The basics of the Molecular Biology Tool

Reach out when struggling with the platform:

Biosustain Benchling support
lims_support@biosustain.dtu.dk

Access Benchling:

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)

Mía López Portillo Ontiveros
RDM/LIMS support

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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
- ✓ Codon optimization
- ✓ Worklists integration
- ✓ *In silico* PCR and digestions
- ✓ Customizable enzyme lists

AA / Protein Analysis

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

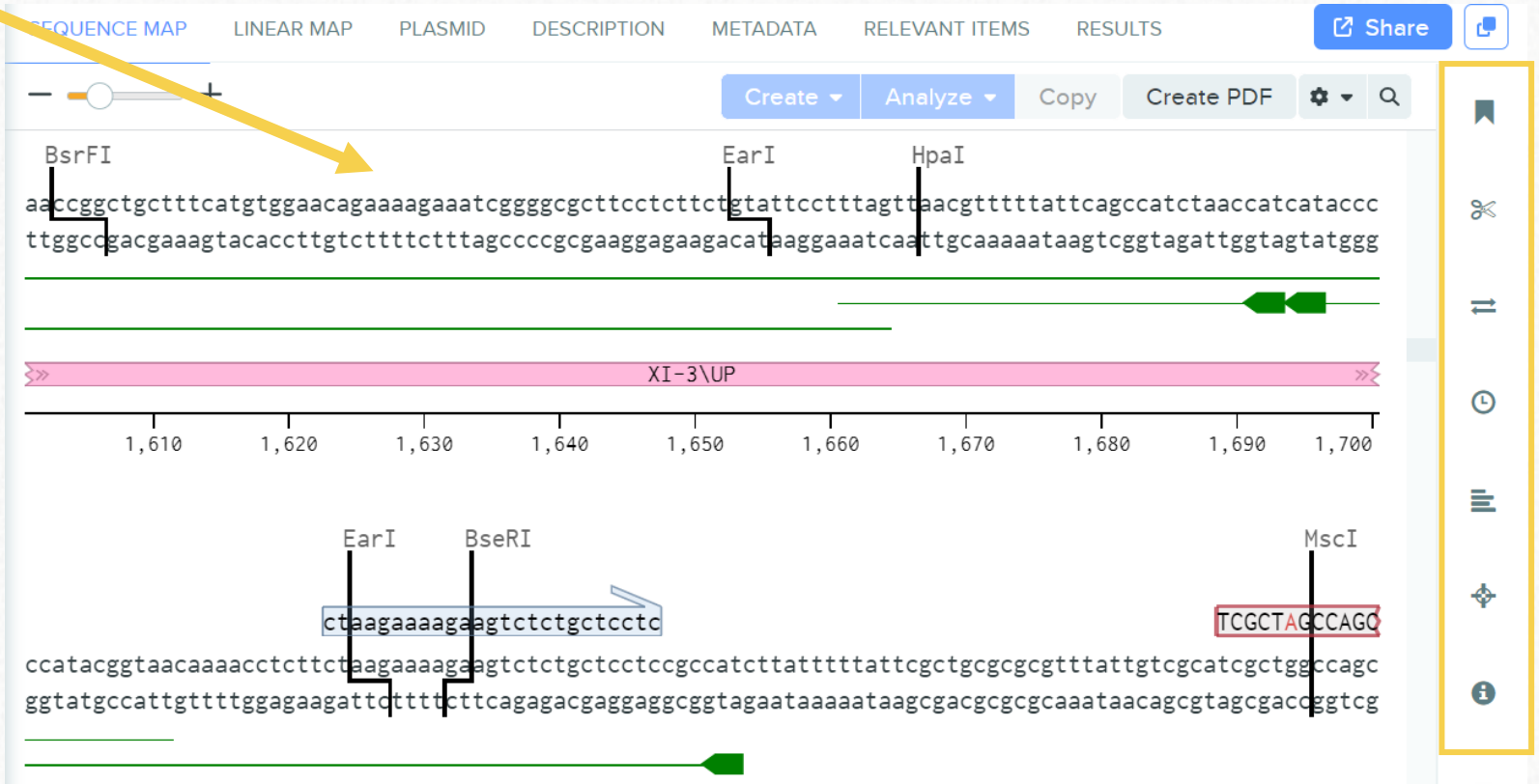
CRISPR

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

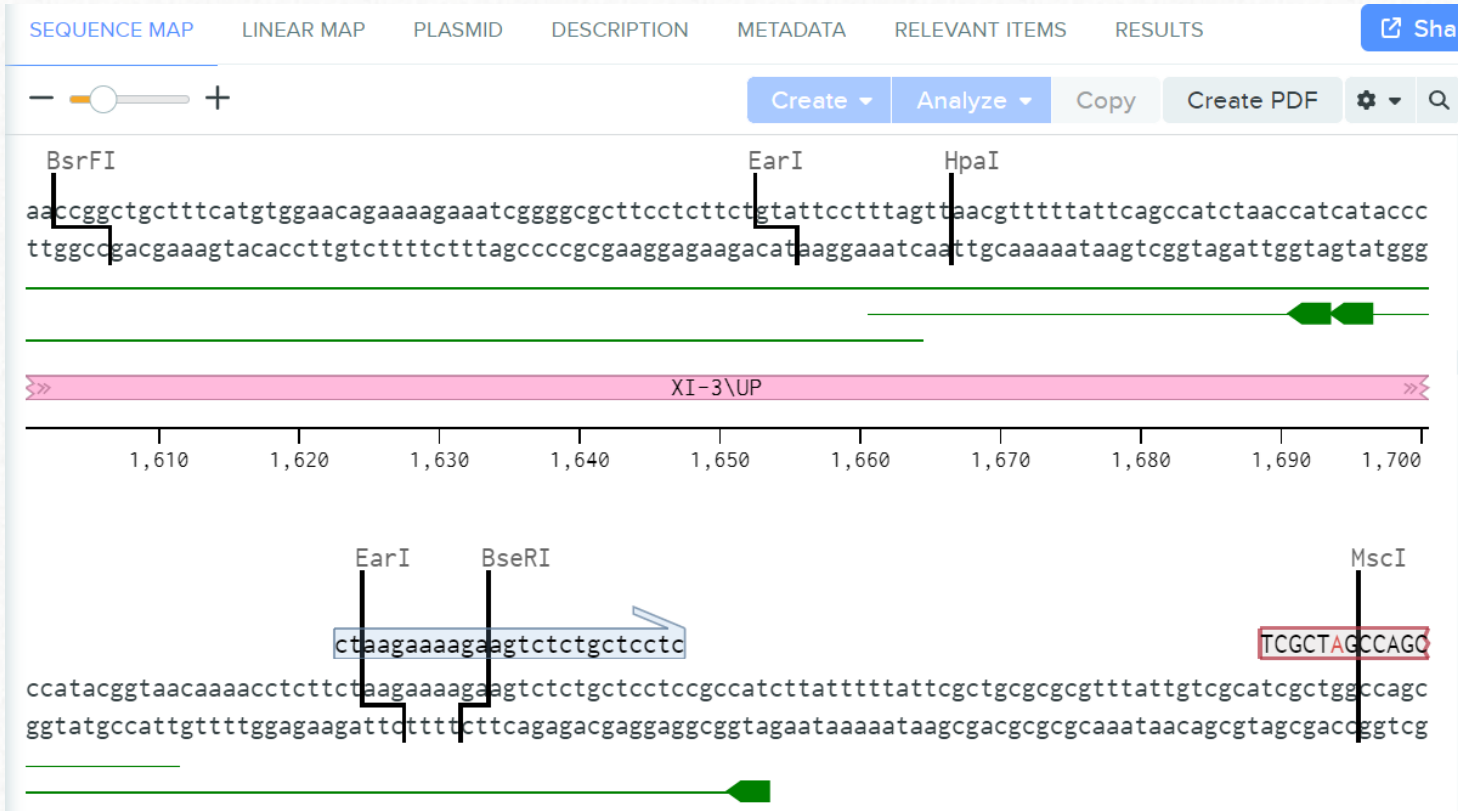
Functionalities and tools overview

Functionalities

Your sequence



Functionalities and tools overview

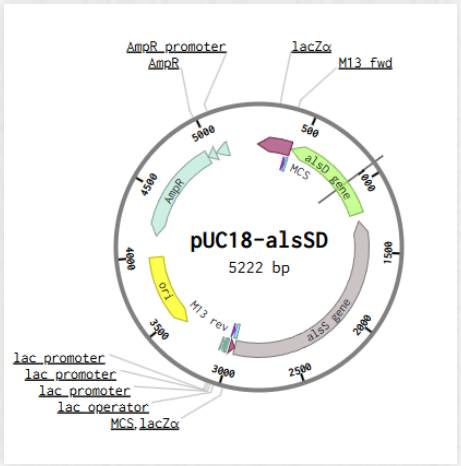


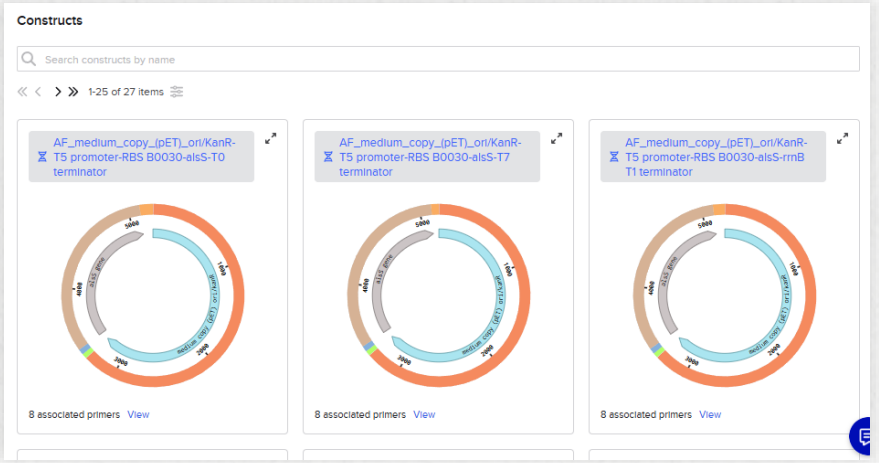
- Features *(annotations and translations)*
- Digests
- Primers
- History
- Alignments
- CRISPR
- Information *(topology, tags)*

2. Training overview

Training goals:

The basics of…

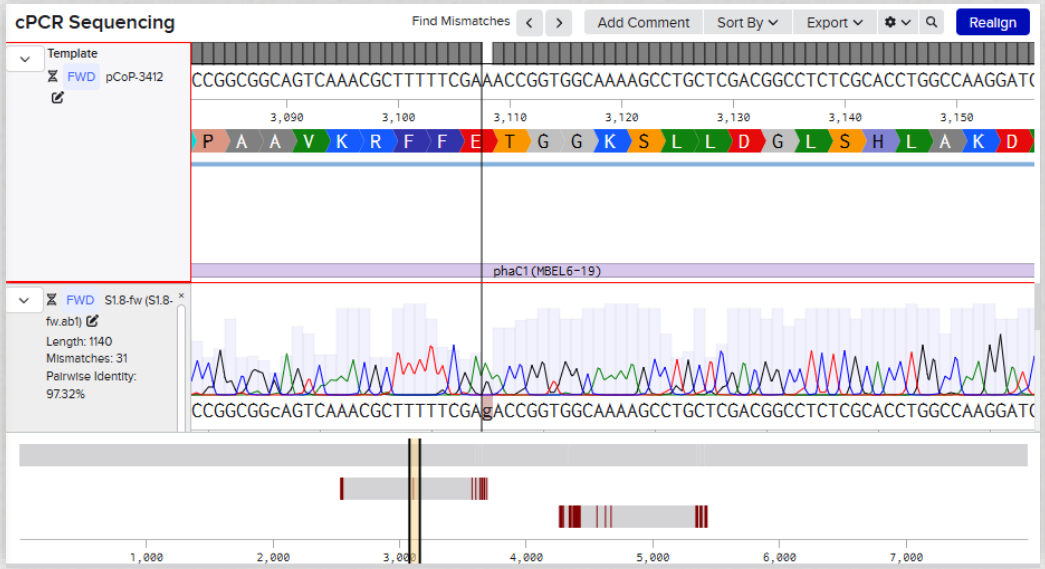


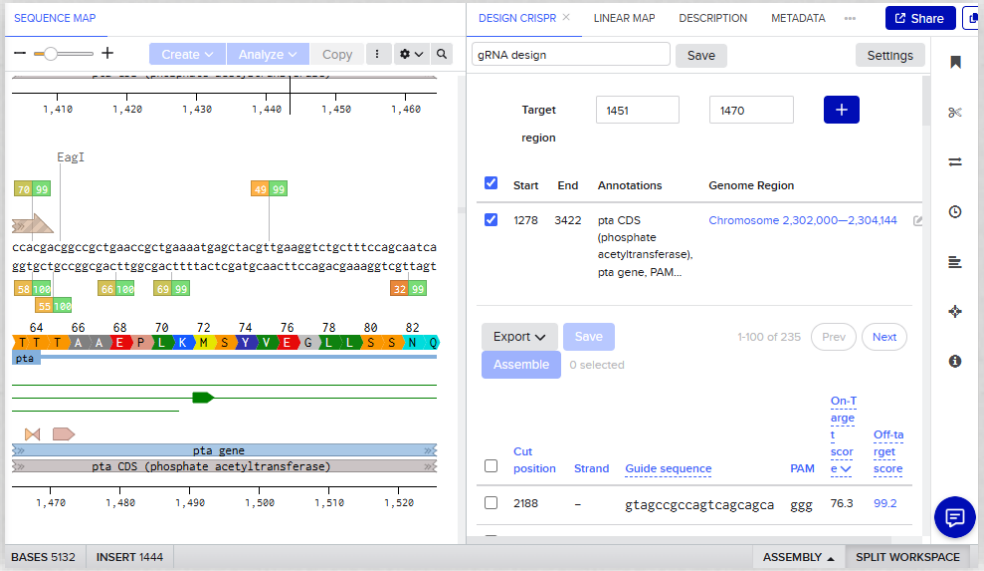


- ❑ How to navigate the **sequence visualization** window and the workspace
- ❑ How to **assemble** simple constructs
- ❑ How to perform bulk assemblies with the **Combinatorial Assembly** tool

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

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***

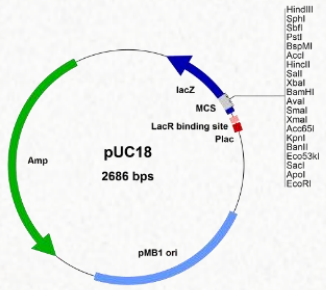
1

Cloning vector design → Creation of expression vector library → Gene knockout using CRISPR/Cas9 → Sequence alignment

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

alsS *alsD*

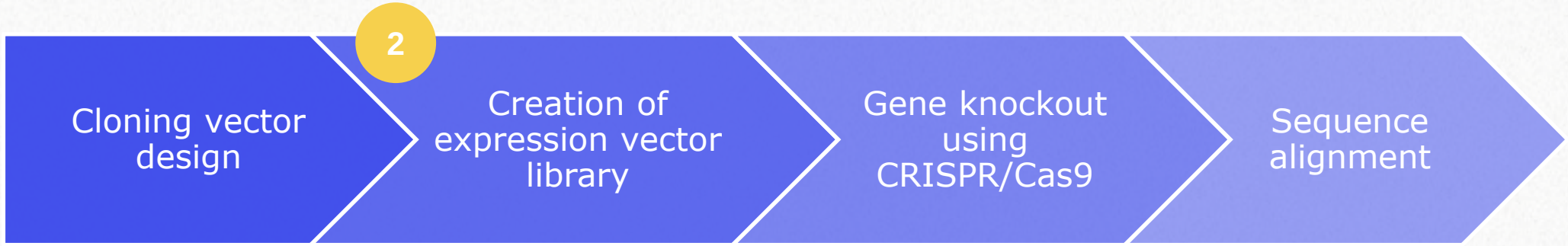
pUC18 cloning vector

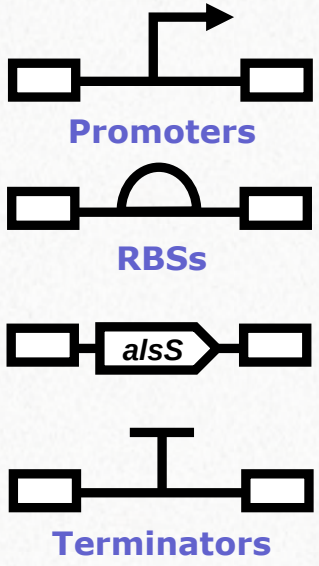


- ✓ Primer design
- ✓ *In silico* PCR
- ✓ Virtual digestion
- ✓ RE-based cloning

Today's work example:

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

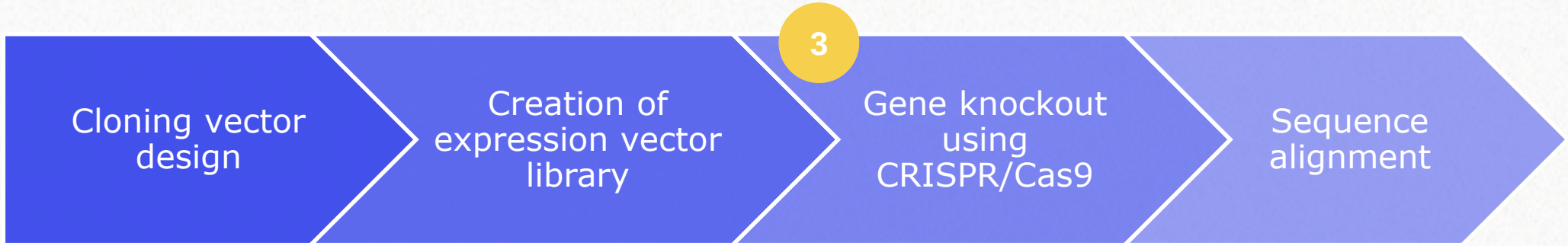




✓ Combinatorial cloning: **Golden Gate**

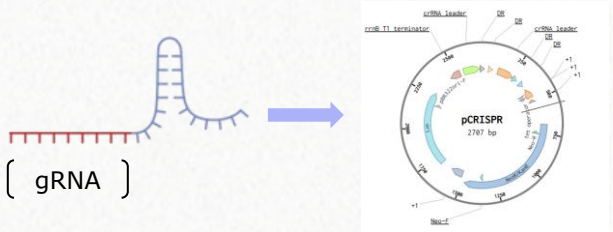
Today's work example:

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



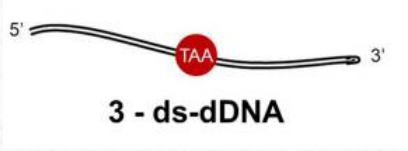
Target: *pta* in *E. coli*

gRNA design + assembly into pCRISPR



- ✓ gRNA design
- ✓ HR template design

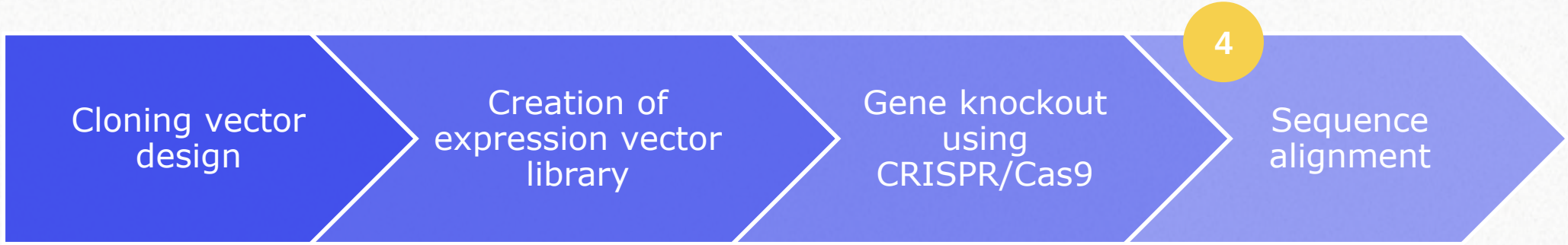
HR template design to KO *pta*



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.

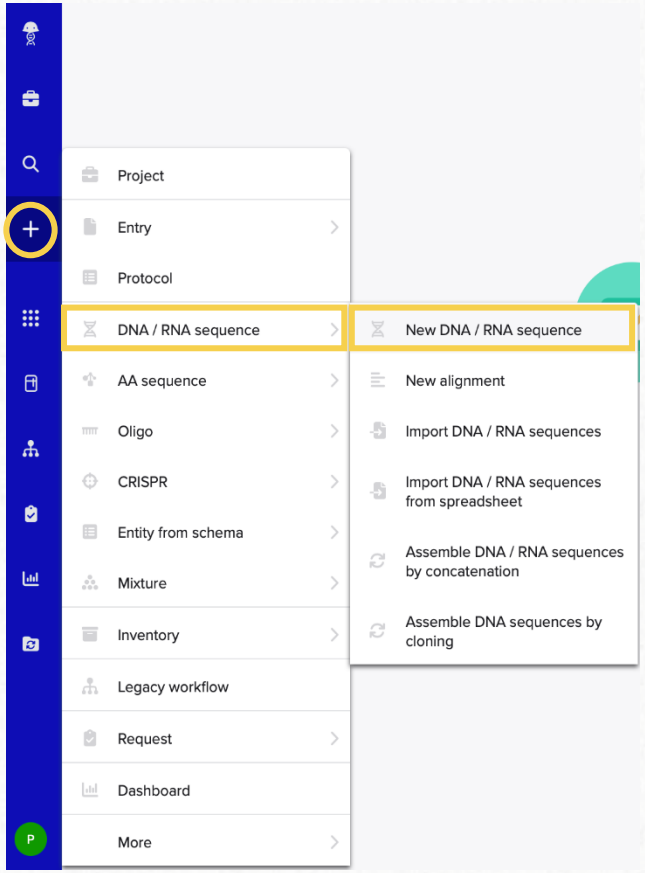
3. The basics of sequences

3.1 Sequence creation and import

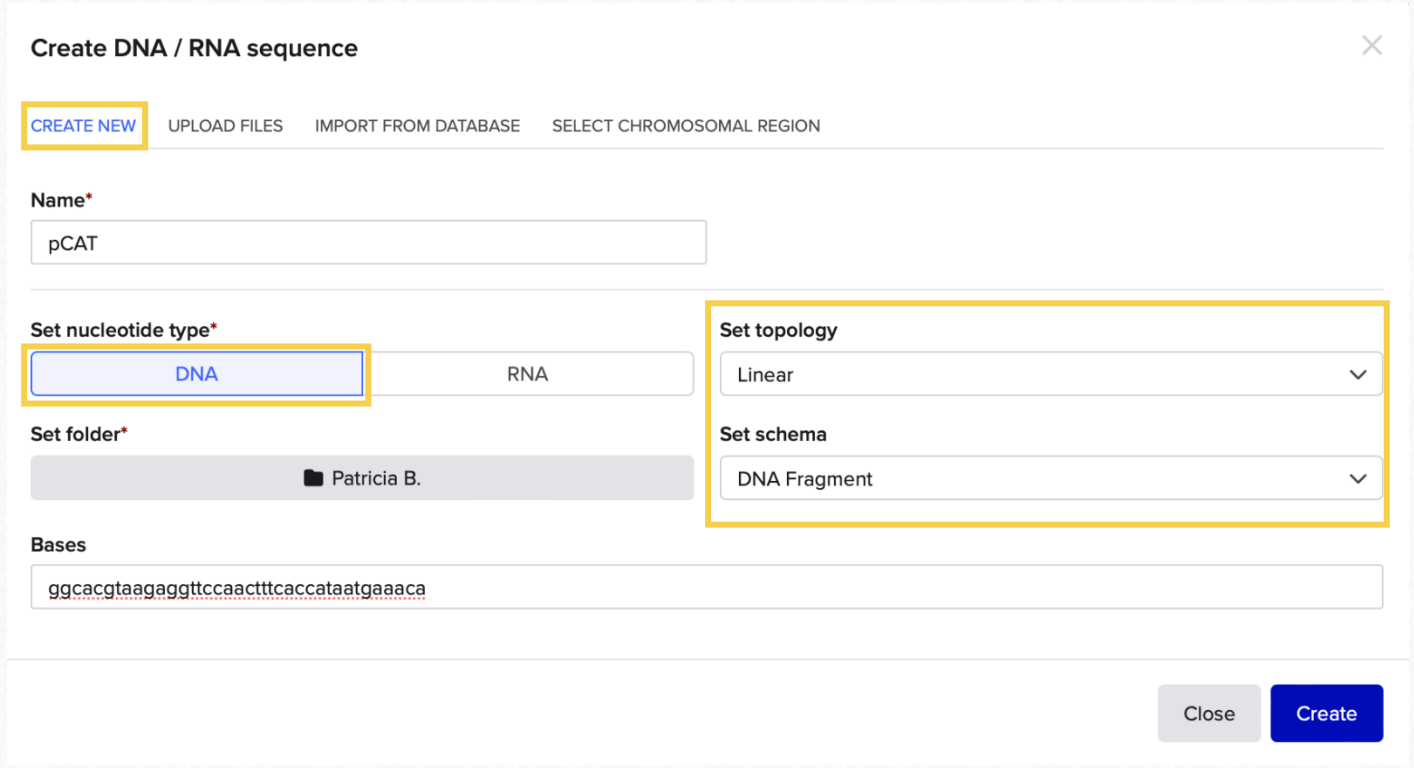
Create and import a sequence

*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.

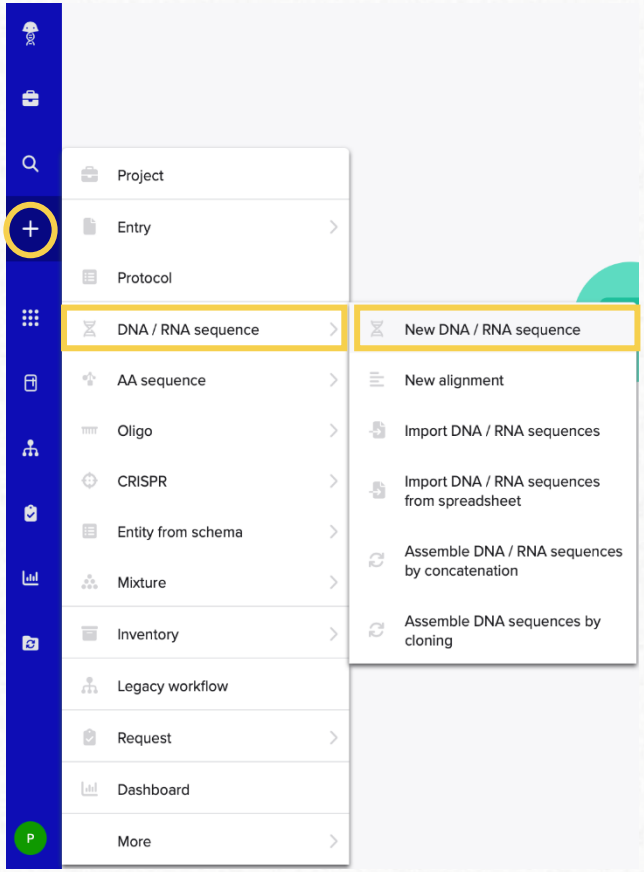


You can leave the **Bases** field **empty** and add your sequence later. This can be useful if you wish to copy and paste a sequence with its annotations.

