alignment on the template sequence. [Show details](#)

Template(s)  Alignment file 2\_circular

Non-template sequence(s)  EF71215631\_EF71215631

Choose an alignment program.

The fields below are set to the values you chose for your most recent alignment. These values may not reflect the selections you made when you last performed this particular alignment.

Auto (MAFFT)

Alignments performed via MAFFT v

Automatically reindex alignment if needed.

If you check this box, Benchling will automatically reset the origin of the template sequence to better accommodate the corresponding non-template sequences.


# Tips and tricks

## Autoindexing when creating alignments

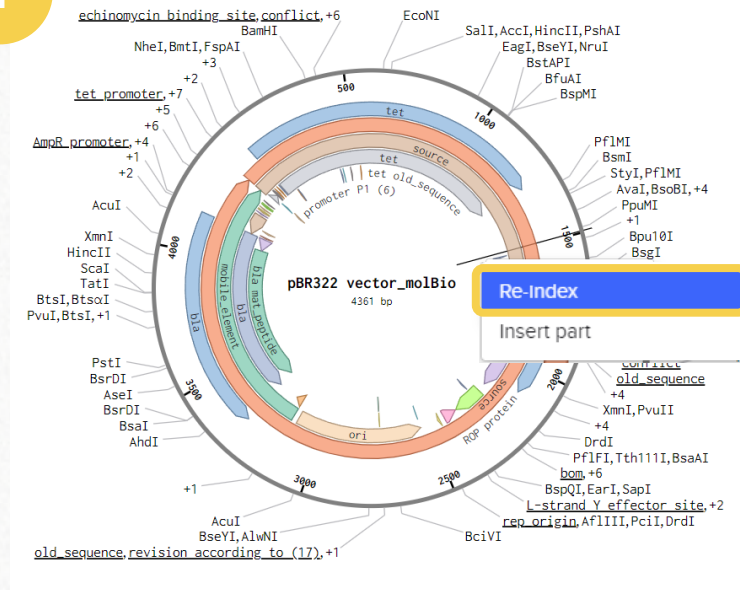
When creating an alignment of circular sequences, Benchling by default performs an **auto indexing** of these sequences.

To change this, after creating the alignment, you will have to realign the file and unmark the “automatically reindex” box.

### Pro TIP:

- ✓ You can always re-index a circular plasmid by right-clicking on any part of the sequence. For linear sequences, the index can be changed using the “information” tab on the right panel. 
- ✓ Make sure to have your sequences correctly indexed before performing an alignment to avoid further complications.

1



2

**Re-index** ✕

Enter the desired location for the current base (e.g. enter 100 for the current base to become the 100<sup>th</sup> base).

Current Location	1504	
New Location	<input style="width: 80%;" type="text" value="1"/>	

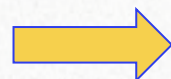
Re-index
Cancel

# 10. Resources





# Questions?



Contact [lims\\_support@biosustain.dtu.dk](mailto:lims_support@biosustain.dtu.dk)



# More resources

## Benchling Learning Labs

Benchling provides a **learning platform** that offers role-specific courses that can be taken in a **flexible-pace** structure.

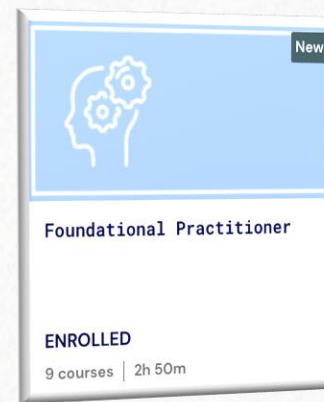
<https://www.benchling.com/learning-labs>



**Molecular Biology Tools**

FREE

8 courses | 2h 45m



**Foundational Practitioner**

ENROLLED

9 courses | 2h 50m

## Welcome to Benchling Learning Labs!

The destination to achieve your Benchling learning goals

Course Catalog

Get Certified

Email Us



### Practitioner

Essential skills for all Benchling R&D Cloud users, covering core applications and best practices.



### Administrator

Additional training for Benchling Administrators, covering roles, permissions, configurations, and more.



### Developer

Specialized training covering Developer Platform fundamentals such as APIs, Events, and more.



### Consultant

Additional training for consulting partners covering the Benchling Implementation Methodology.

## More resources

### Benchling Help Center

Benchling provides some short guides on main functionalities

<https://help.benchling.com/hc/en-us>