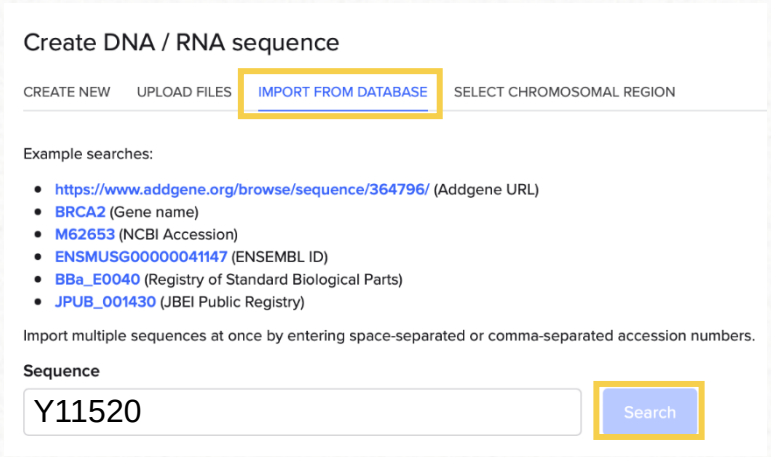
Create and import a sequence

*How to import of sequences **from a database***

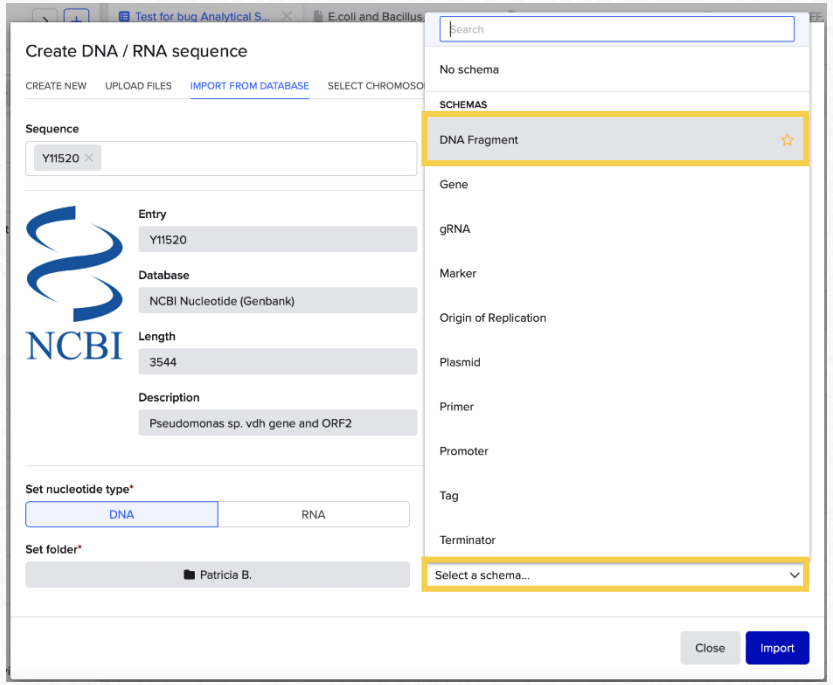
1. Create a new DNA sequence



2. You can write or paste a valid accession number from databases like GenBank, Addgene or the iGEM Registry



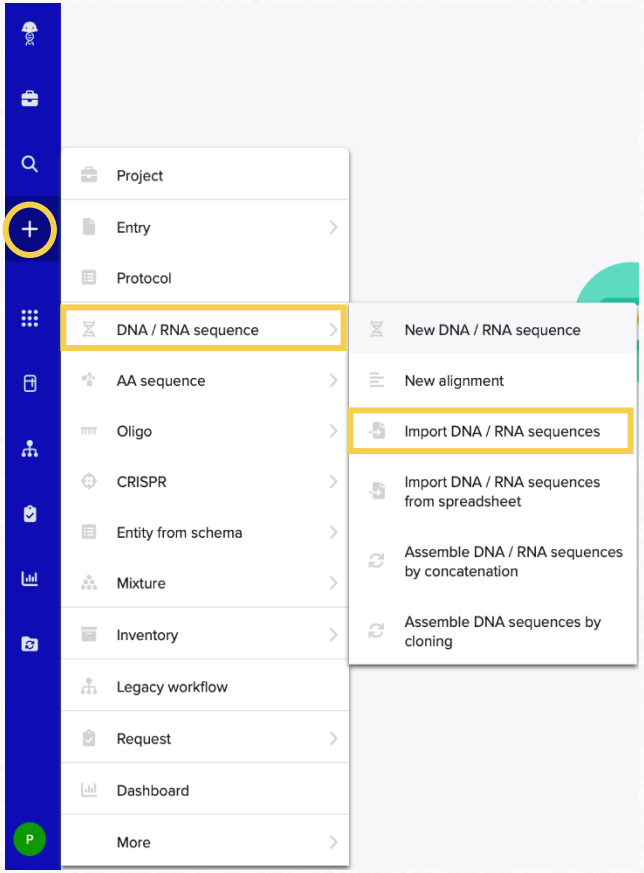
3. If the ID is valid, Benchling will show you the gene's description. You can set its schema and import it.



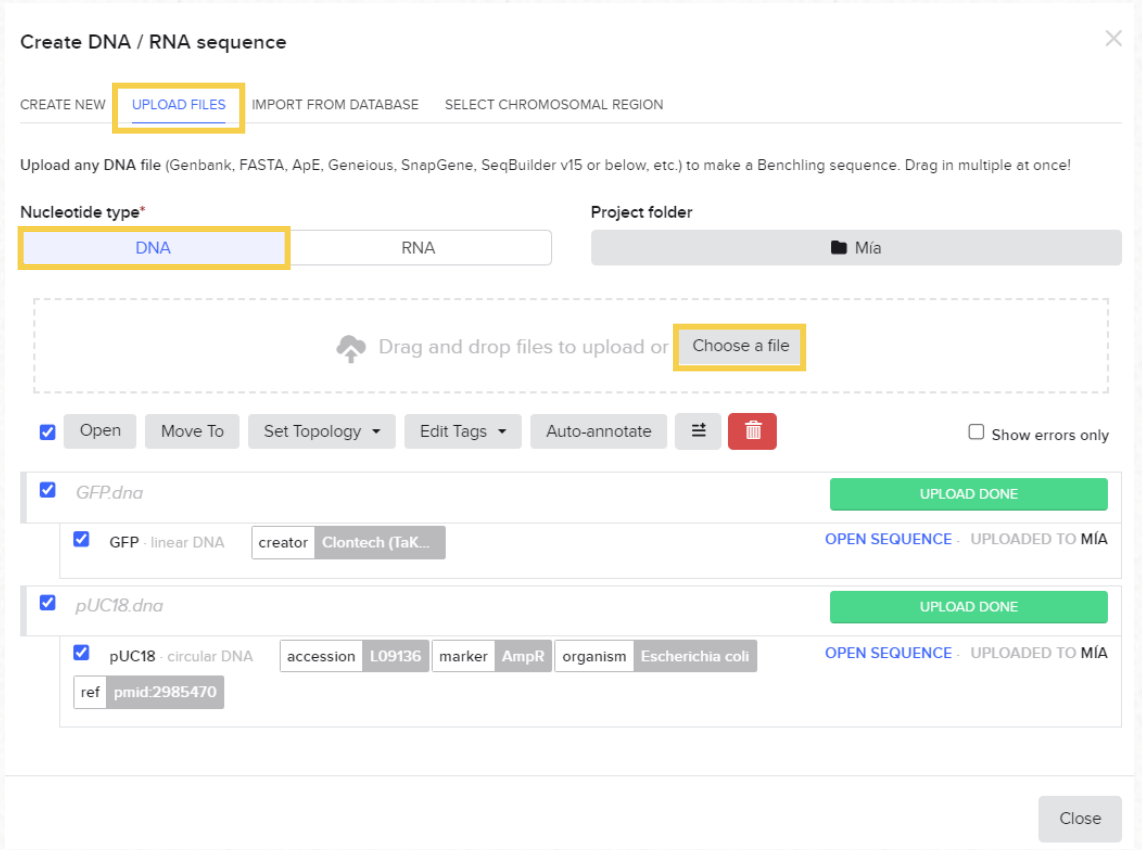
Create and import a sequence

*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.

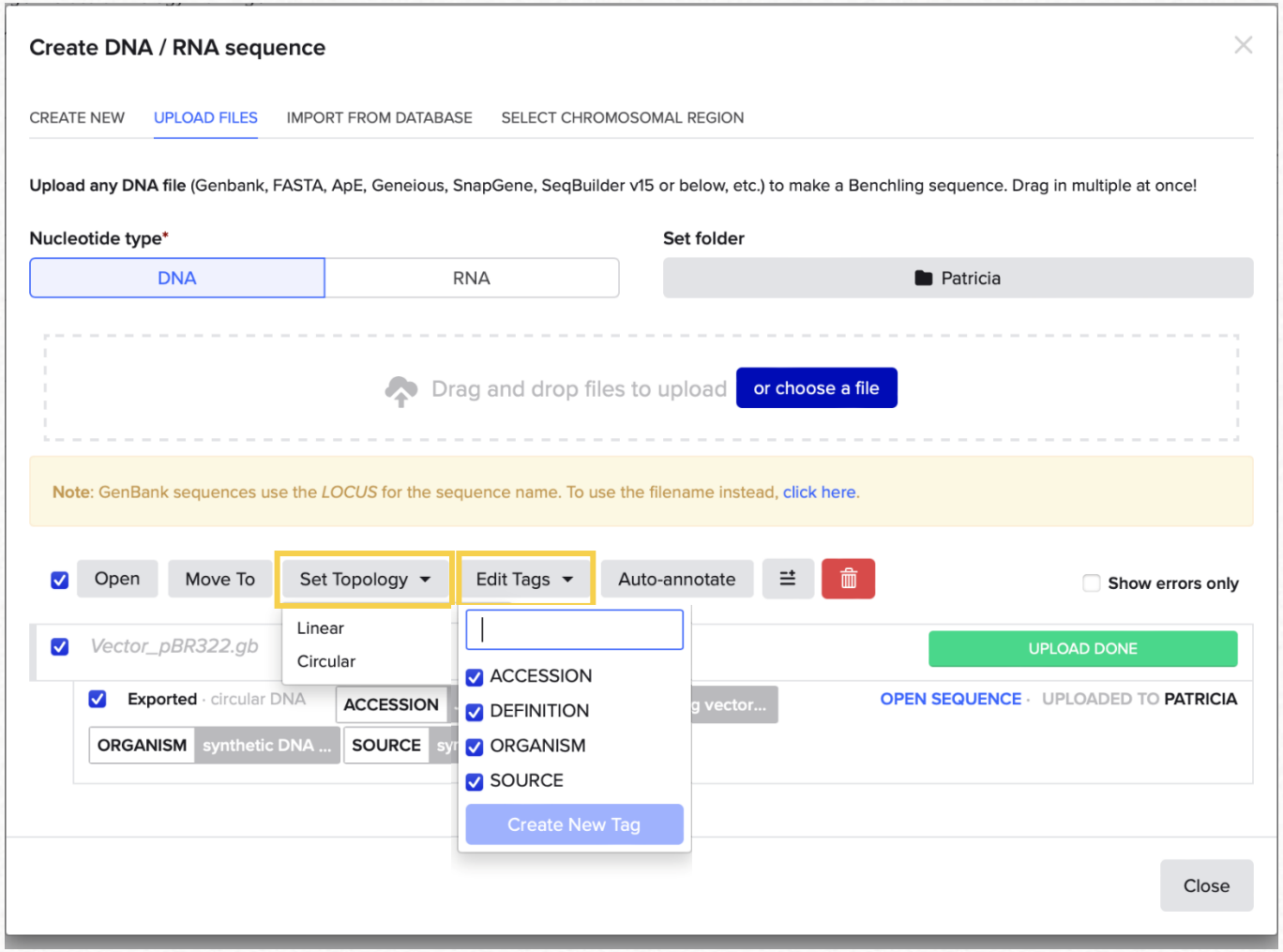


Remember to set the folder **before** uploading your files.

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***

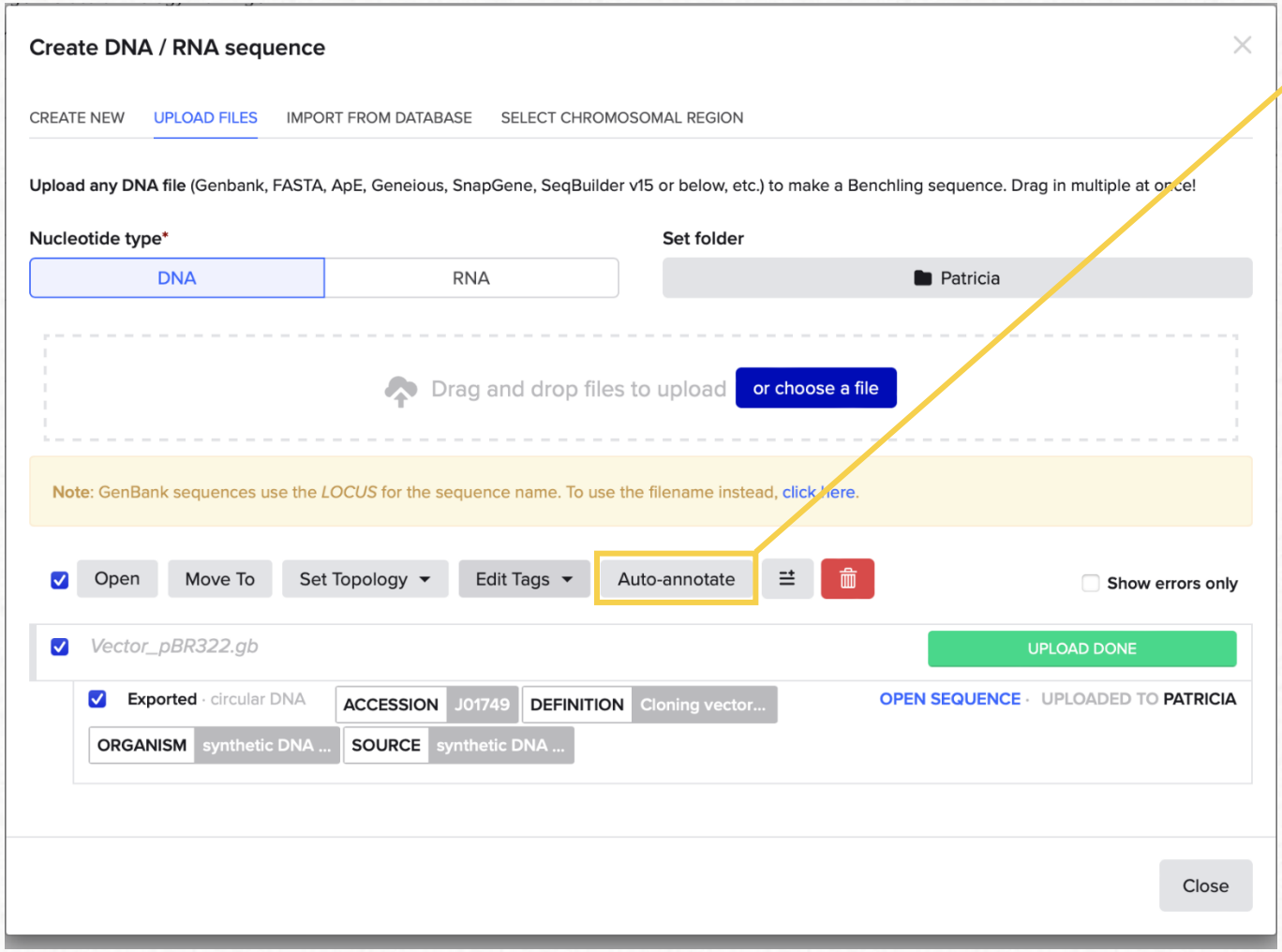


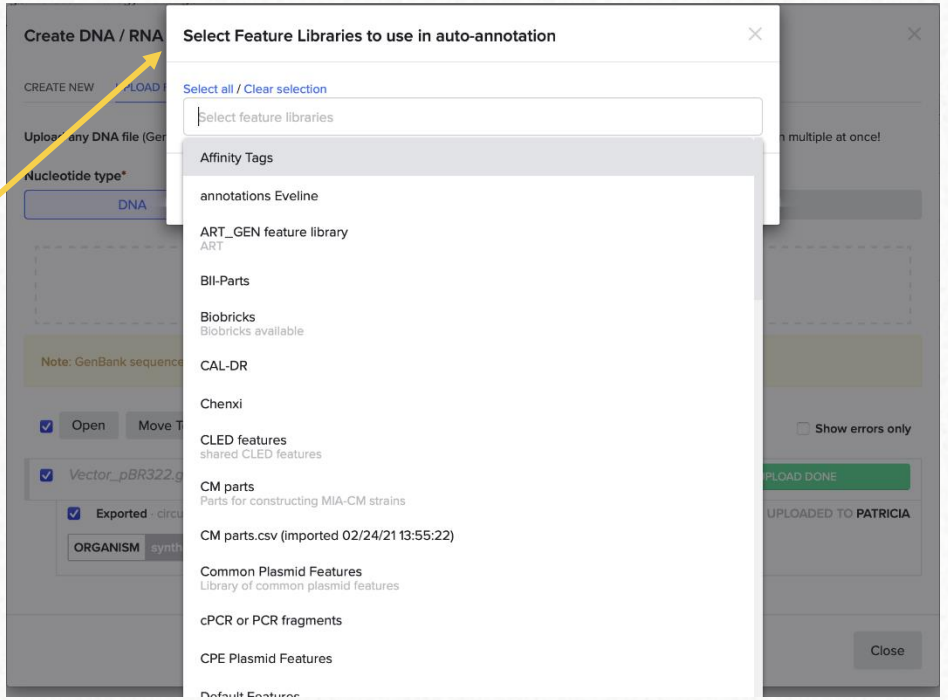
When uploading a sequence, it is possible to:

- **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***



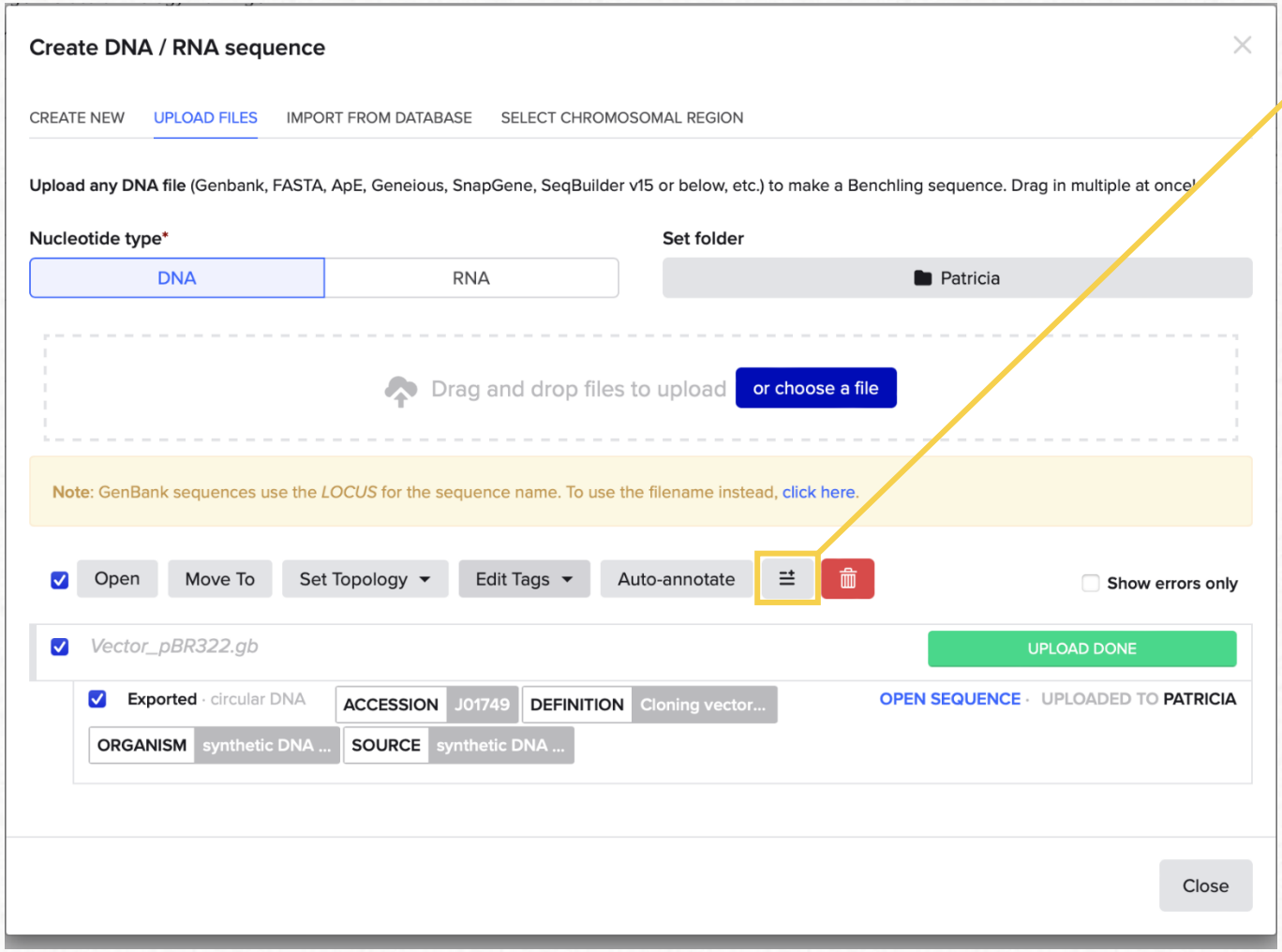


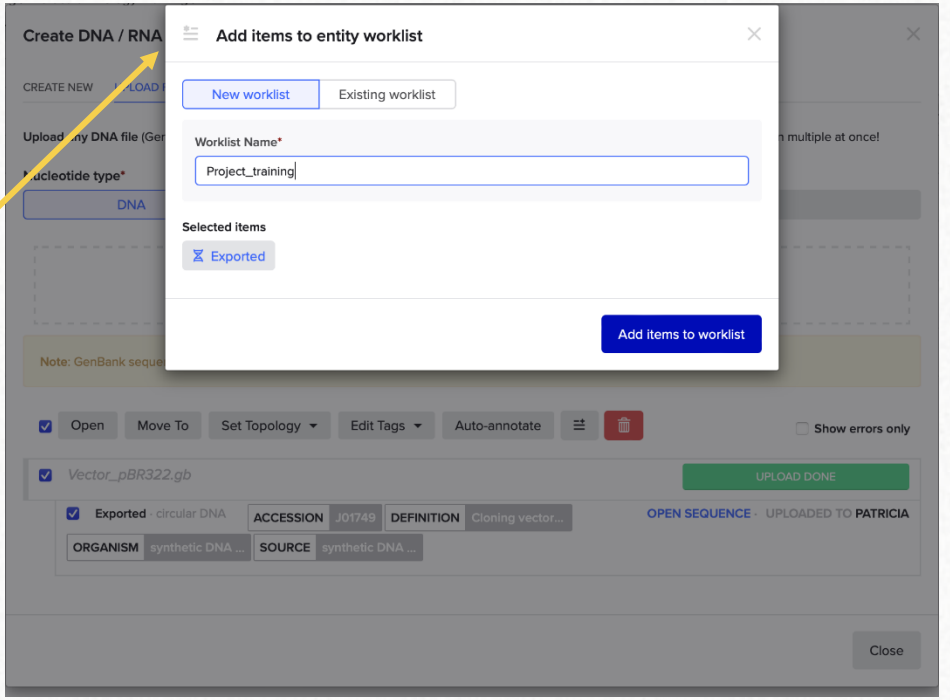
You can **auto – annotate** the sequence from an existing list of features.

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

First steps: Create and import the building blocks to create the DNA construct

*Import of sequences **from a file***





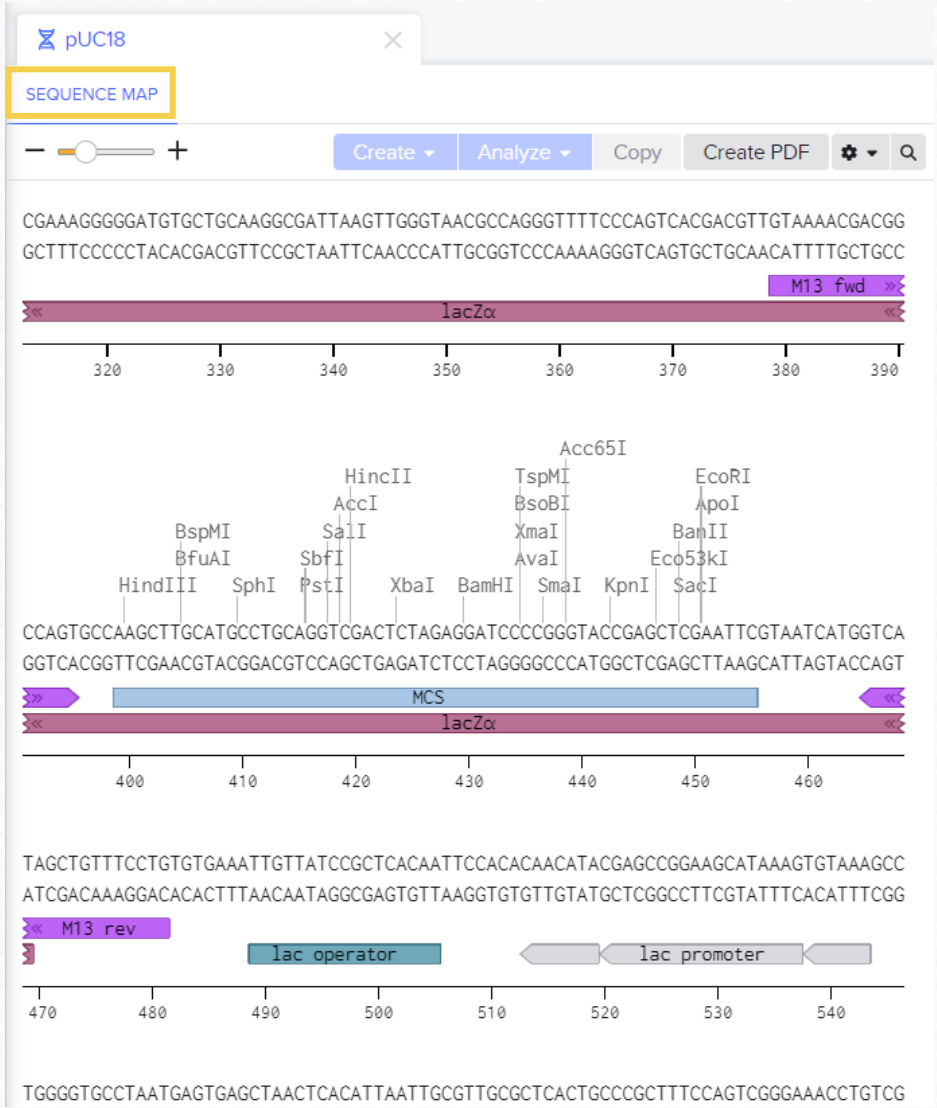
You can also **create worklists or add to existing ones** to find your currently used entities faster.

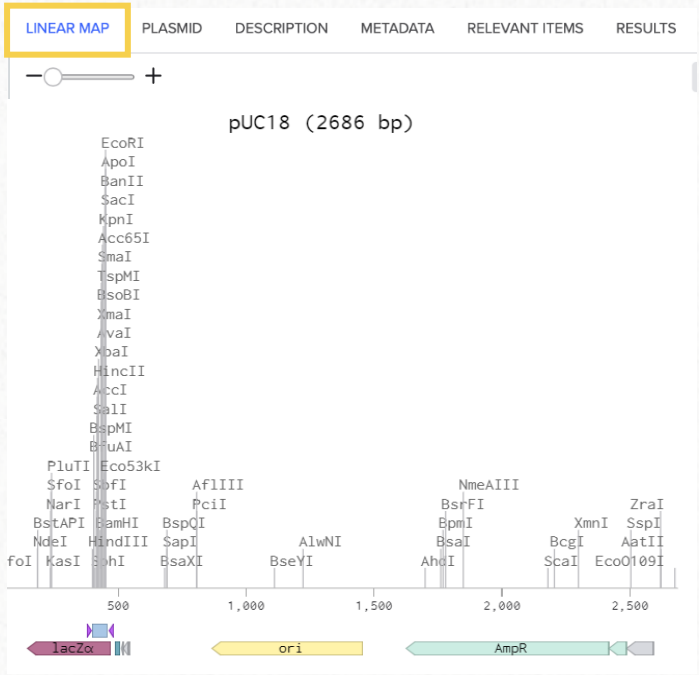
3. The basics of sequences

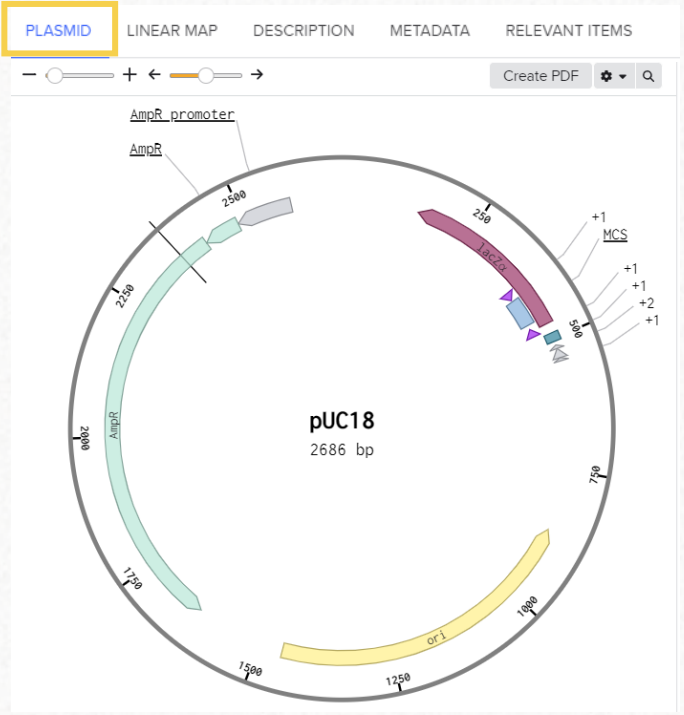
3.2 Sequence visualization

View, annotate and edit your sequences

*Different **viewing options:***



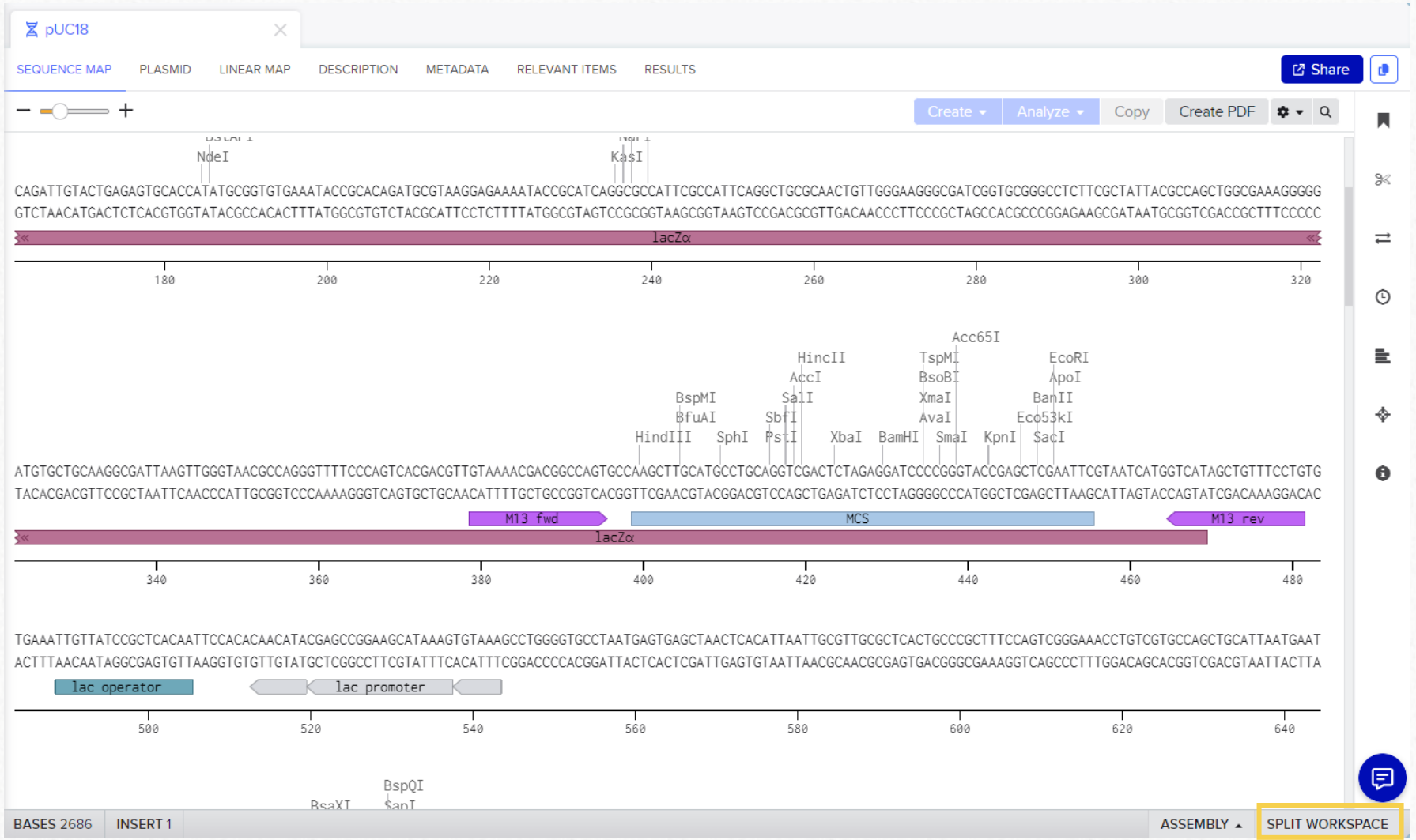




- ✓ For circular sequences, a plasmid viewing option is available
- ✓ You can click on the different elements or annotations in any of the views to select the corresponding sequence fragment

View, annotate and edit your sequences

*Different **viewing options:***

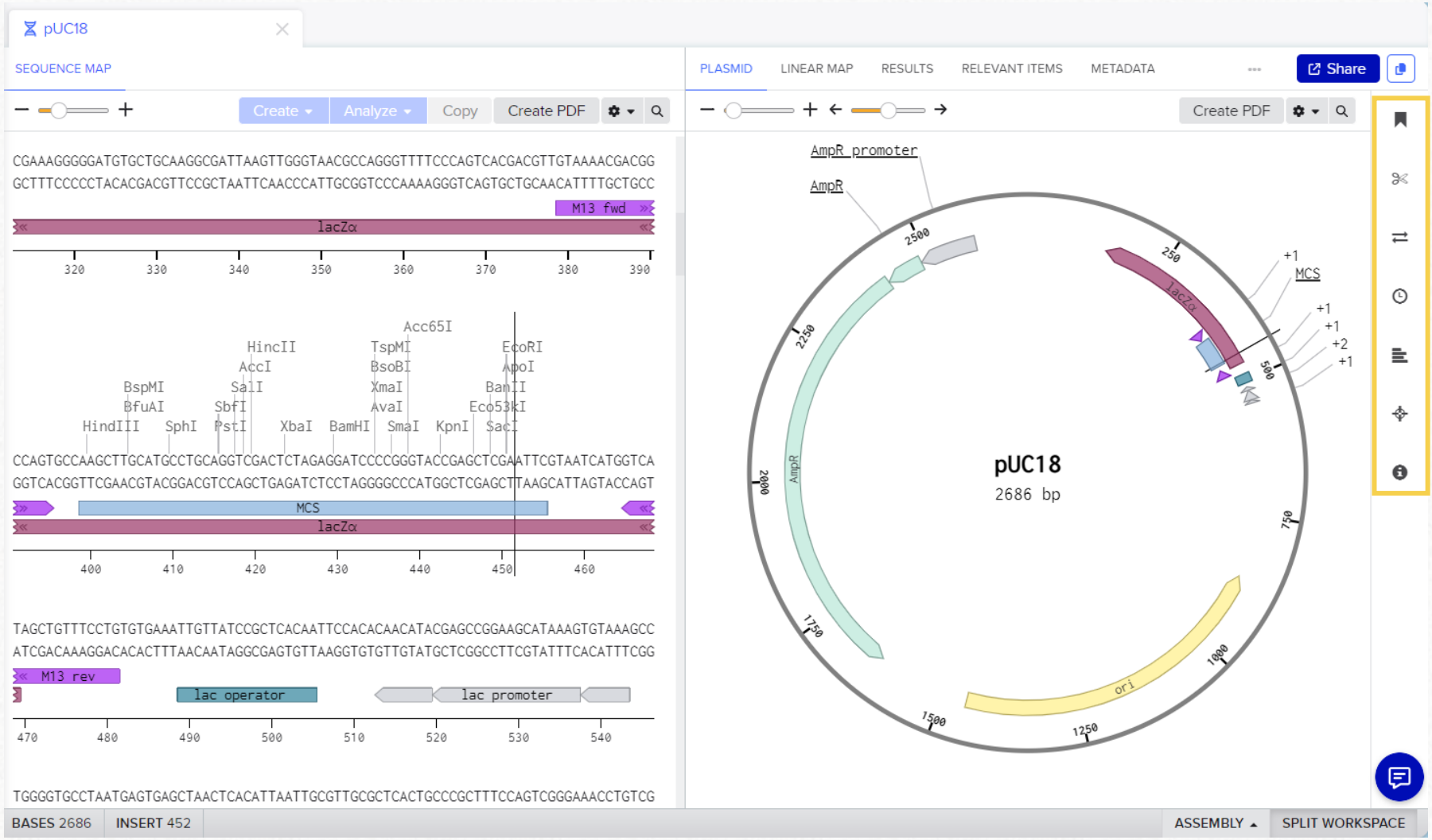


PRO TIP:

Click on *"**split workspace**"* to change the viewing mode to split screen/full screen

View, annotate and edit your sequences

*Sequence **navigation:***

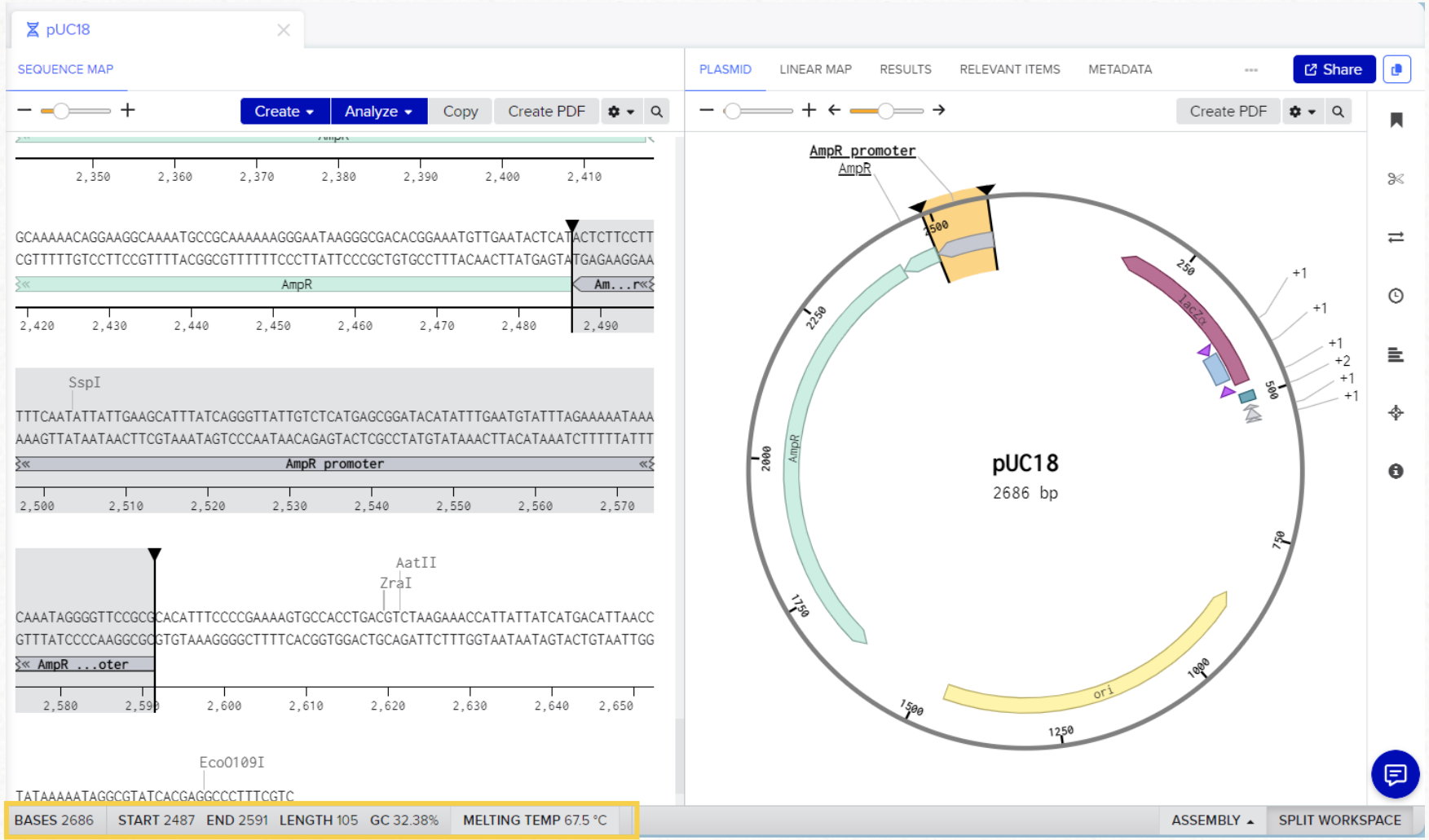


Functionalities

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

View, annotate and edit your sequences

*Sequence **navigation:***



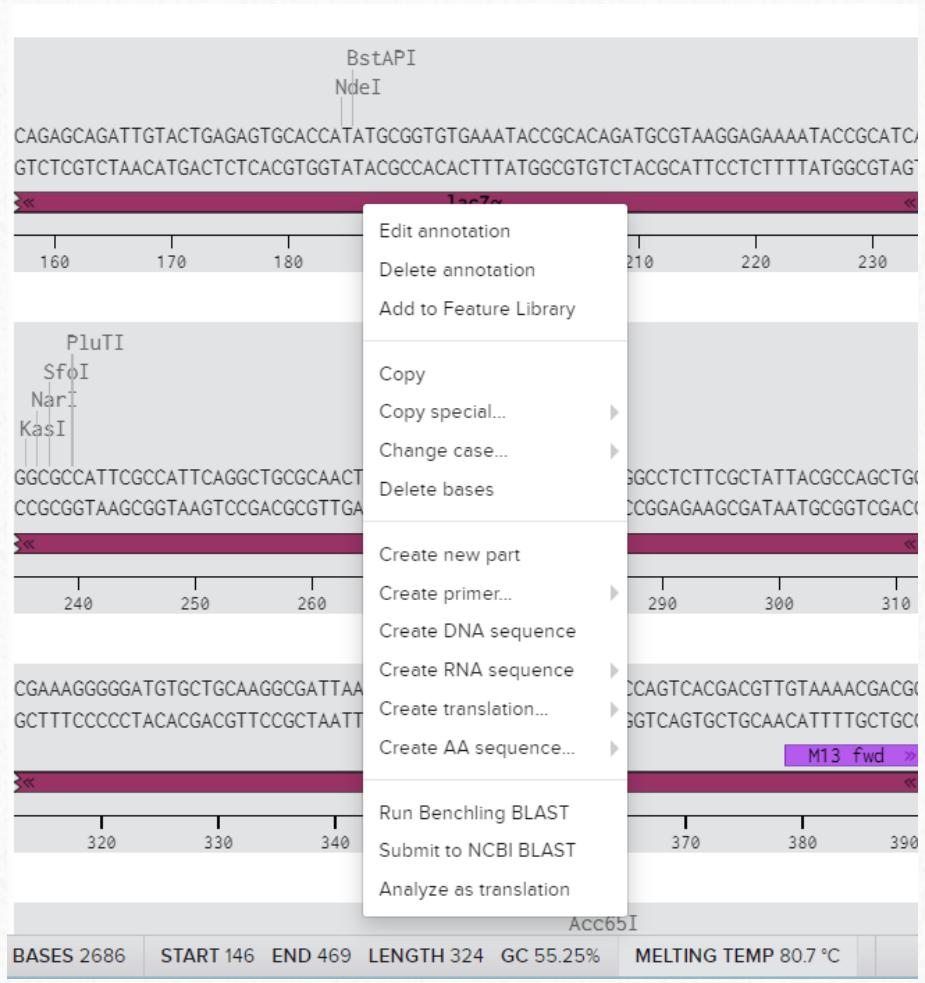
- ✓ Click on any element or annotation in any of the views to select the corresponding sequence fragment
- ✓ See the **electrochemical properties** of the fragment on the bottom

PRO TIP:

Click on "*melting temperature*" to access the parameter settings. Different calculation algorithms are available.

View, annotate and edit your sequences

*Sequence **navigation:***



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

PRO TIP:

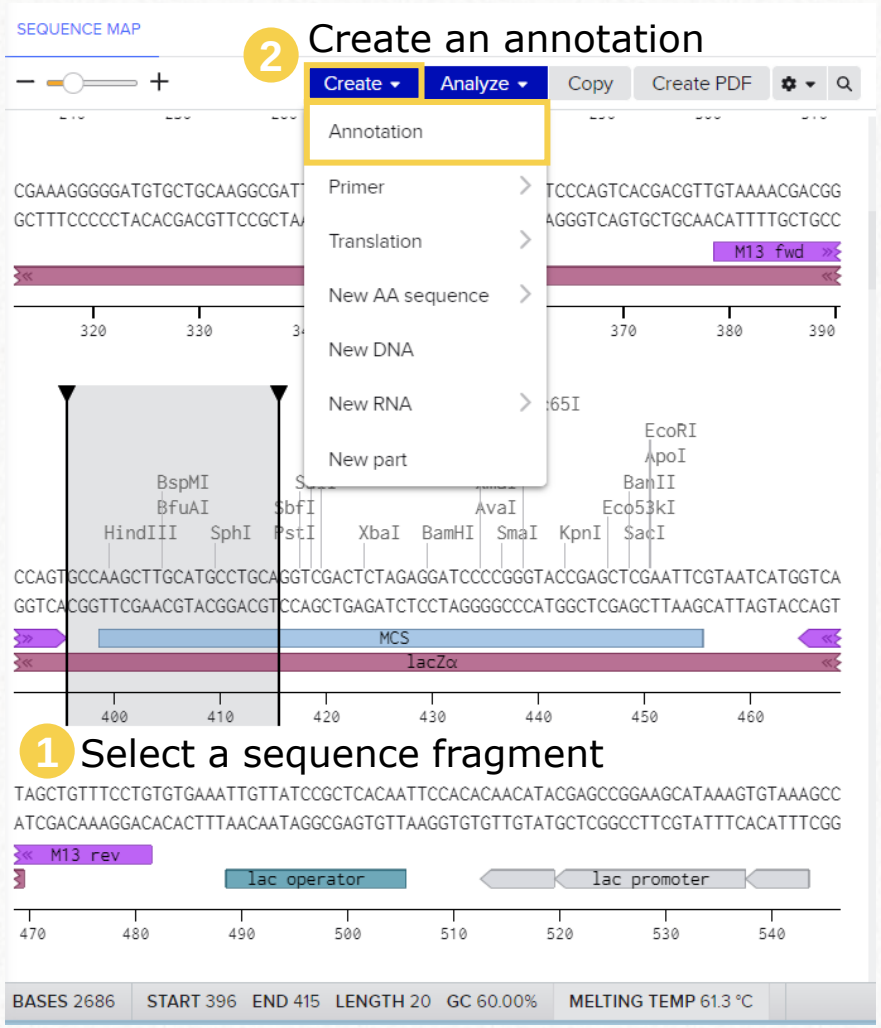
Click directly on any part of the sequence (not a fragment) and paste or write new bases directly.

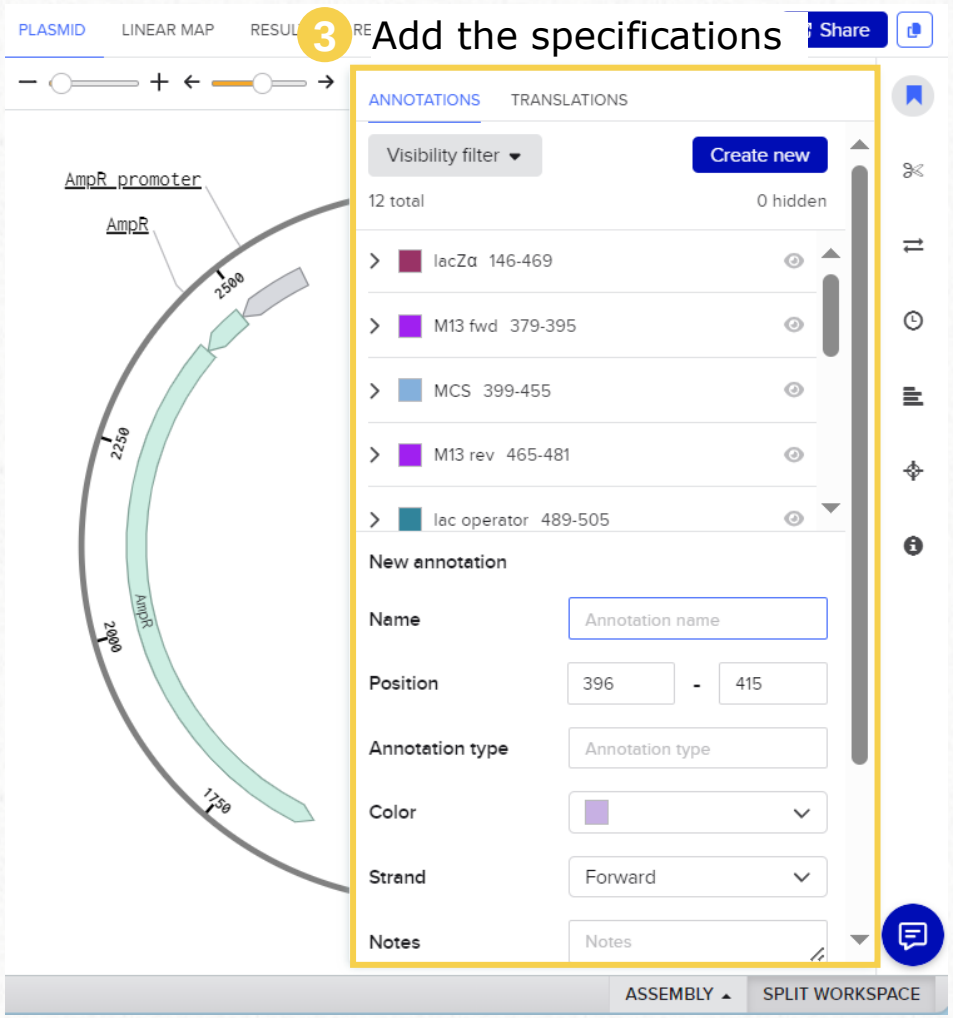
3. The basics of sequences

3.3 Sequence annotation

View, annotate and edit your sequences

*Sequence **annotations***





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

View, annotate and edit your sequences

*Sequence **annotations***

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

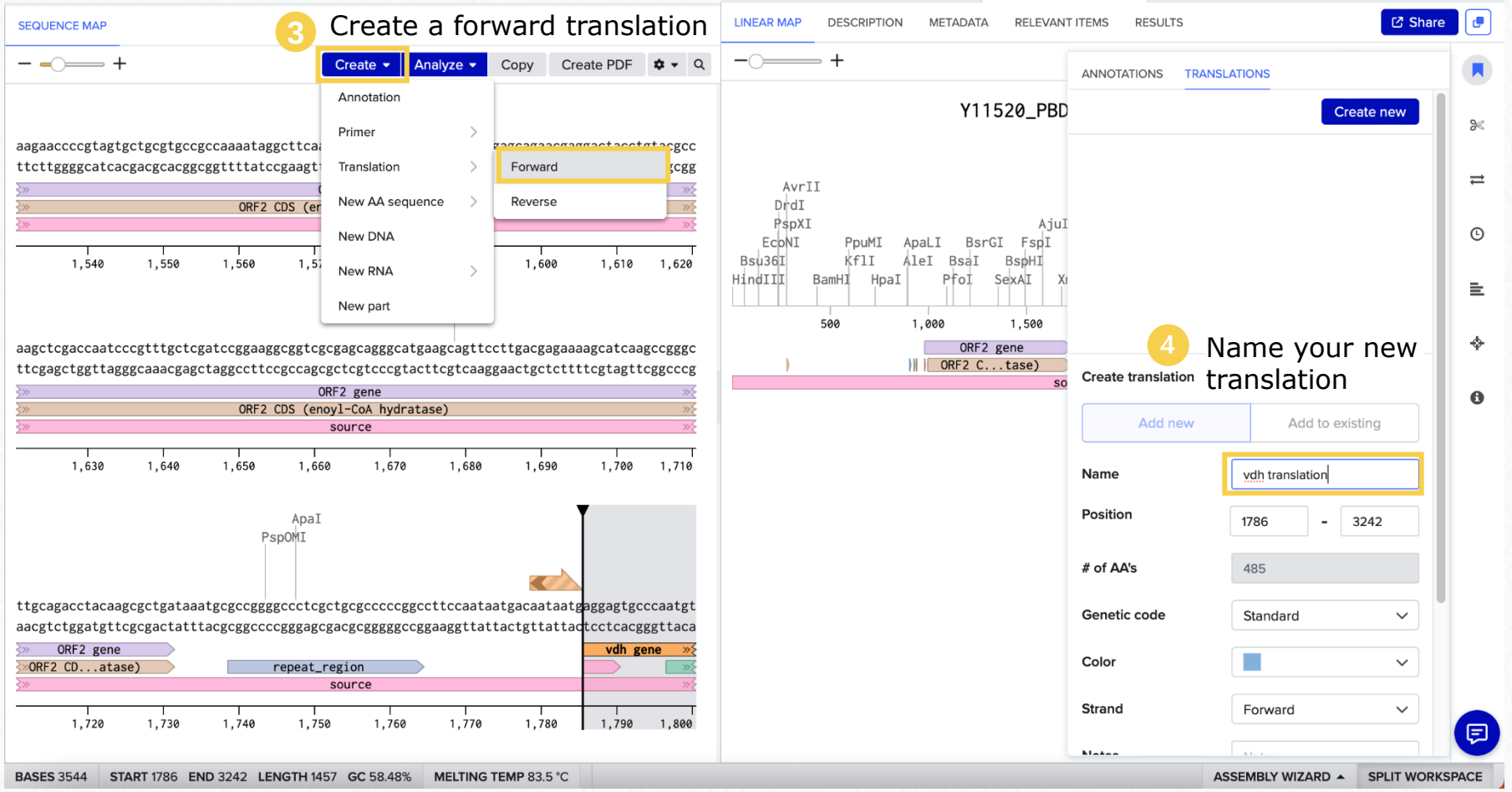
3.4 Codon optimization

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)



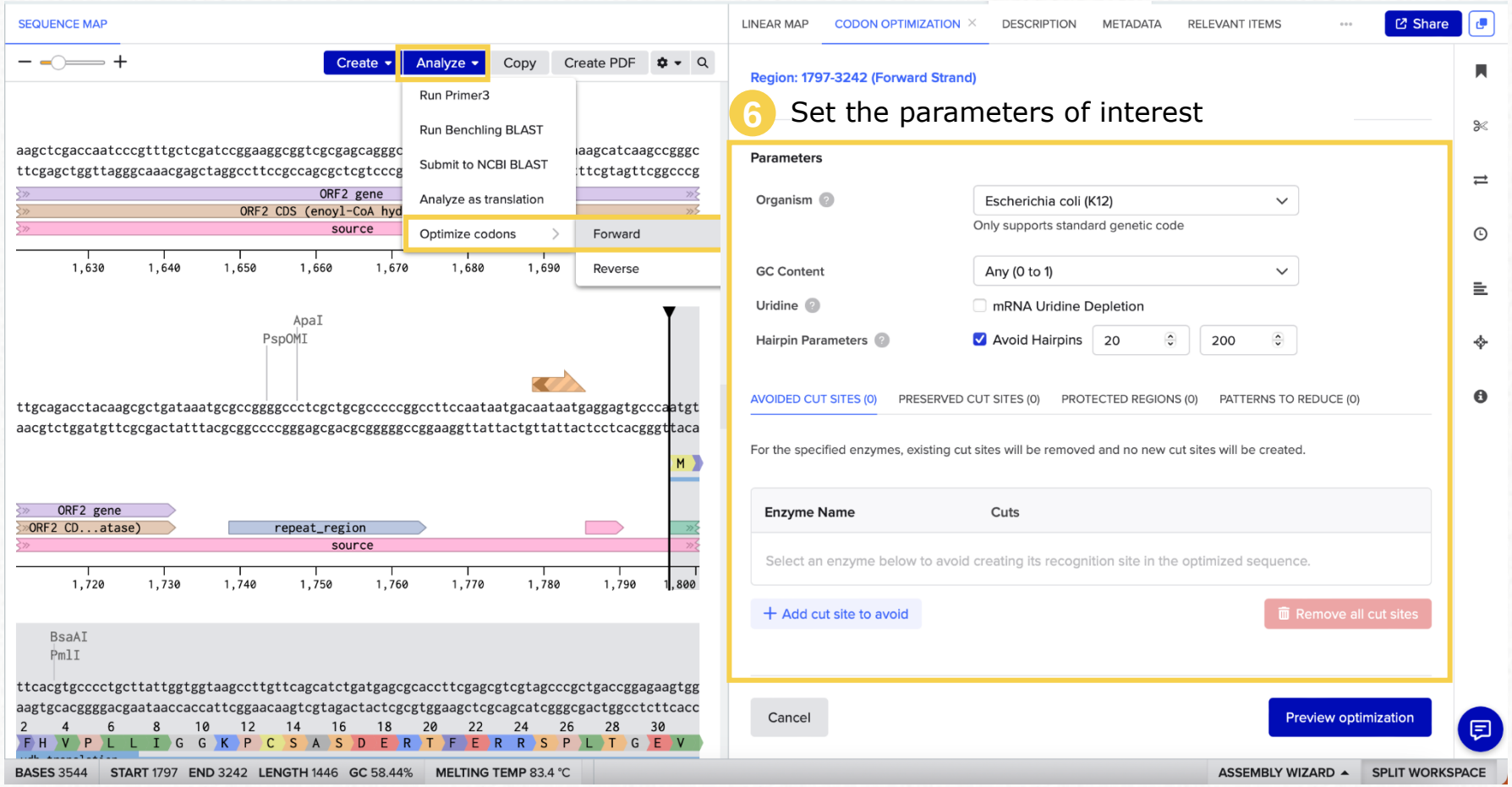
✓ Before codon optimization, the DNA sequence must be translated

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

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

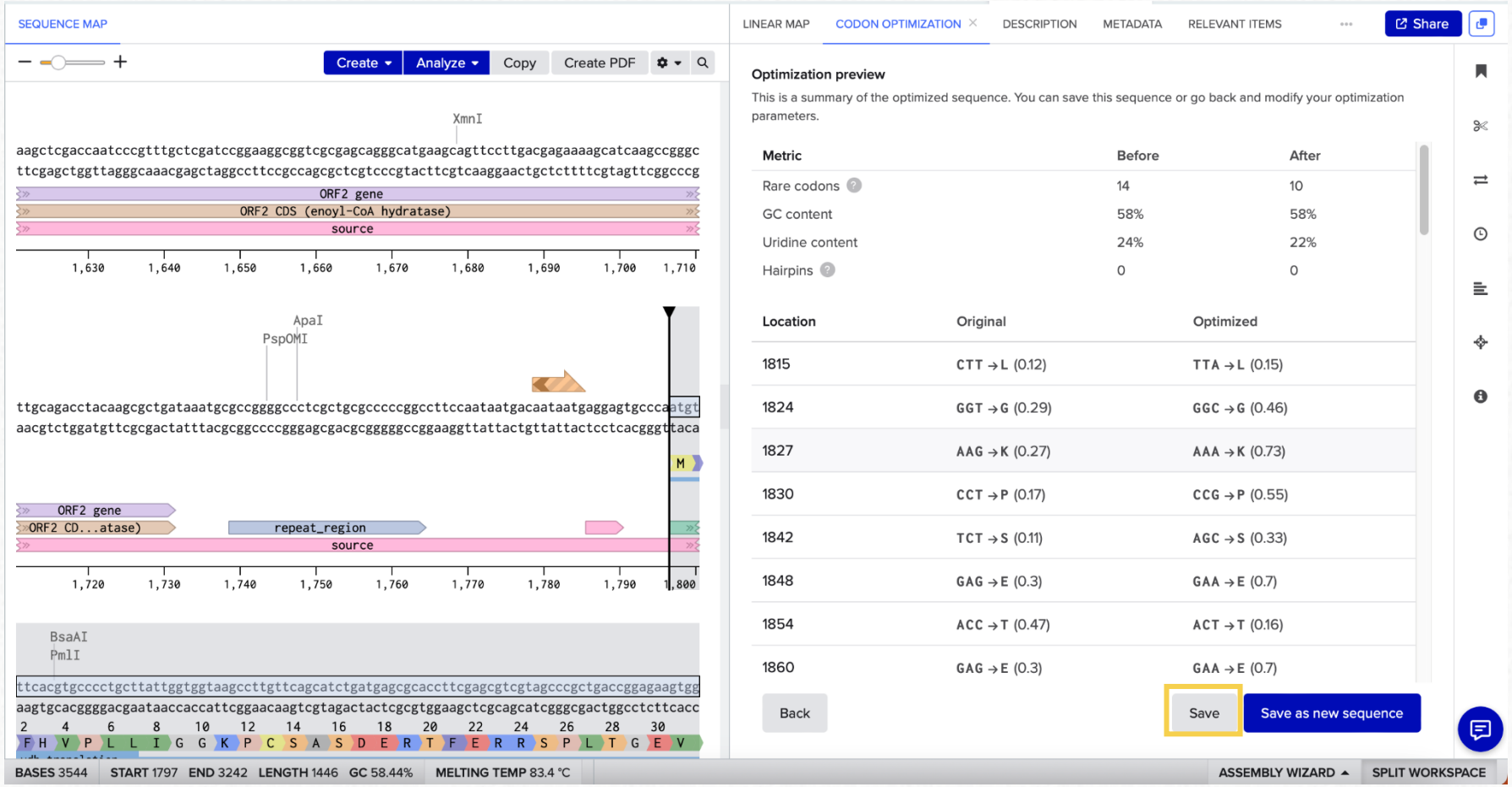


- ✓ When codon optimizing, its possible to select the GC content and other details
- ✓ You can select cut sites to avoid or remove in your optimized sequence

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



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

4. Benchling access and folder setup

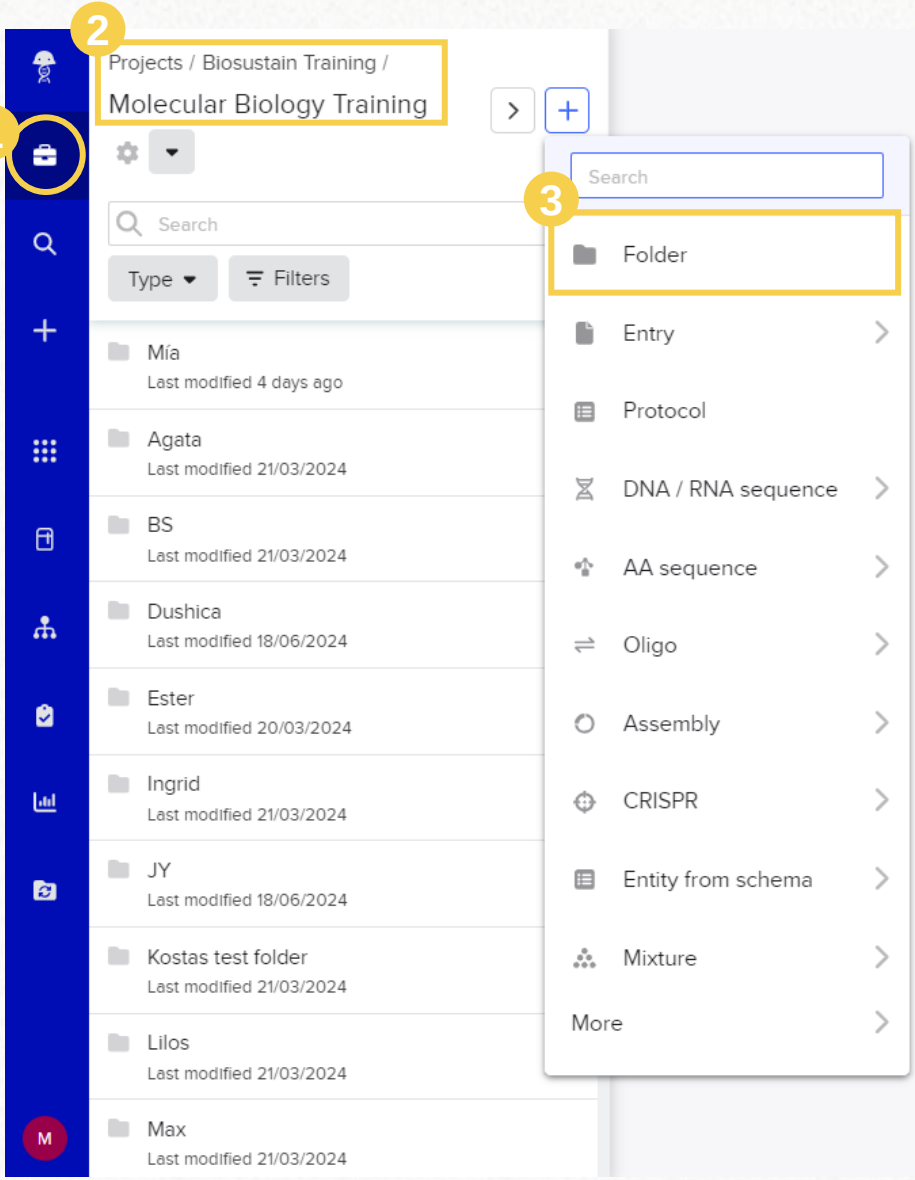
LET'S MOVE TO BENCHLING TO START THE HANDS-ON!

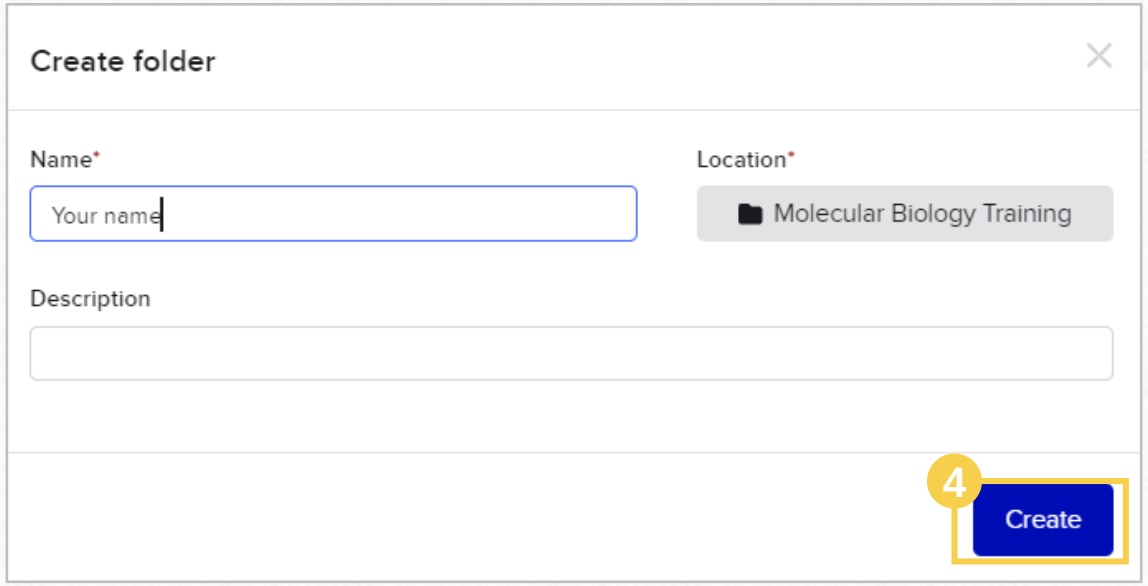
Access Benchling:

biosustain.benchling.com

(login with DTU credentials)

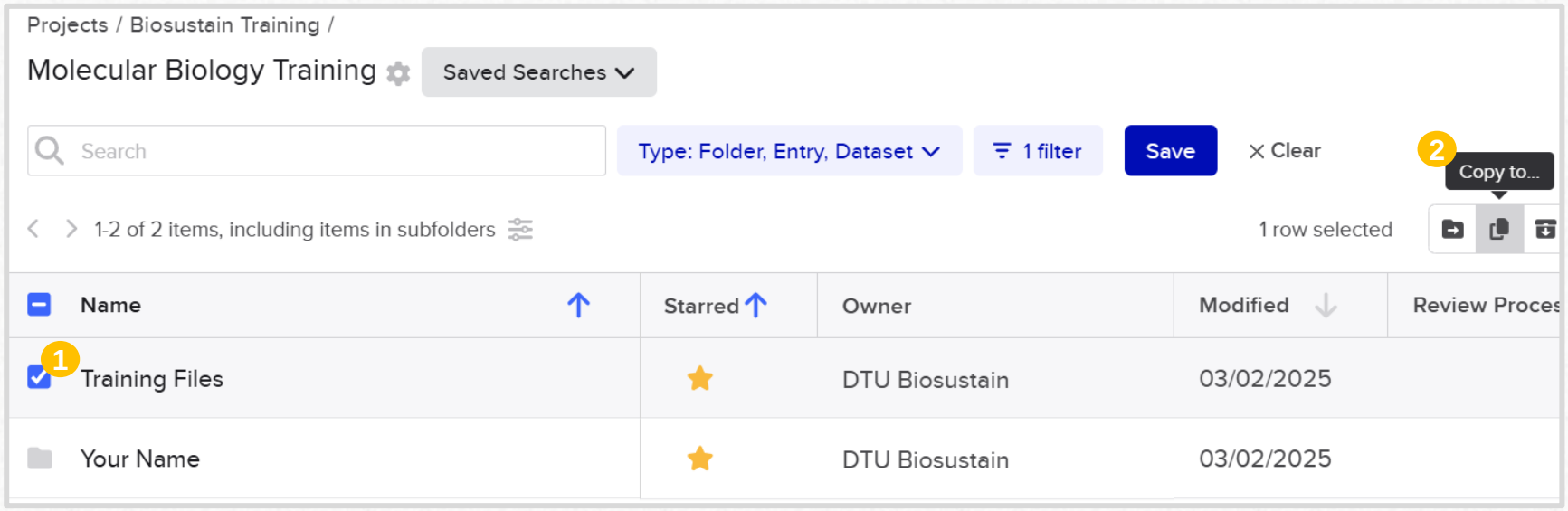
Create a training folder to work in

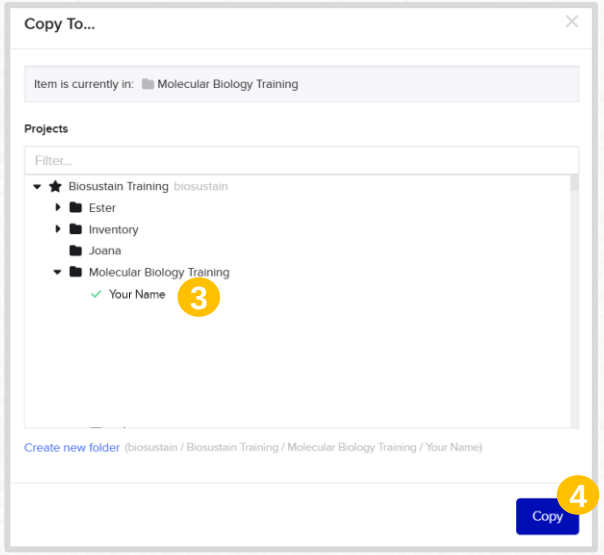




✓ Remember to select your own training folder when creating or importing sequences

Copy the *Training Files* folder into your own

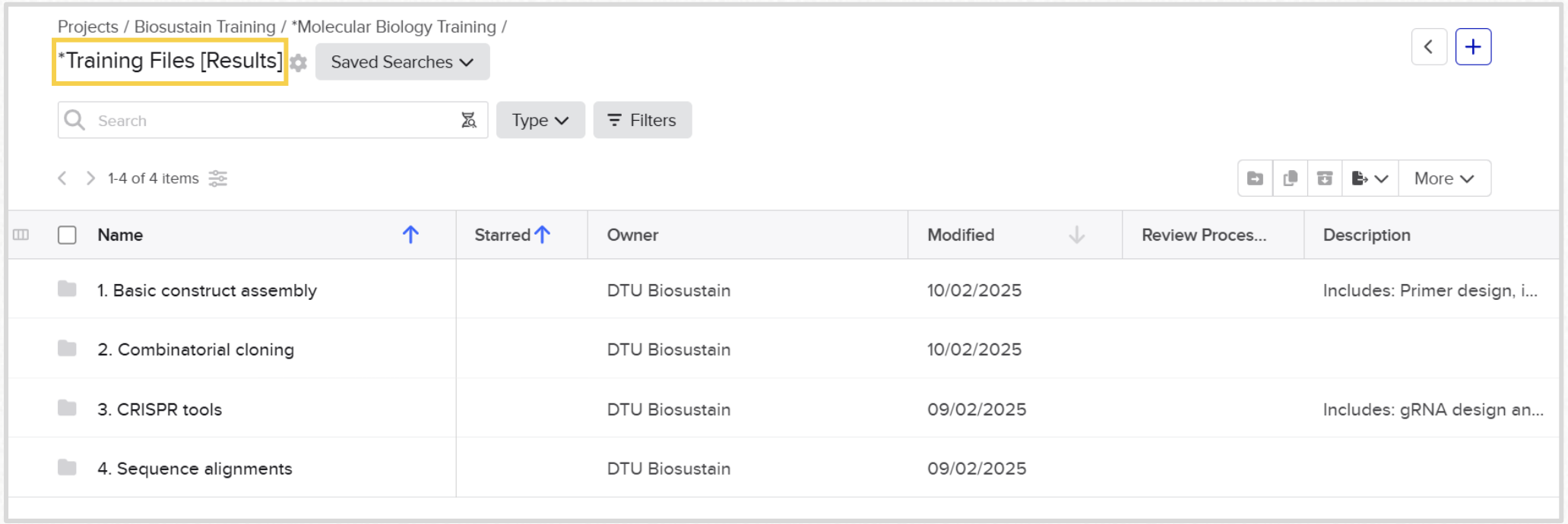




Do not modify the ***Training Files*** folder! Make sure you are **copying it**, and **not moving** its contents.

The *Training Files [Results]* folder

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.



Do not modify the contents of this folder!

5. Basic construct assembly

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

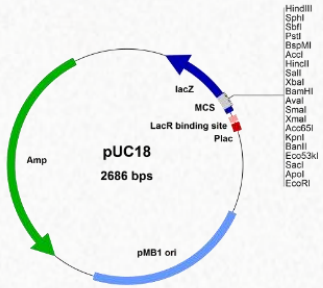
1

Cloning vector design → Creation of expression vector library → Gene knockout using CRISPR/Cas9 → Sequence alignment

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

alsS *alsD*

pUC18 cloning vector

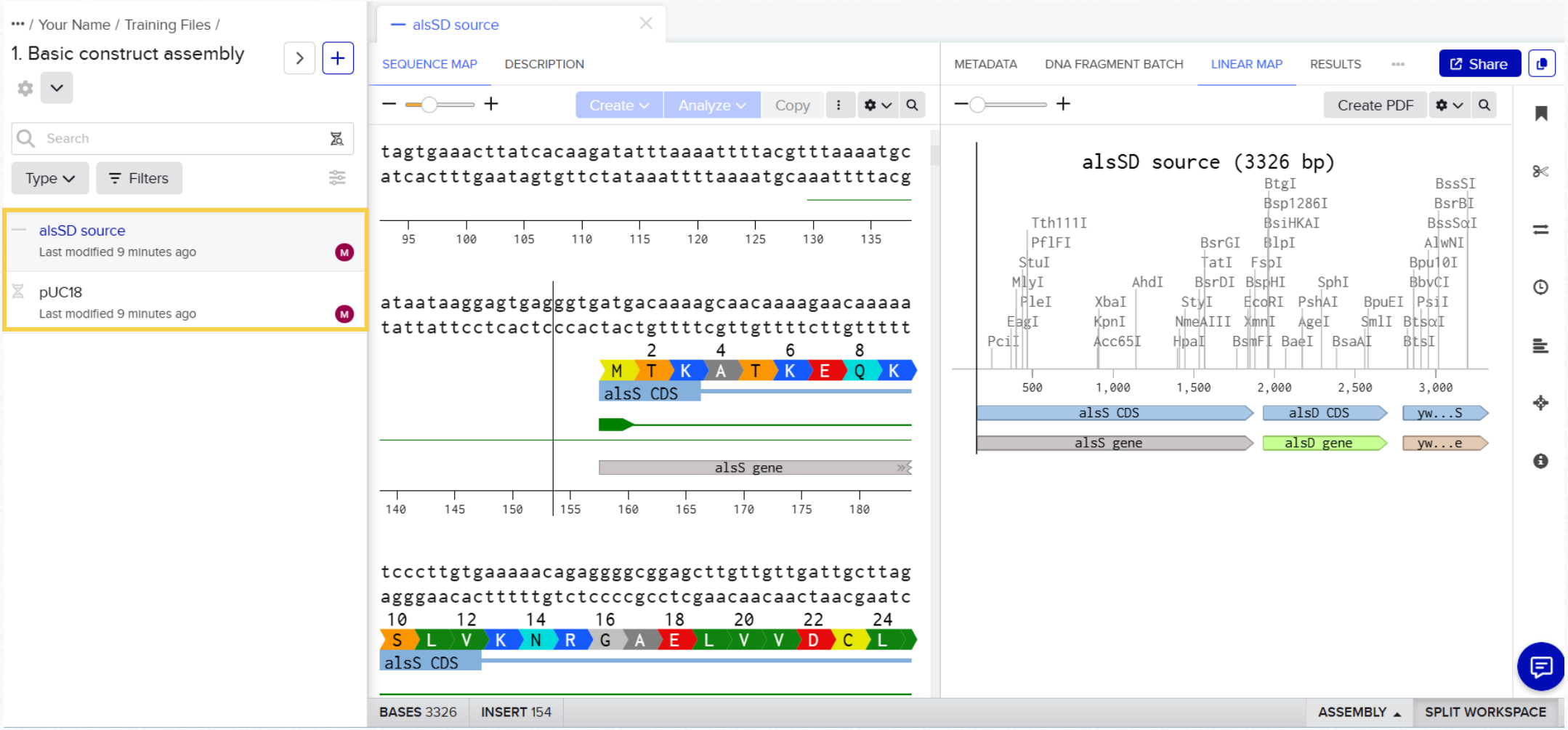


- ✓ Primer design
- ✓ *In silico* PCR
- ✓ Virtual digestion
- ✓ RE-based cloning

Expected output:

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

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



5. Basic construct assembly

5.1 Primer design

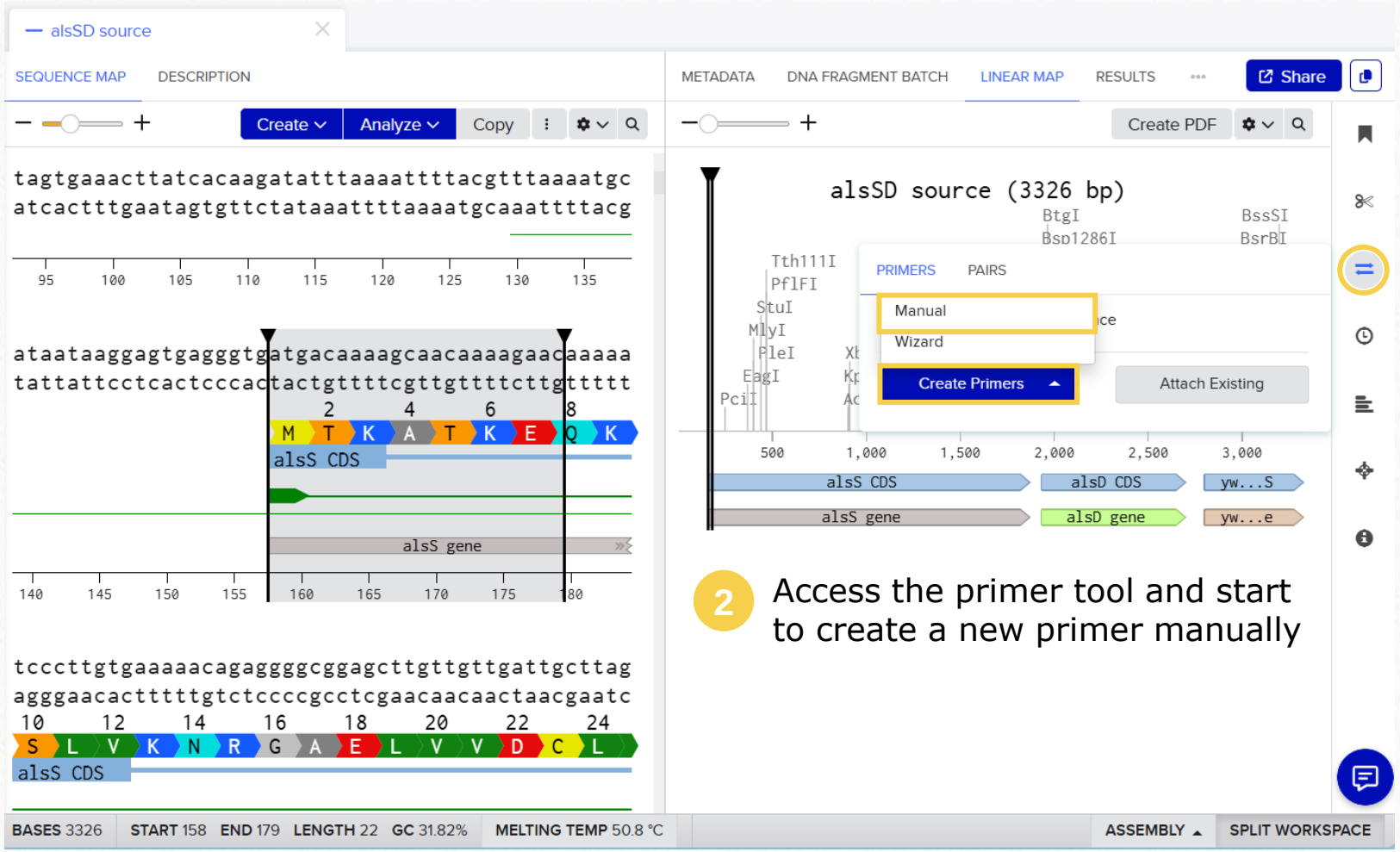
5.1.1 Manual primer design

Construct design

Manual primer creation

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

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



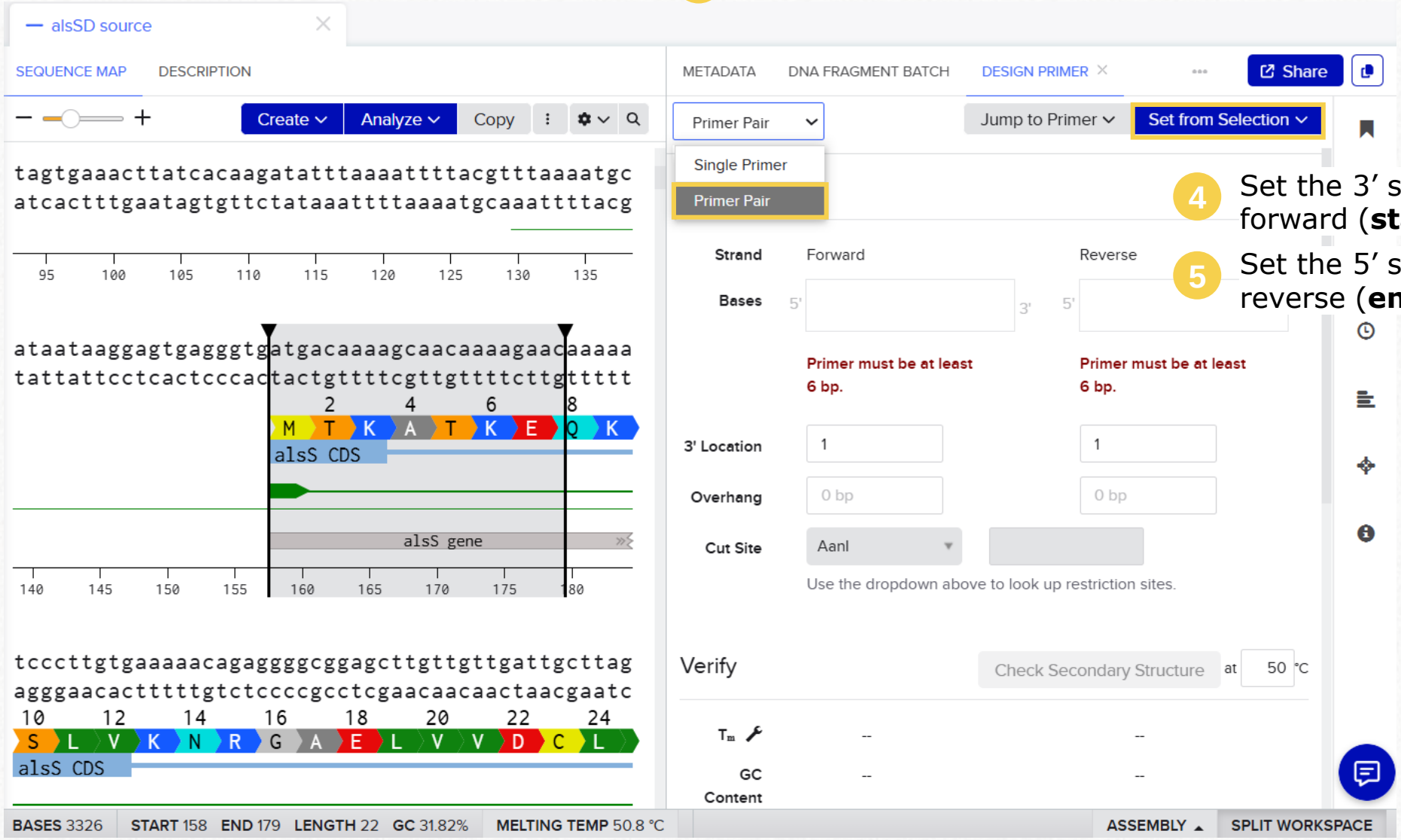
2. Access the primer tool and start to create a new primer manually

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

Construct design

Manual primer creation

3 Select **primer pair** creation



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

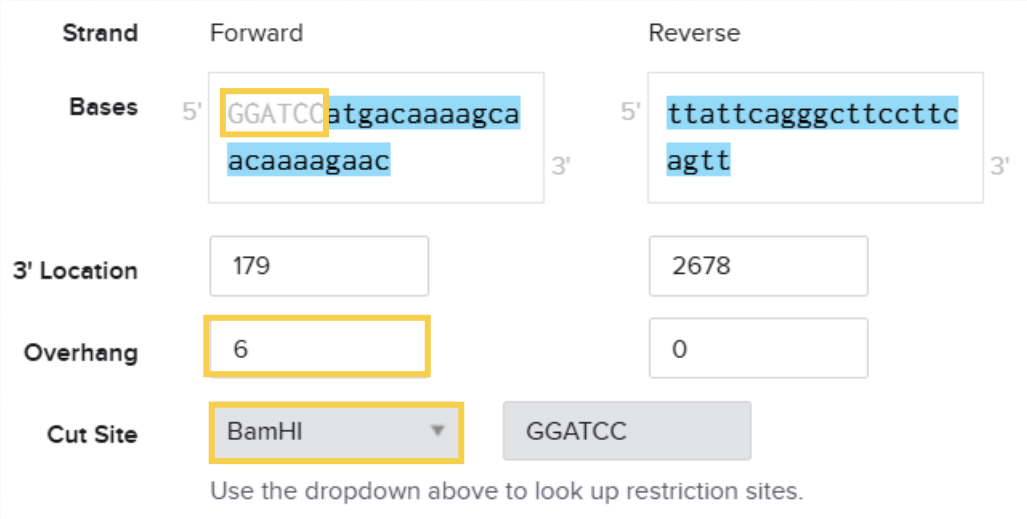
5 Set the 5’ selected bases as reverse (**end of alsD**)

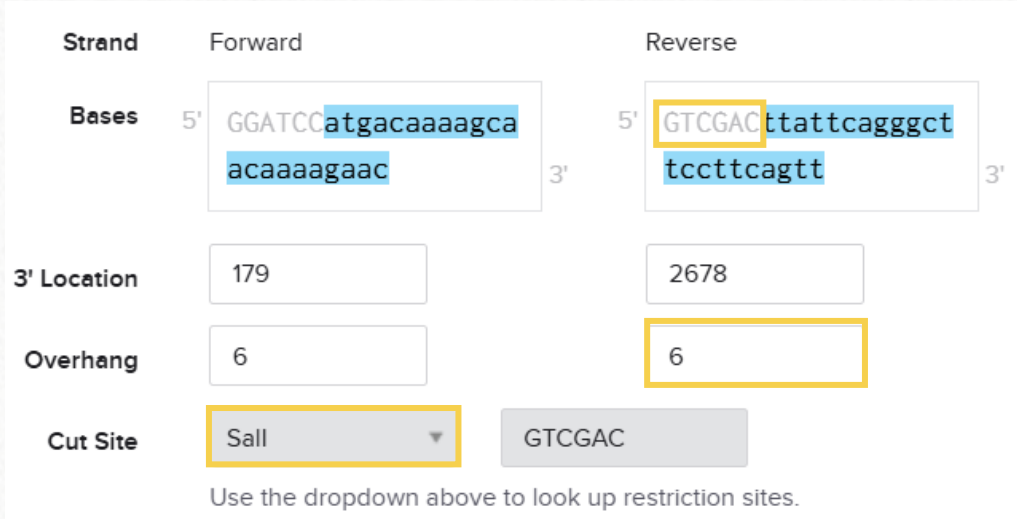
✓ Make sure to select the **start** of **alsS** and the end of **alsD**

Construct design

Manual primer creation

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



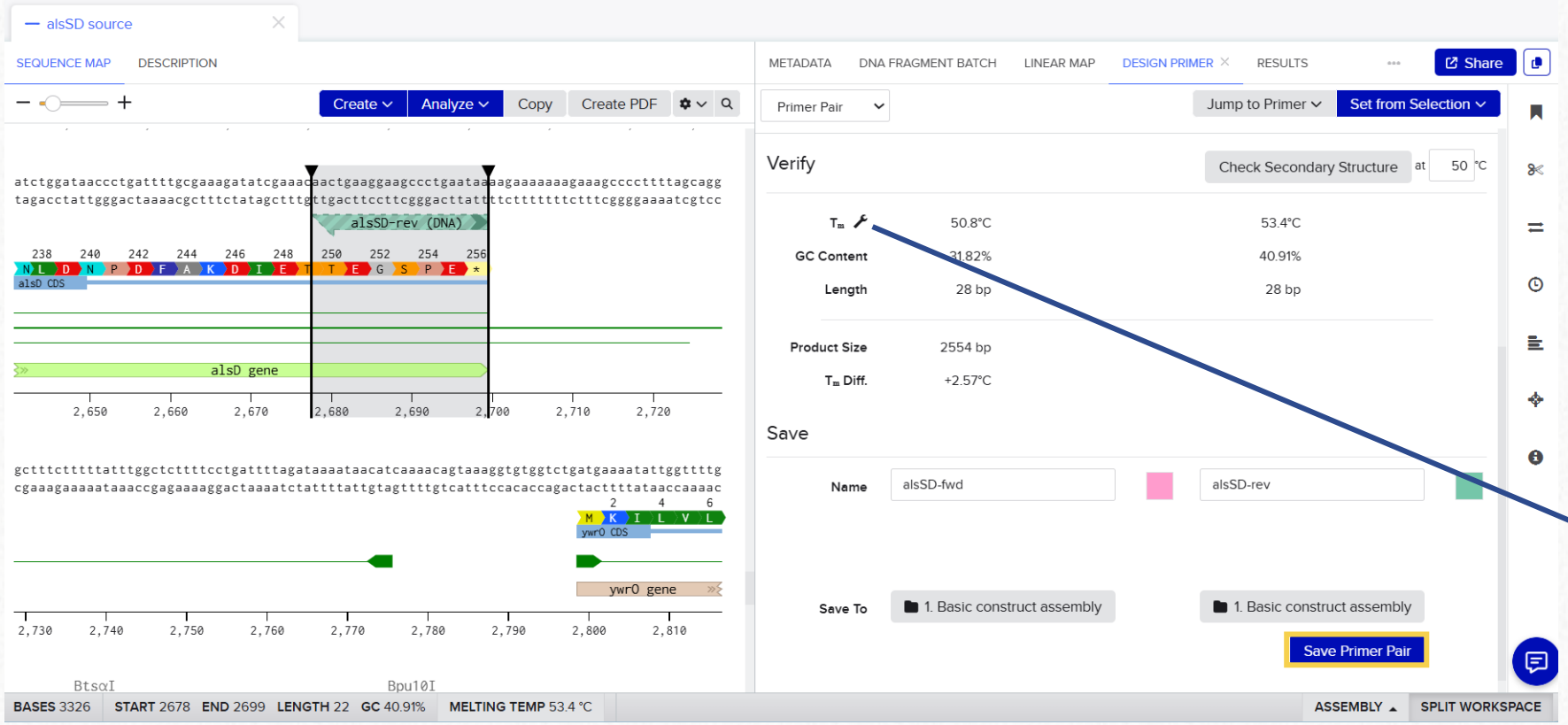


8. Repeat the process to add a **SalI** site at the beginning of the reverse primer

Construct design

Manual primer creation

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



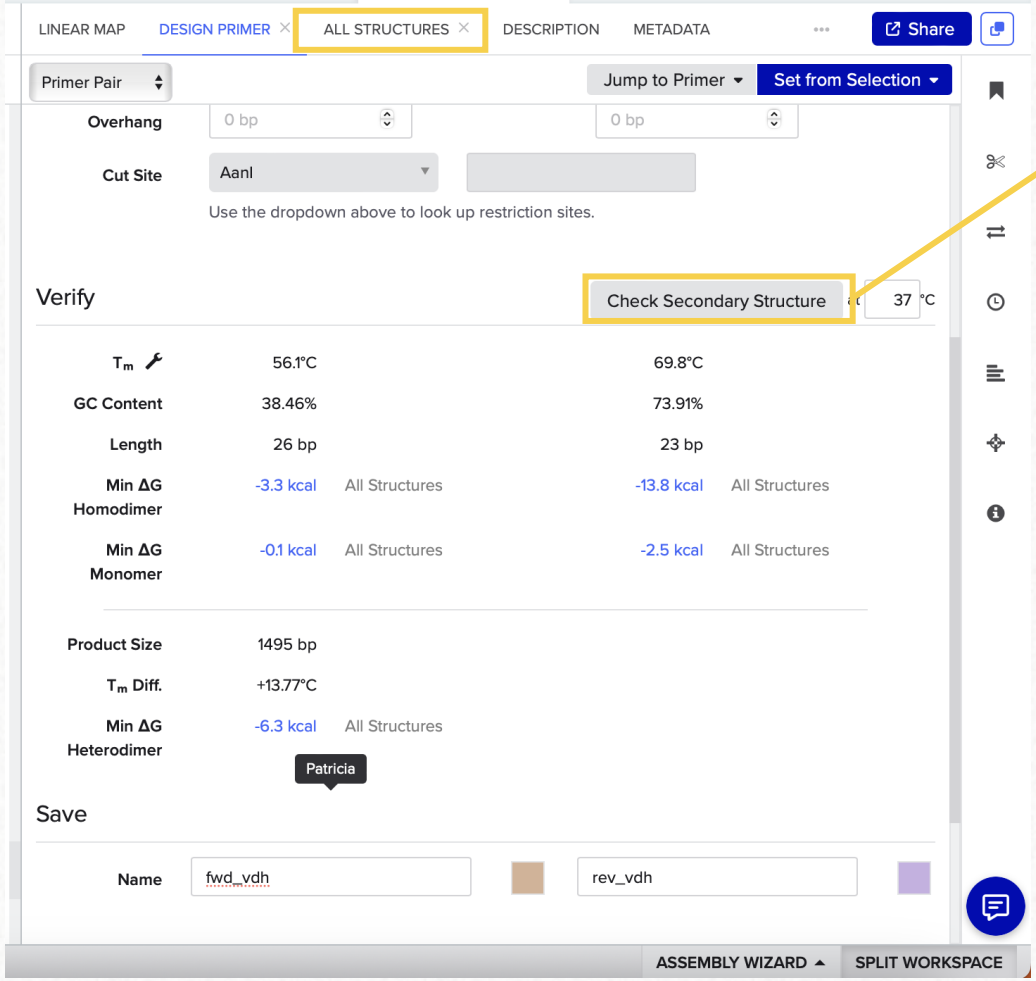
✓ Make sure to check that the melting temperatures of your primer pair are within an acceptable range

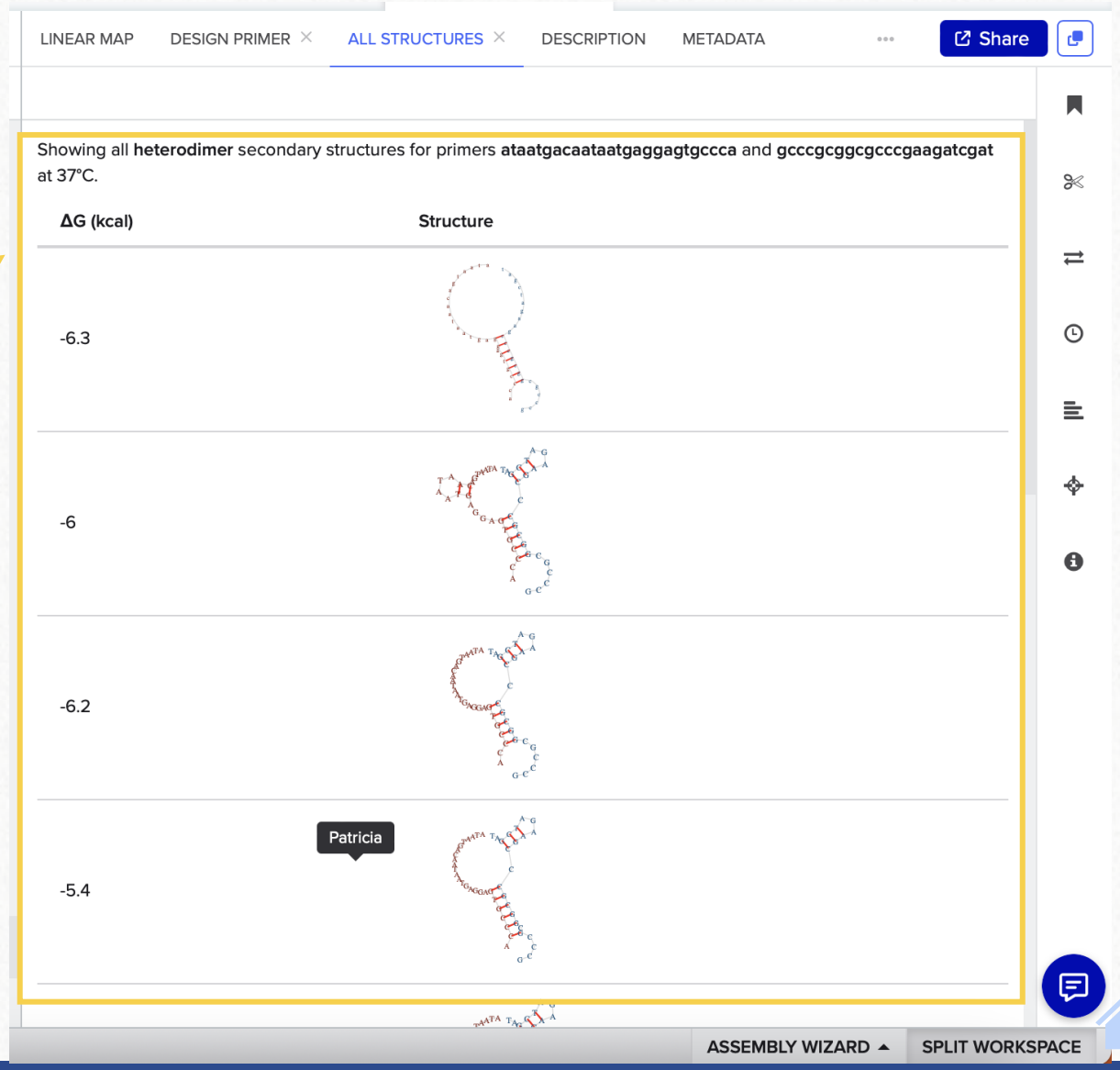
PRO TIP: *You can adjust the default parameters for thermodynamic calculations*

Construct design

Manual primer creation

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





5. Basic construct assembly

5.1 Primer design

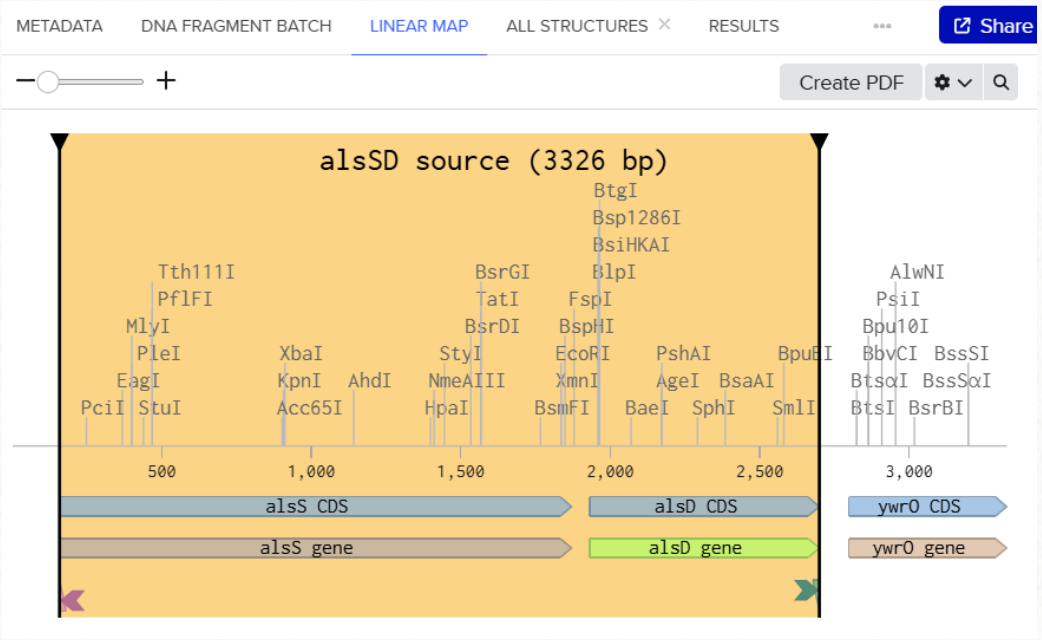
5.1.2 Primer wizard

Construct design

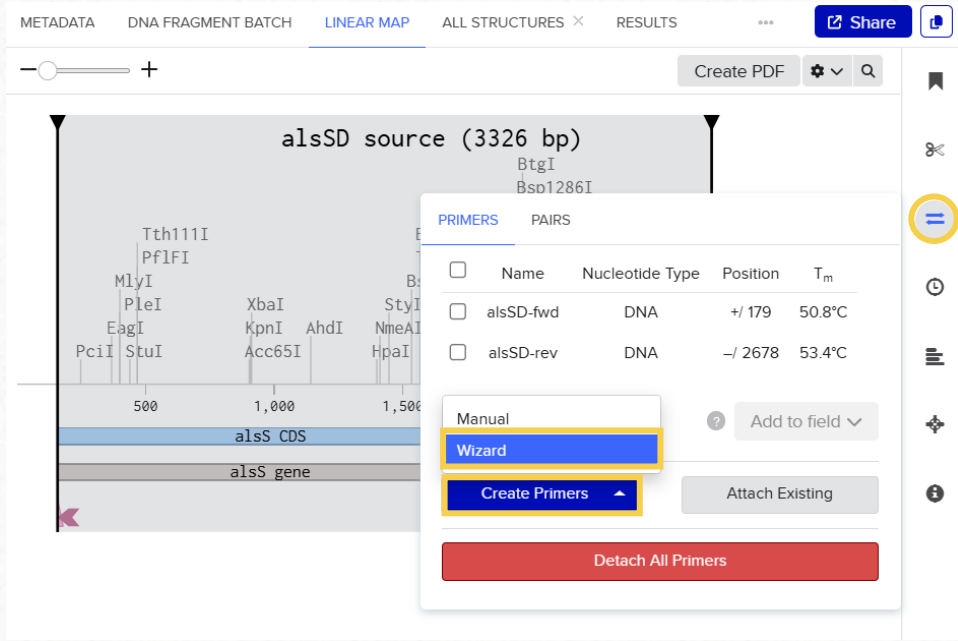
*Automatic primer creation – **Primer Wizard***

✓ 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*



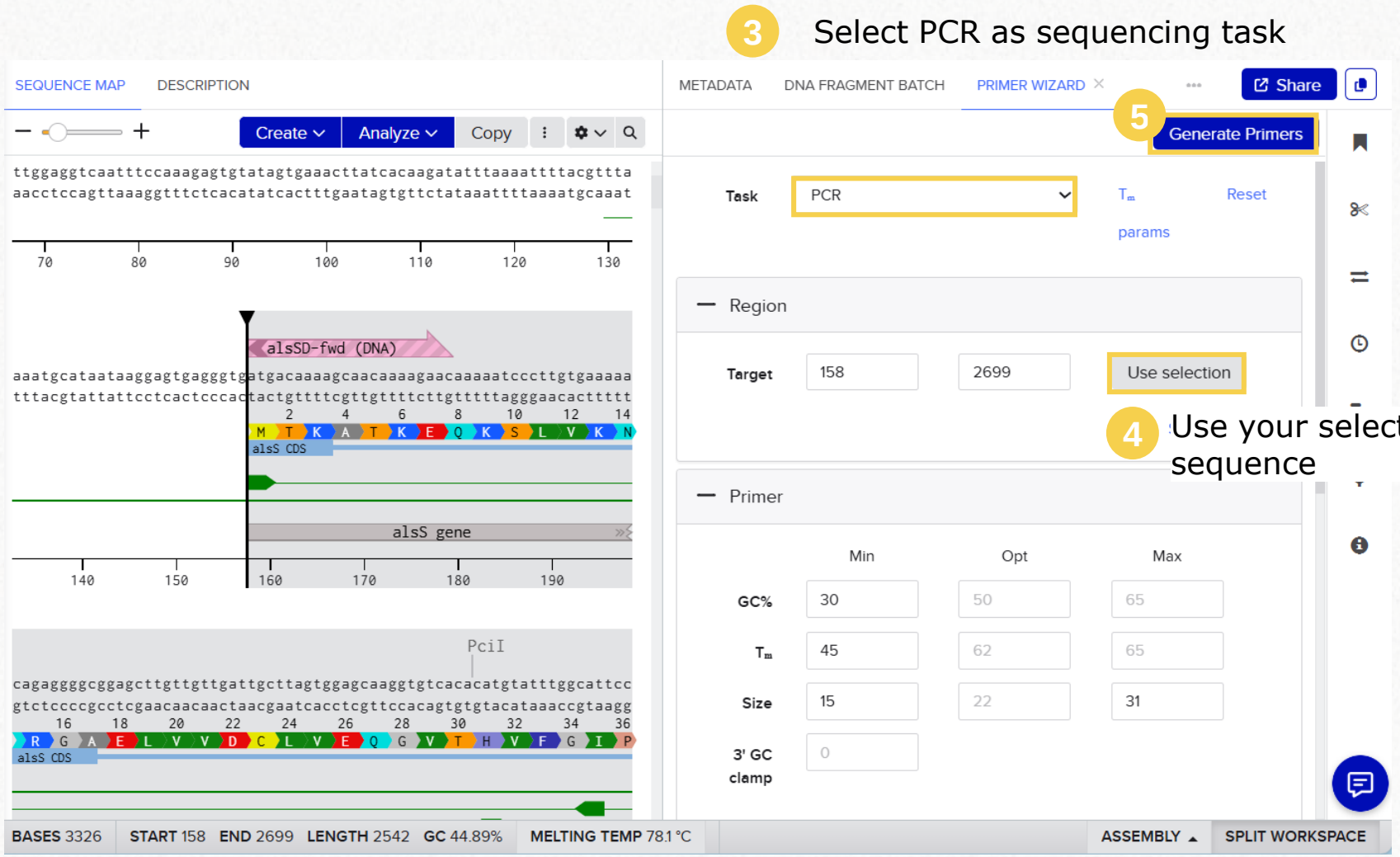
2 Access the primer tool and select *wizard*



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

Construct design

*Automatic primer creation – **Primer Wizard***



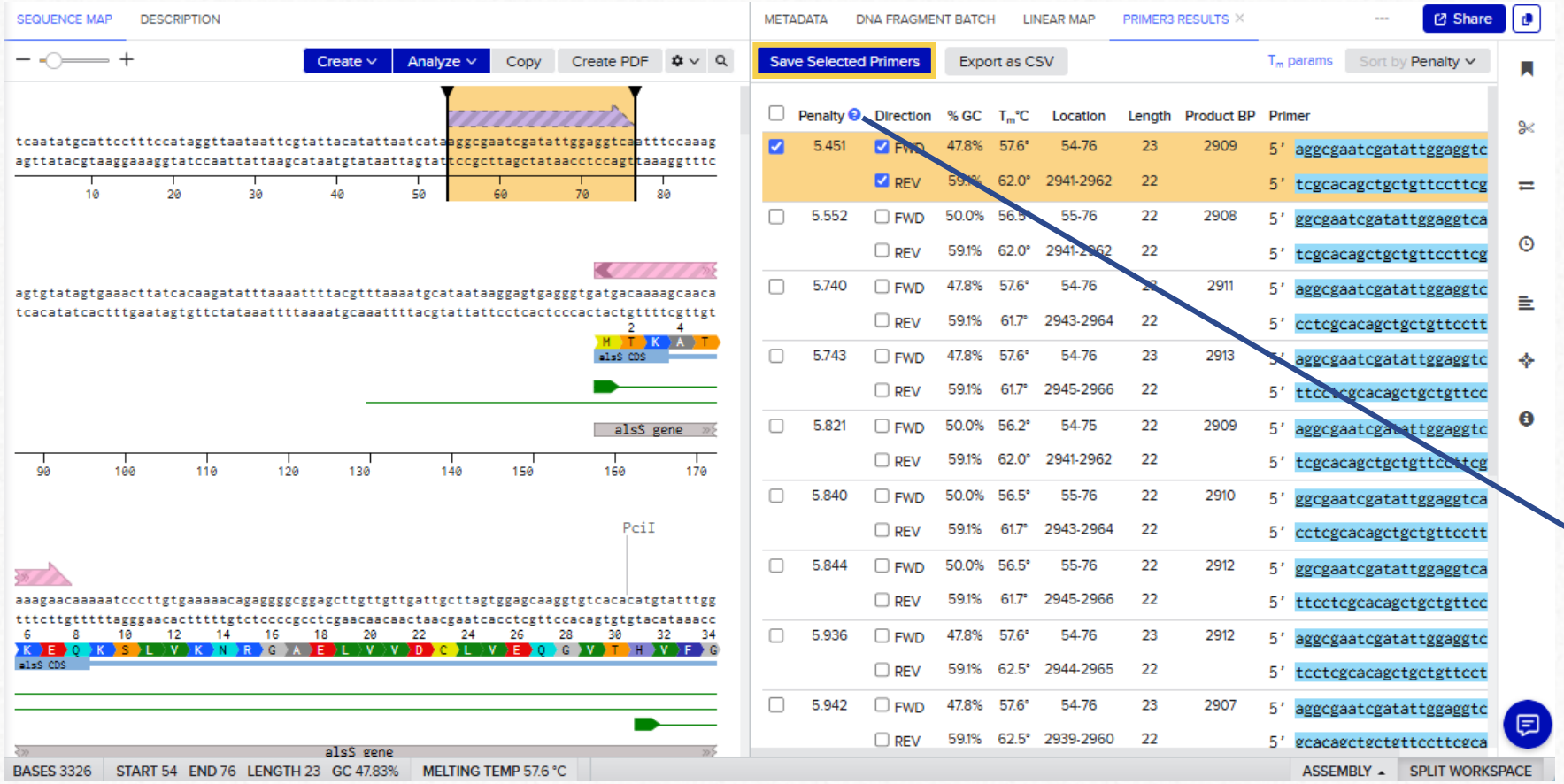
- ✓ Primer Wizard allows for different sequencing tasks
- ✓ Primer Wizard is powered by Primer3

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

Construct design

*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.



Penalty	Direction	% GC	T_m °C	Location	Length	Product BP	Primer
5.451	FWD	47.8%	57.6°	54-76	23	2909	5′ aggcgaatcgatattggaggtc
	REV	59.1%	62.0°	2941-2962	22		5′ tcgcacagctgctgttccttcg
5.552	FWD	50.0%	56.5°	55-76	22	2908	5′ ggcgaatcgatattggaggtca
	REV	59.1%	62.0°	2941-2962	22		5′ tcgcacagctgctgttccttcg
5.740	FWD	47.8%	57.6°	54-76	23	2911	5′ aggcgaatcgatattggaggtc
	REV	59.1%	61.7°	2943-2964	22		5′ cctcgcacagctgctgttcctt
5.743	FWD	47.8%	57.6°	54-76	23	2913	5′ aggcgaatcgatattggaggtc
	REV	59.1%	61.7°	2945-2966	22		5′ ttcctcgcacagctgctgttcc
5.821	FWD	50.0%	56.2°	54-75	22	2909	5′ aggcgaatcgatattggaggtc
	REV	59.1%	62.0°	2941-2962	22		5′ tcgcacagctgctgttccttcg
5.840	FWD	50.0%	56.5°	55-76	22	2910	5′ ggcgaatcgatattggaggtca
	REV	59.1%	61.7°	2943-2964	22		5′ cctcgcacagctgctgttcctt
5.844	FWD	50.0%	56.5°	55-76	22	2912	5′ ggcgaatcgatattggaggtca
	REV	59.1%	61.7°	2945-2966	22		5′ ttcctcgcacagctgctgttcc
5.936	FWD	47.8%	57.6°	54-76	23	2912	5′ aggcgaatcgatattggaggtc
	REV	59.1%	62.5°	2944-2965	22		5′ tcctcgcacagctgctgttcct
5.942	FWD	47.8%	57.6°	54-76	23	2907	5′ aggcgaatcgatattggaggtc
	REV	59.1%	62.5°	2939-2960	22		5′ gcacagctgctgttccttcgca

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

By default, sorting is done based on Pimer3 penalty score.

The lower the penalty, the better the primer pair

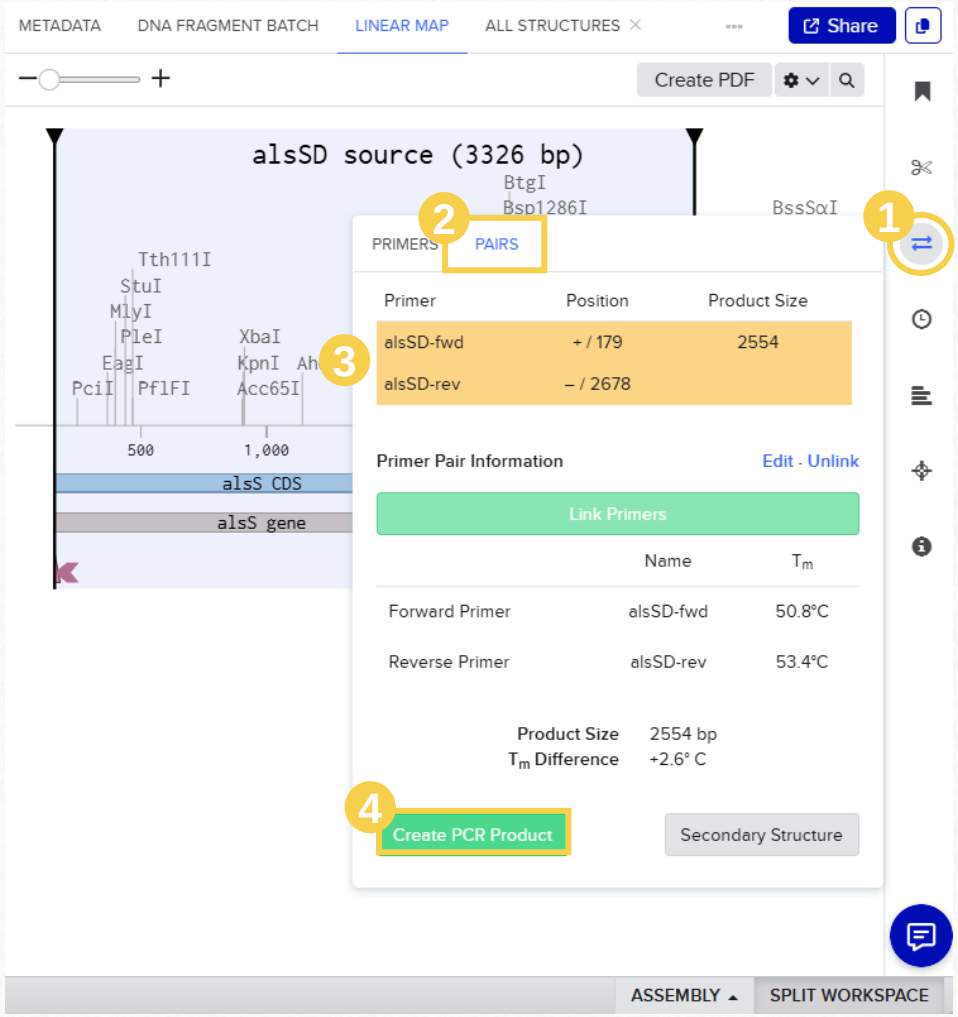
5. Basic construct assembly

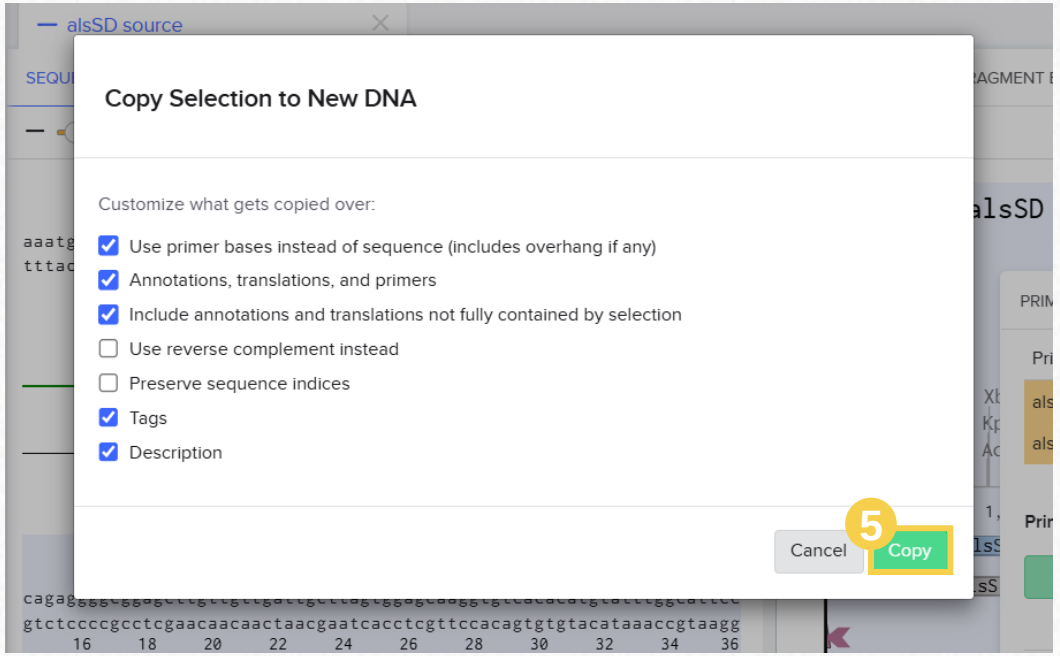
5.2 *In-silico* PCR

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 **SalI** restriction sites.





✓ 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

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

5. Basic construct assembly

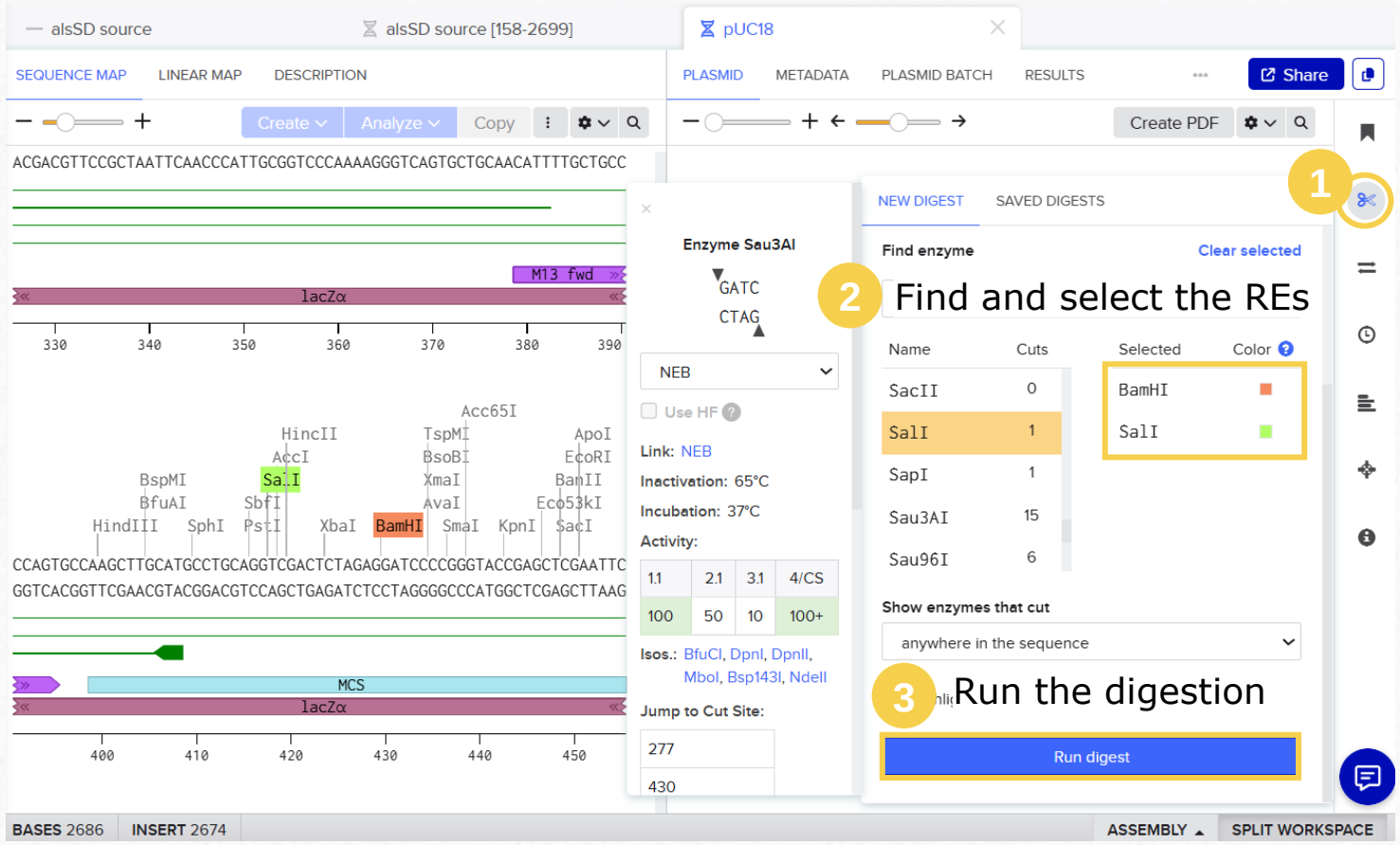
5.3 Virtual digestion

Construct design

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)

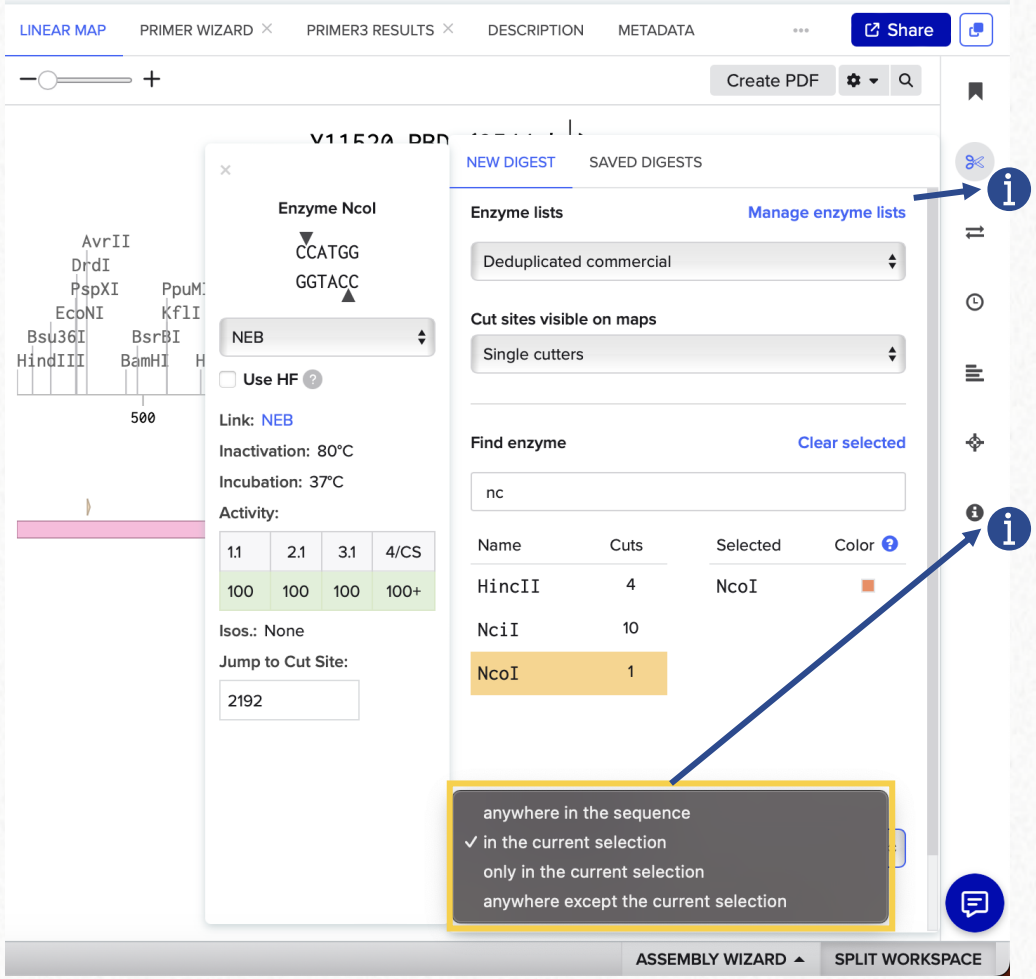
Digestion of the backbone (open the pUC18 sequence)



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

Construct design

Virtual digestion



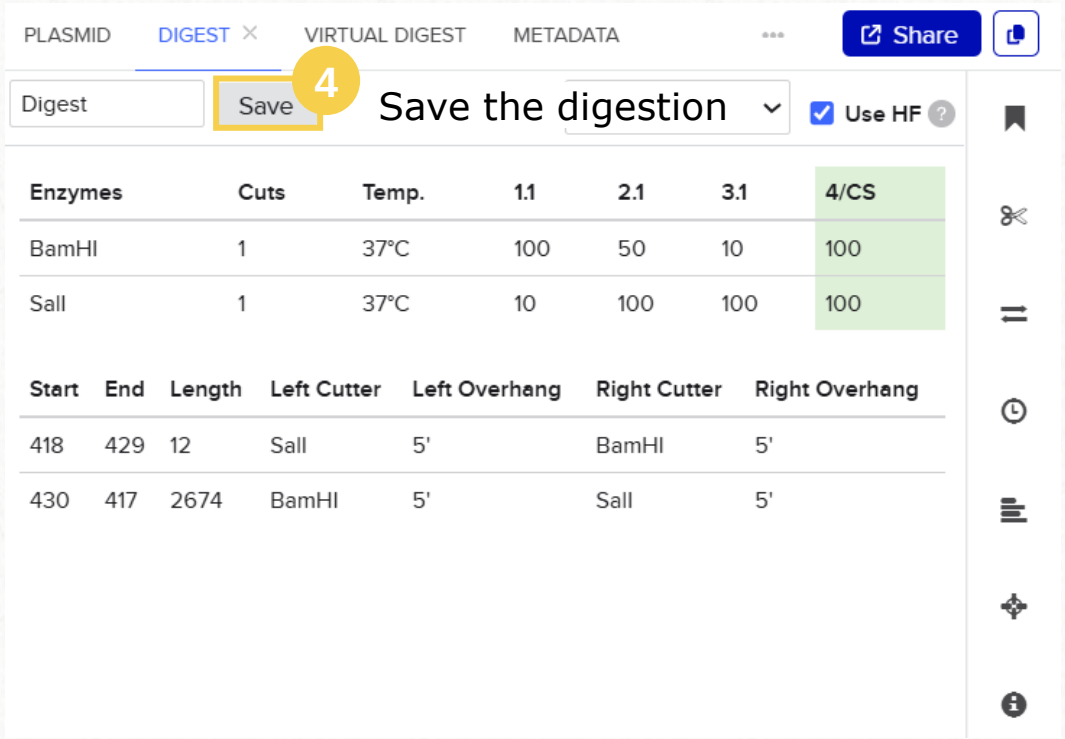
PRO TIP: The enzyme lists available can be managed, similarly to the features libraries and are shared within the Center.

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

Construct design

Virtual digestion

Digestion of the backbone



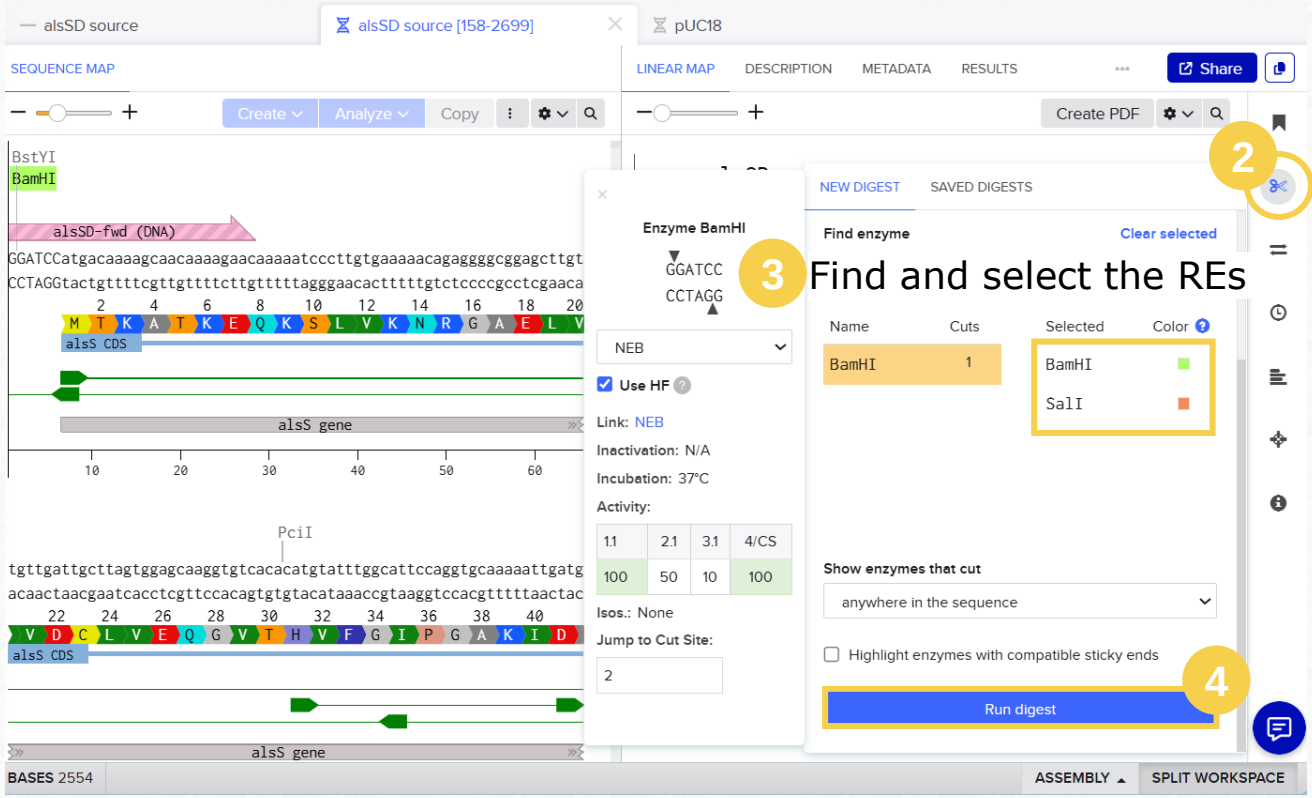
- ✓ A saved digestion will allow you to easily find the fragments you need to work with for the assembly

Construct design

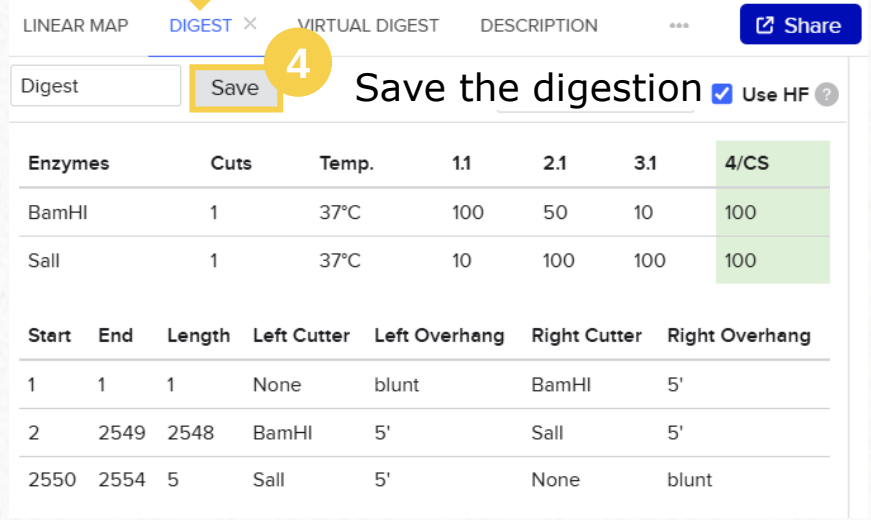
Virtual digestion

Digestion of the insert

1 Open the amplified *alsSD* sequence



The digest tab will open



Save the digestion

Enzymes	Cuts	Temp.	1.1	2.1	3.1	4/CS
BamHI	1	37°C	100	50	10	100
SalI	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'	SalI	5'
2550	2554	5	SalI	5'	None	blunt

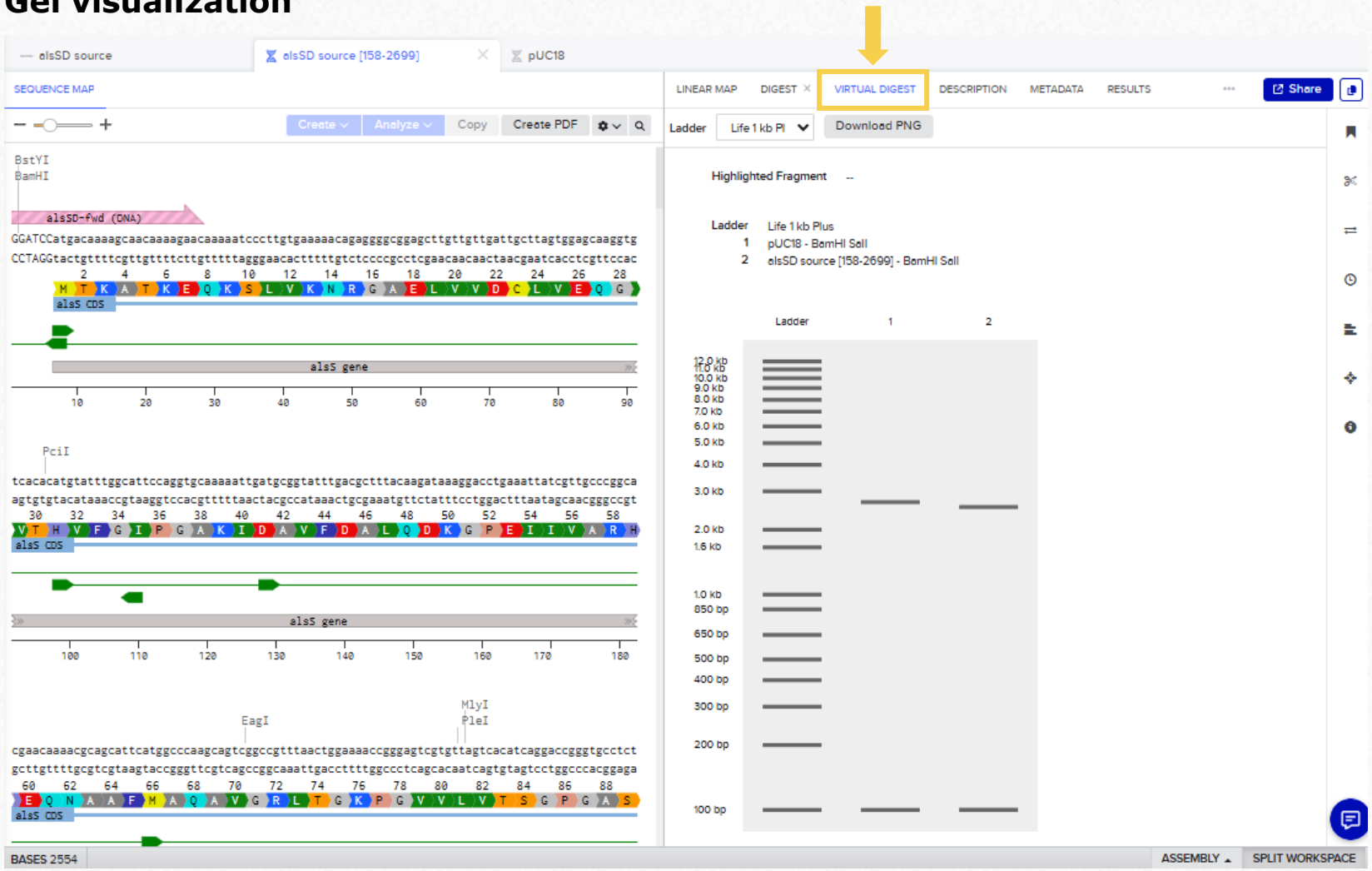
Find and select the REs

Run the digestion

Construct design

Virtual digestion

Gel visualization



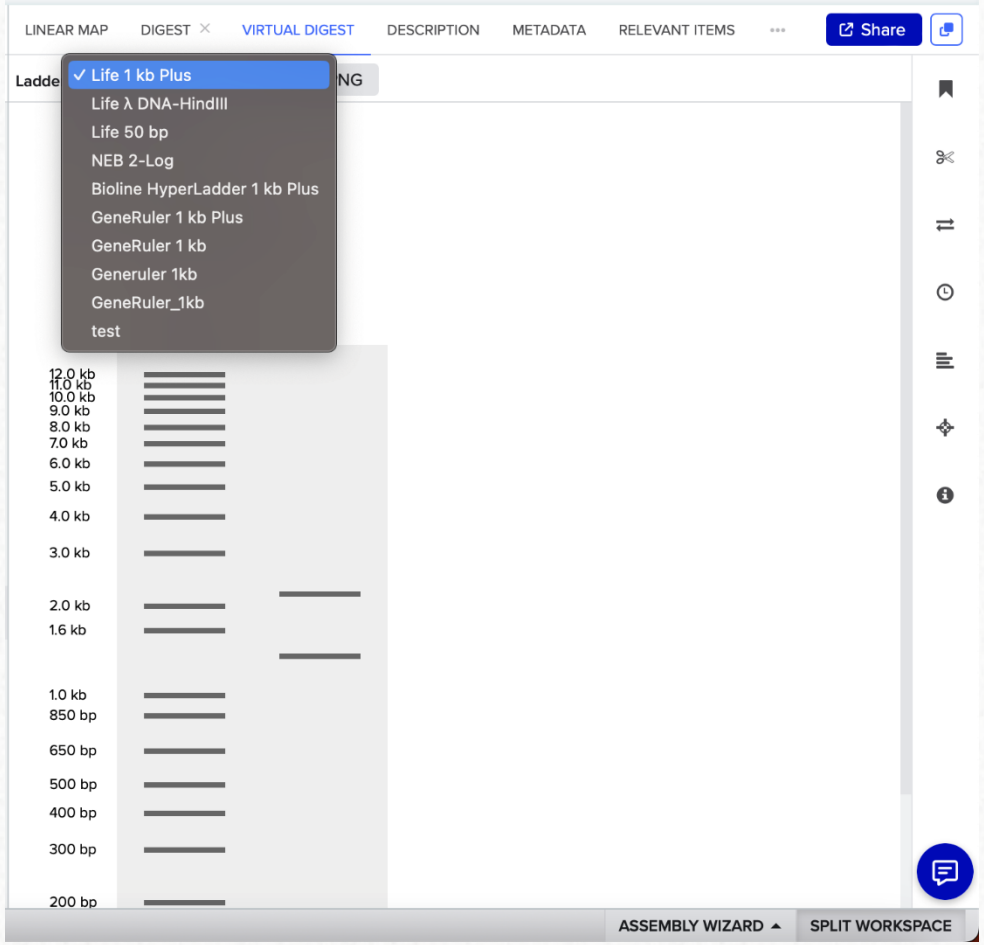
- ✓ After running both digestions, you can easily visualize the resulting fragments in a simulated electrophoresis gel.

- 1st lane: **Ladder**
- 2nd lane: **Backbone**
- 3rd lane: **Insert**

- ✓ If you click on the bands, you can easily select the DNA sequences that correspond to the digested fragments

Construct design

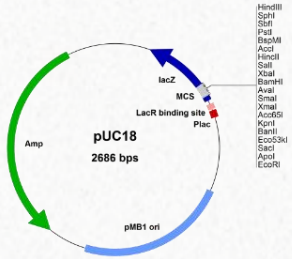
Virtual digestion



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

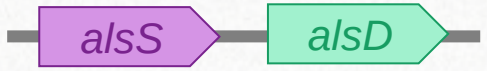
5. Basic construct assembly

5.4 Assembly Wizard



pUC18 vector

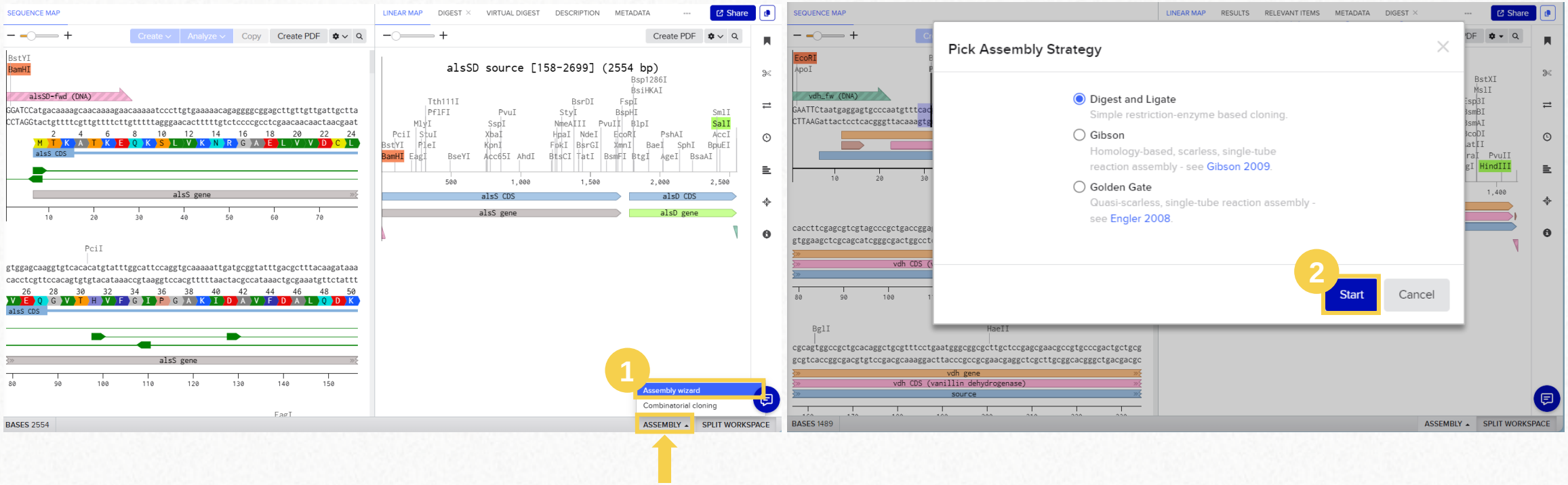
+



Genes of interest

Construct Assembly

Assembly Wizard

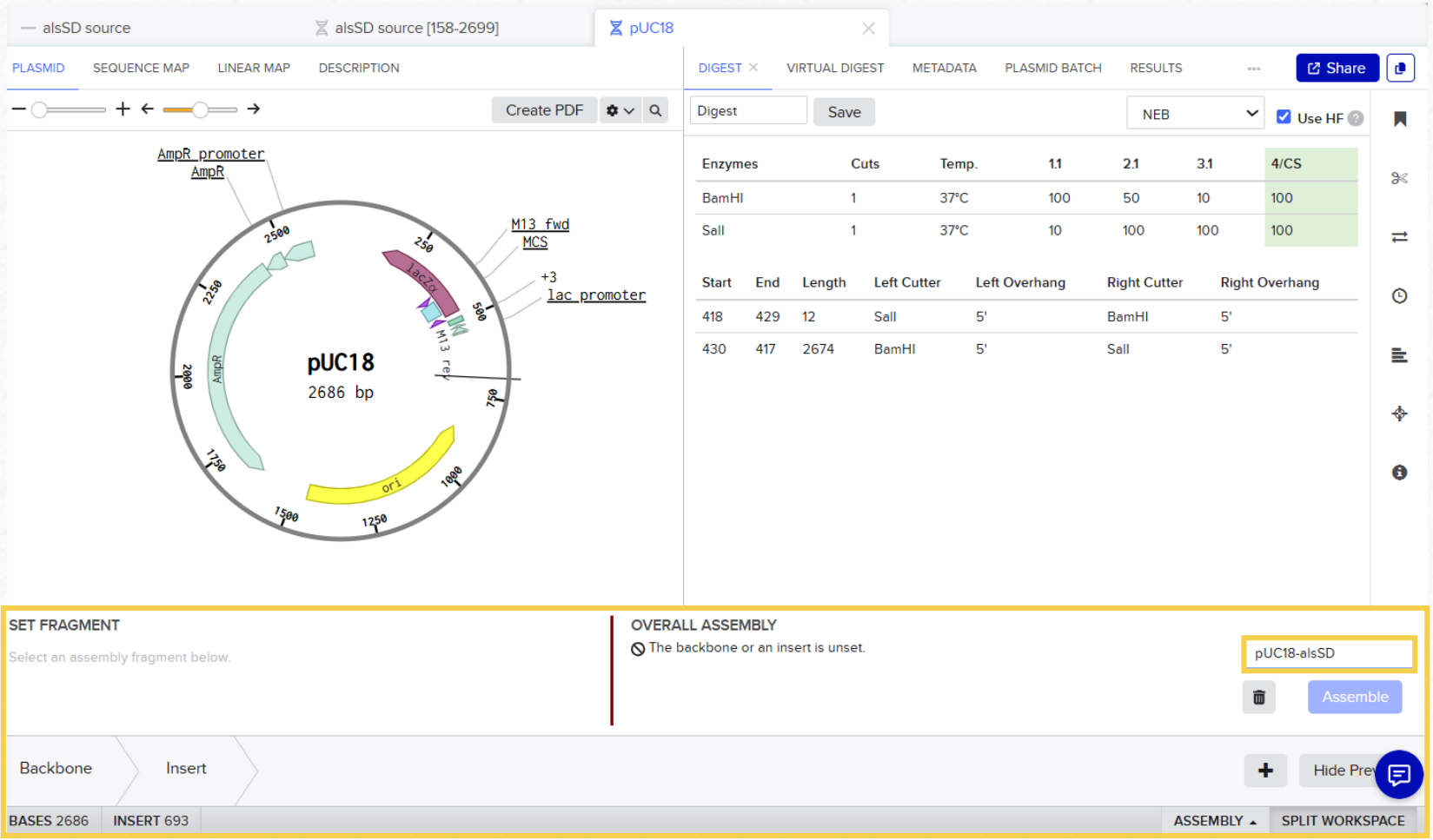


The Assembly Wizard allows you to use the following assembly strategies:

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

Construct Assembly

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

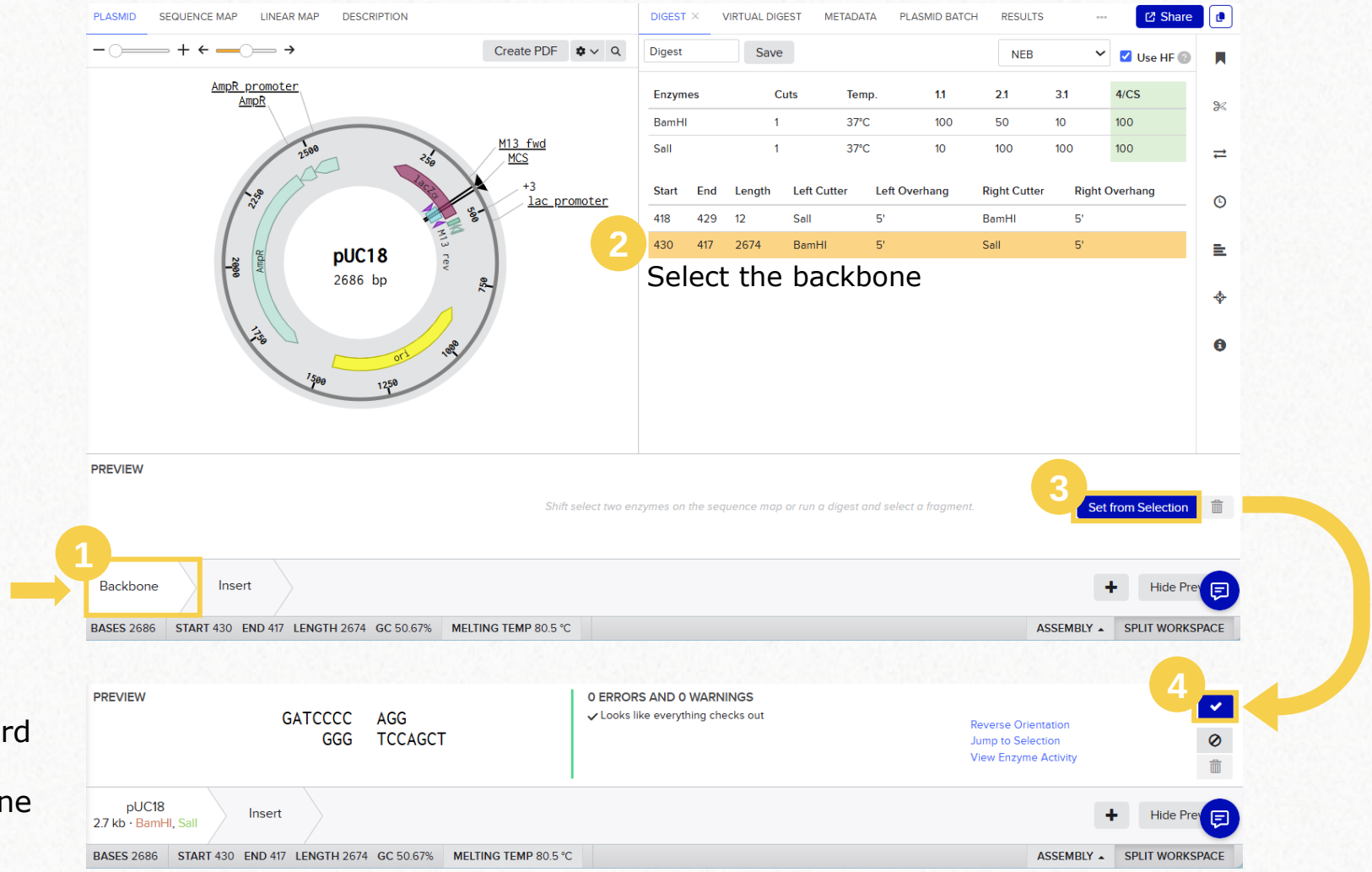


✓ This will remain open even if you go from one file to another

Name your construct

Construct Assembly

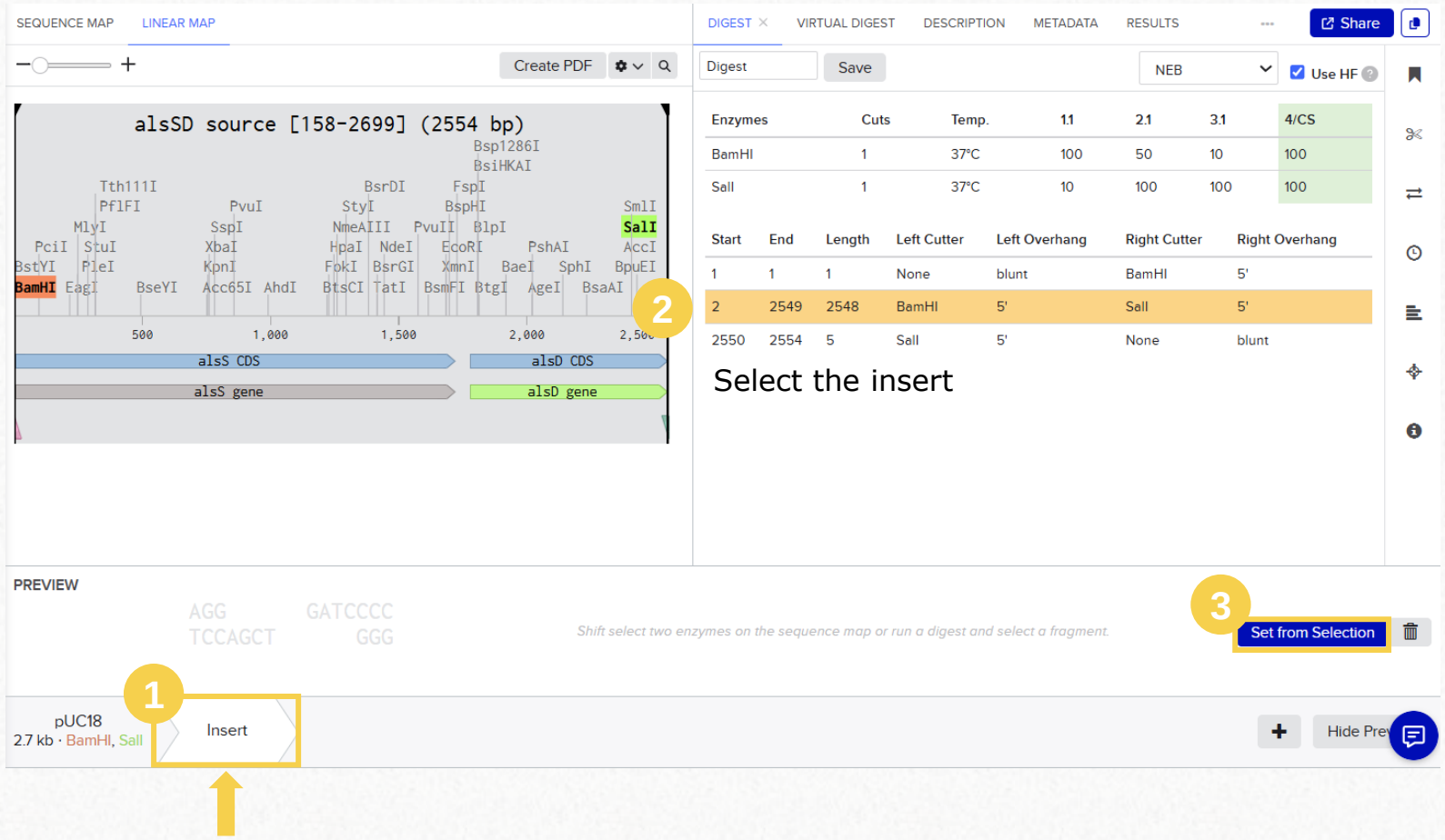
Digest and Ligate: Add the backbone



✓ The Assembly Wizard shows the digested ends of the backbone

Construct Assembly

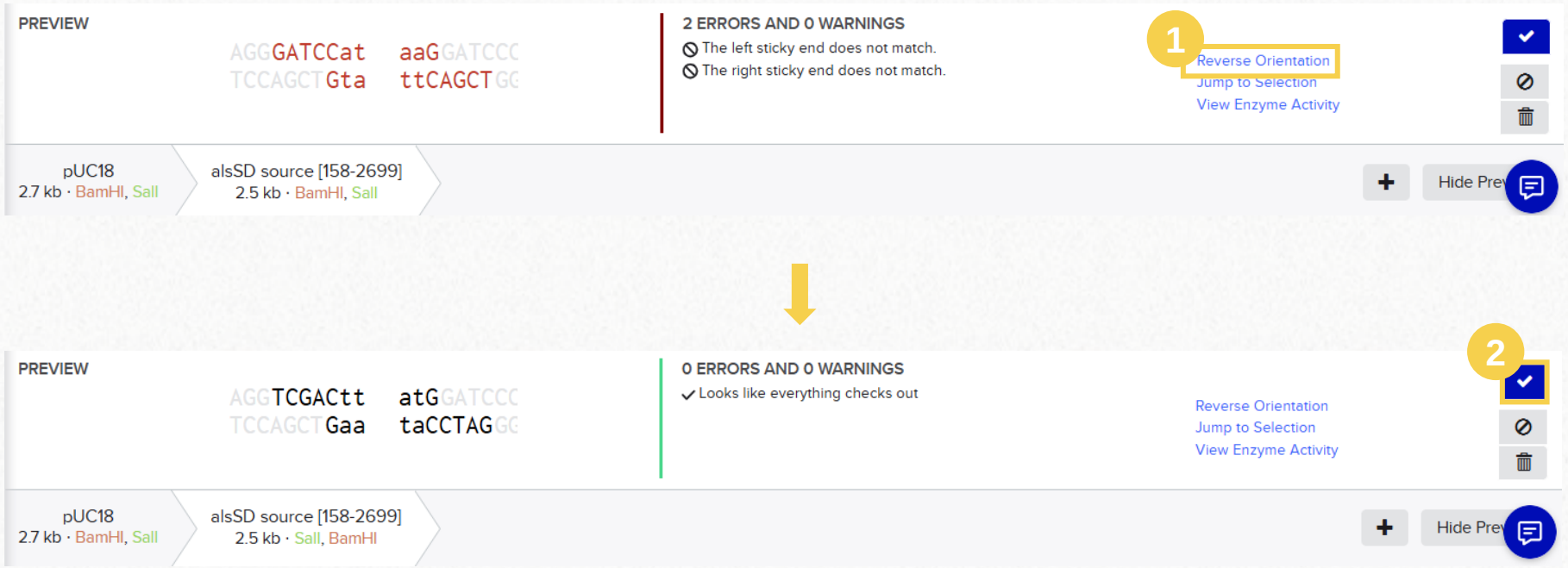
Digest and Ligate: Add the insert



Construct Assembly

Digest and Ligate: Check for compatibility

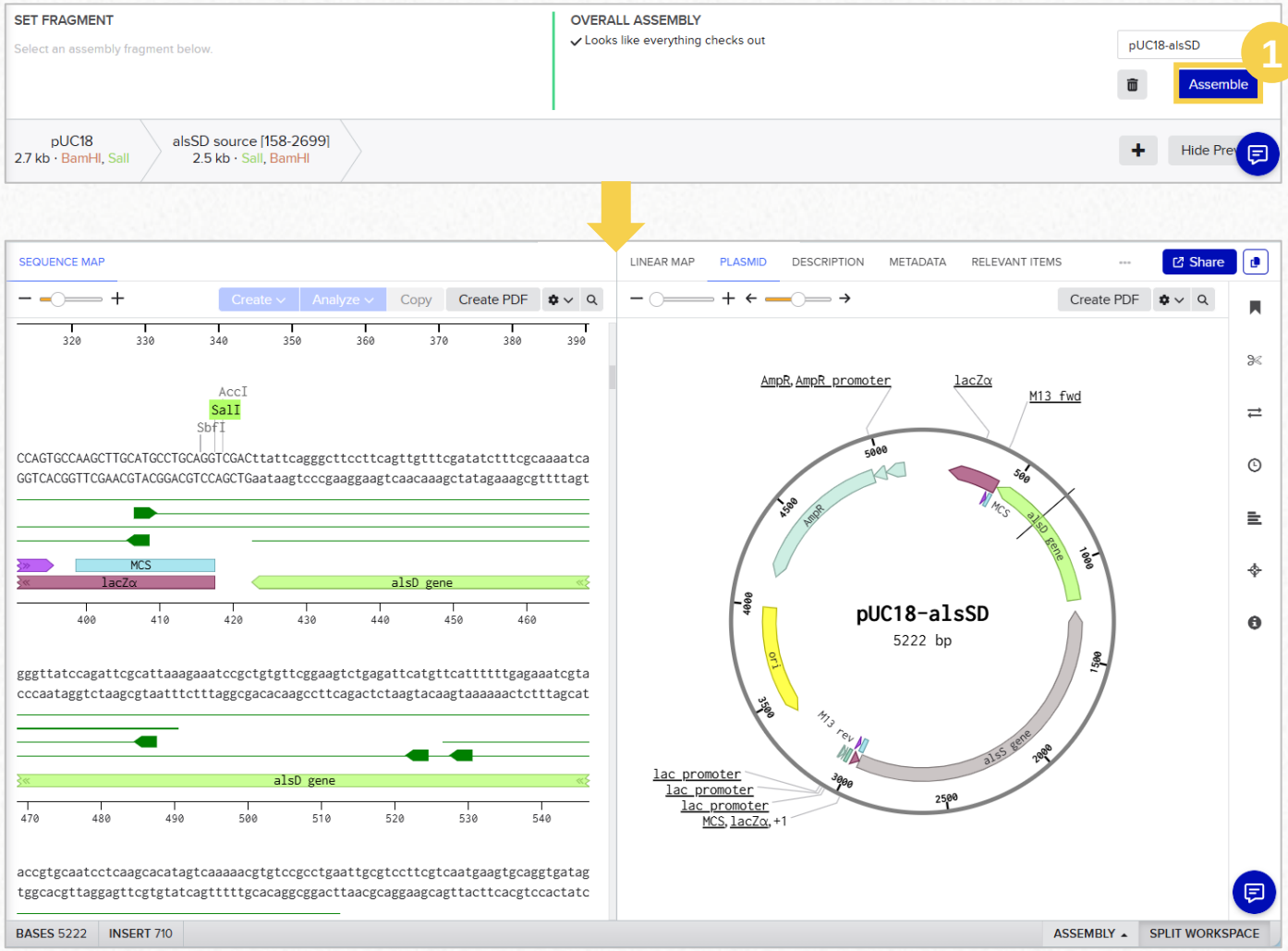
- ✓ The assembly wizard will check for compatibility between sticky ends.
- ✓ Depending on the orientation of your backbone and insert, you might need to make adjustments – such as in this case!



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

Construct Assembly

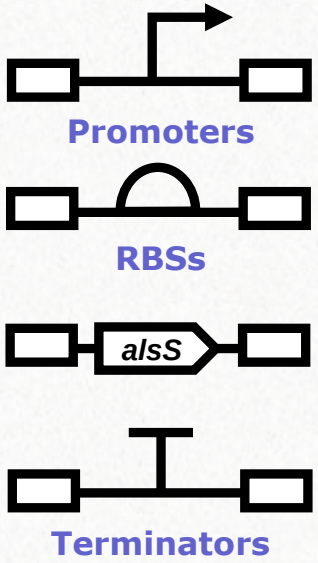
Digest and Ligate: Assemble



- ✓ You will be asked to choose a folder to save the construct in
- ✓ The assembly is now done!

6. Combinatorial cloning: Golden Gate

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

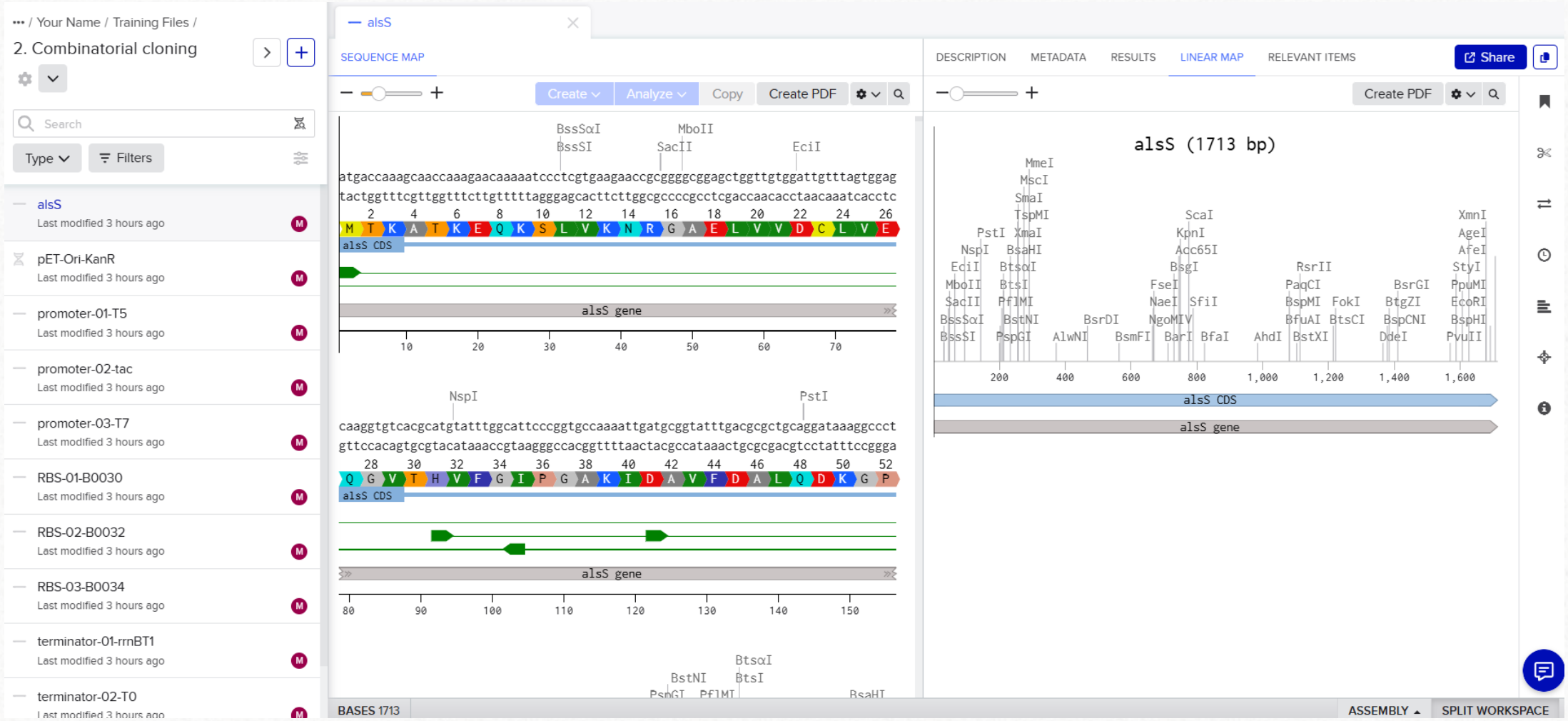


✓ Combinatorial cloning: **Golden Gate**

Expected output:

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

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

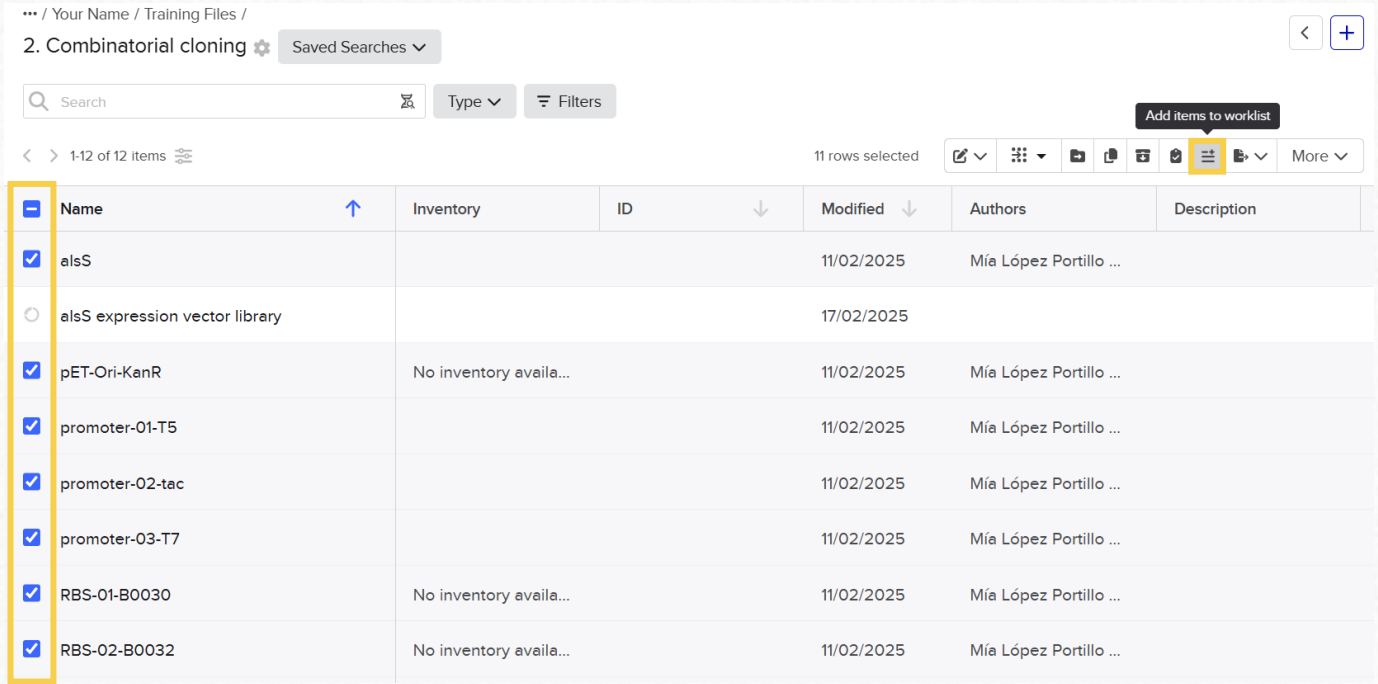


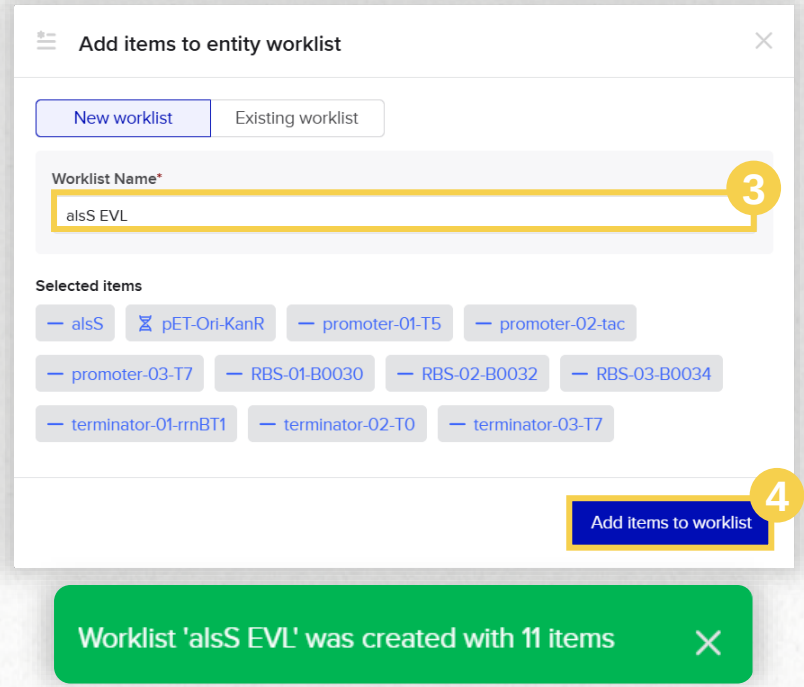
Construct Assembly

Worklist creation

PRO TIP:

Creating a **worklist** can make it easier to find your most used files!





1. Select the sequence files in the **Combinatorial cloning** folder
2. Select "**Add items to worklist**"

Construct Assembly

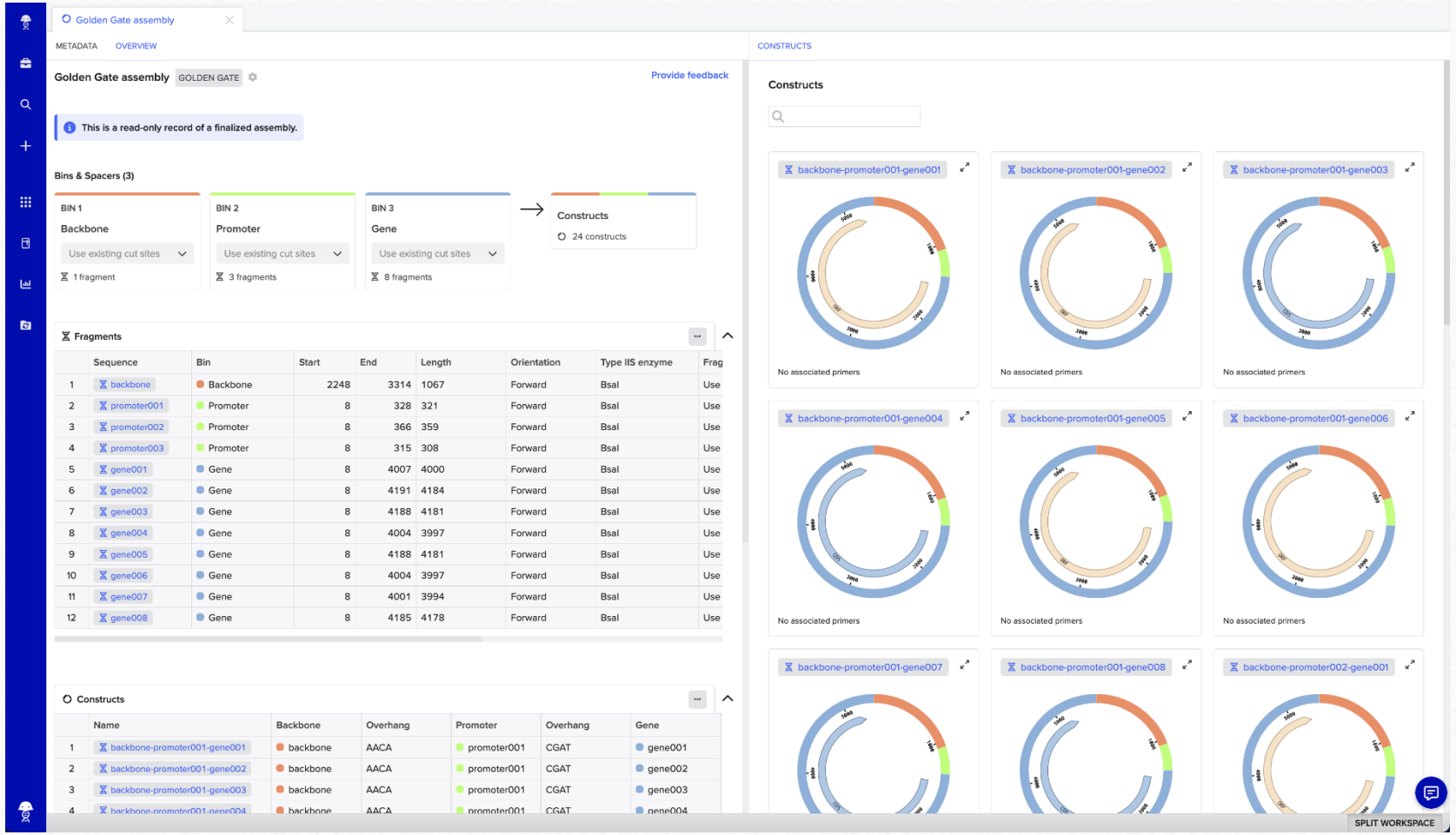
Combinatorial Cloning Tool

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

It allows you to work with several cloning methods:

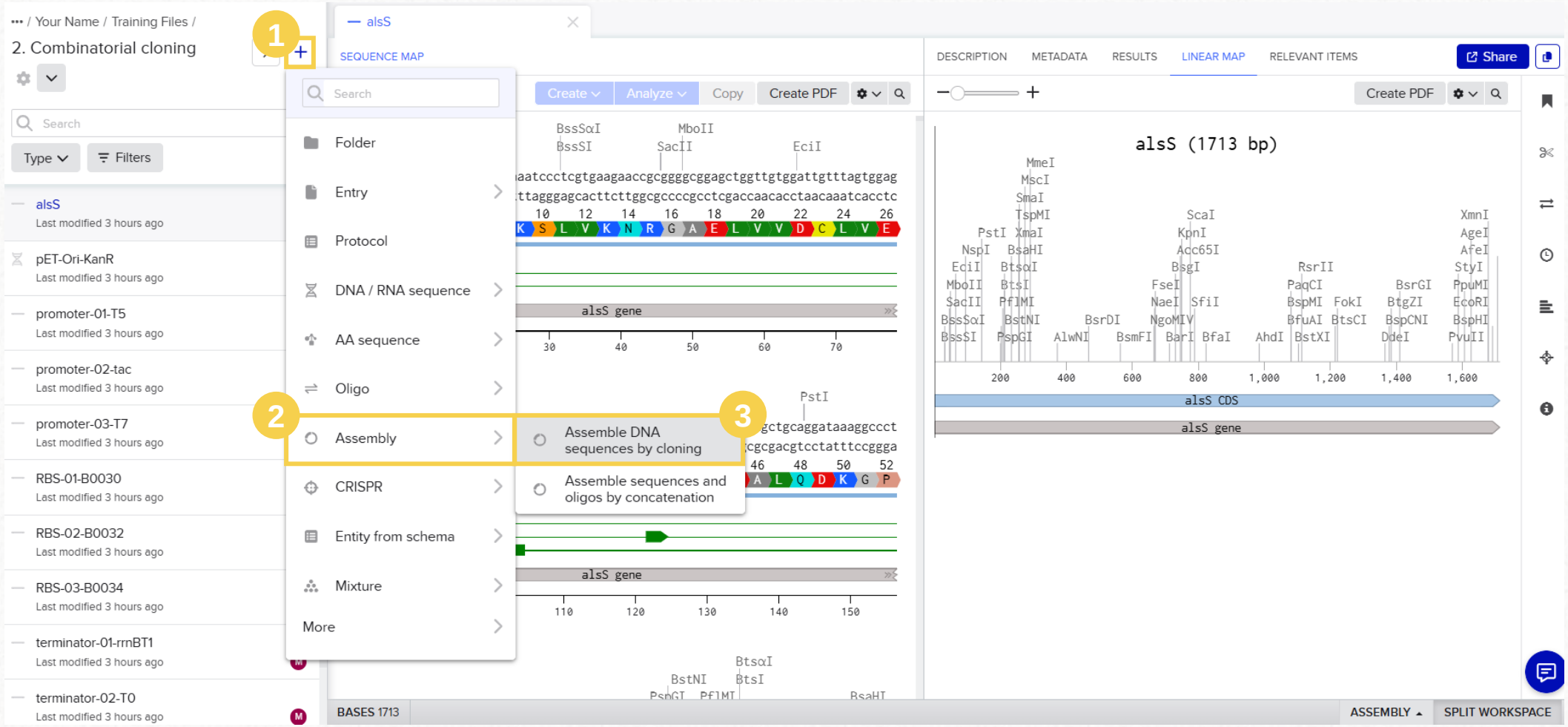
- ✓ **Golden Gate**
- ✓ **Gibson**
- ✓ **Homology**

- ✓ This tool is especially useful for **designing many constructs at once**



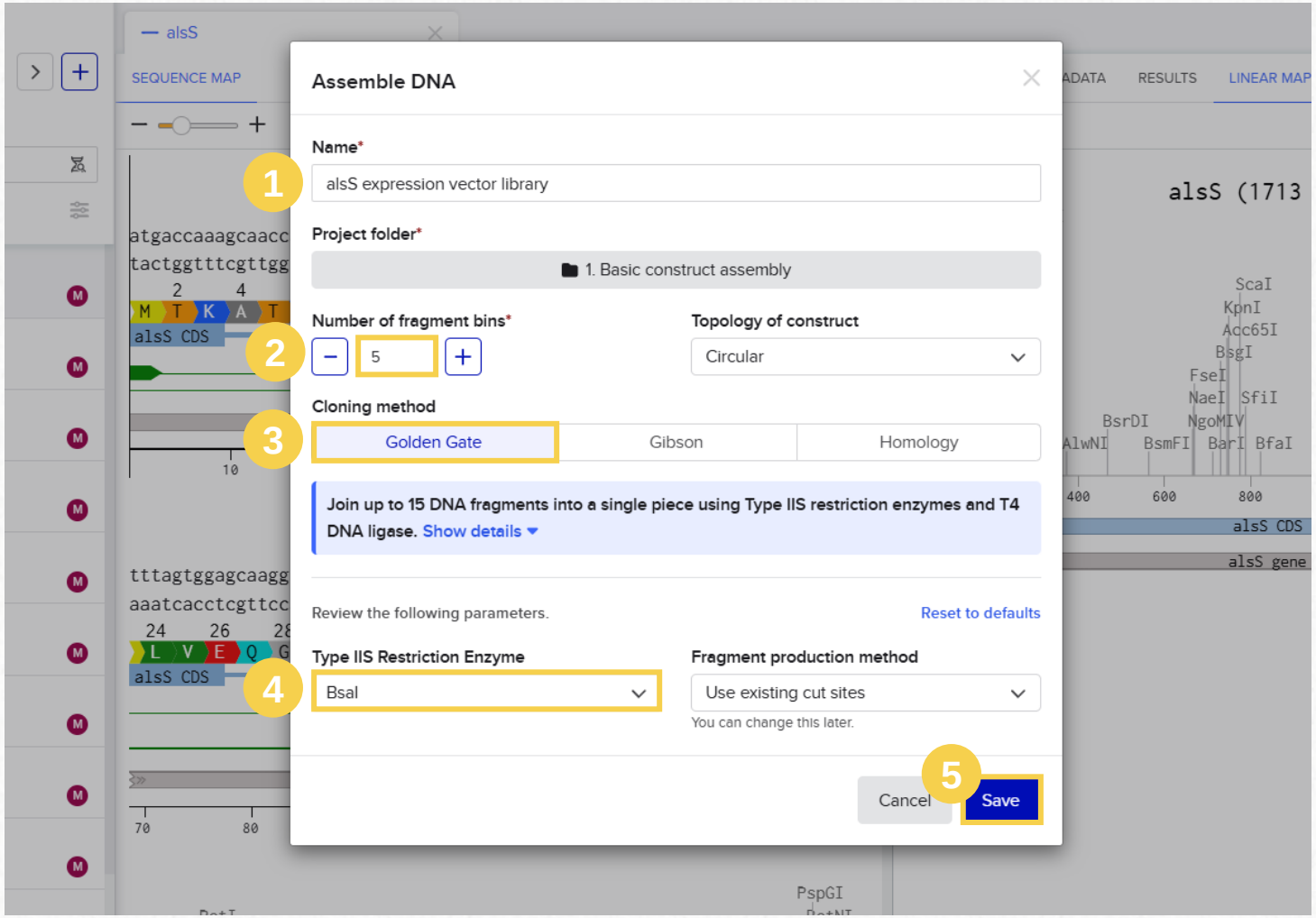
Construct Assembly

Combinatorial Cloning Tool: *How to access it*



Construct Assembly

Combinatorial Cloning Tool: Configuration

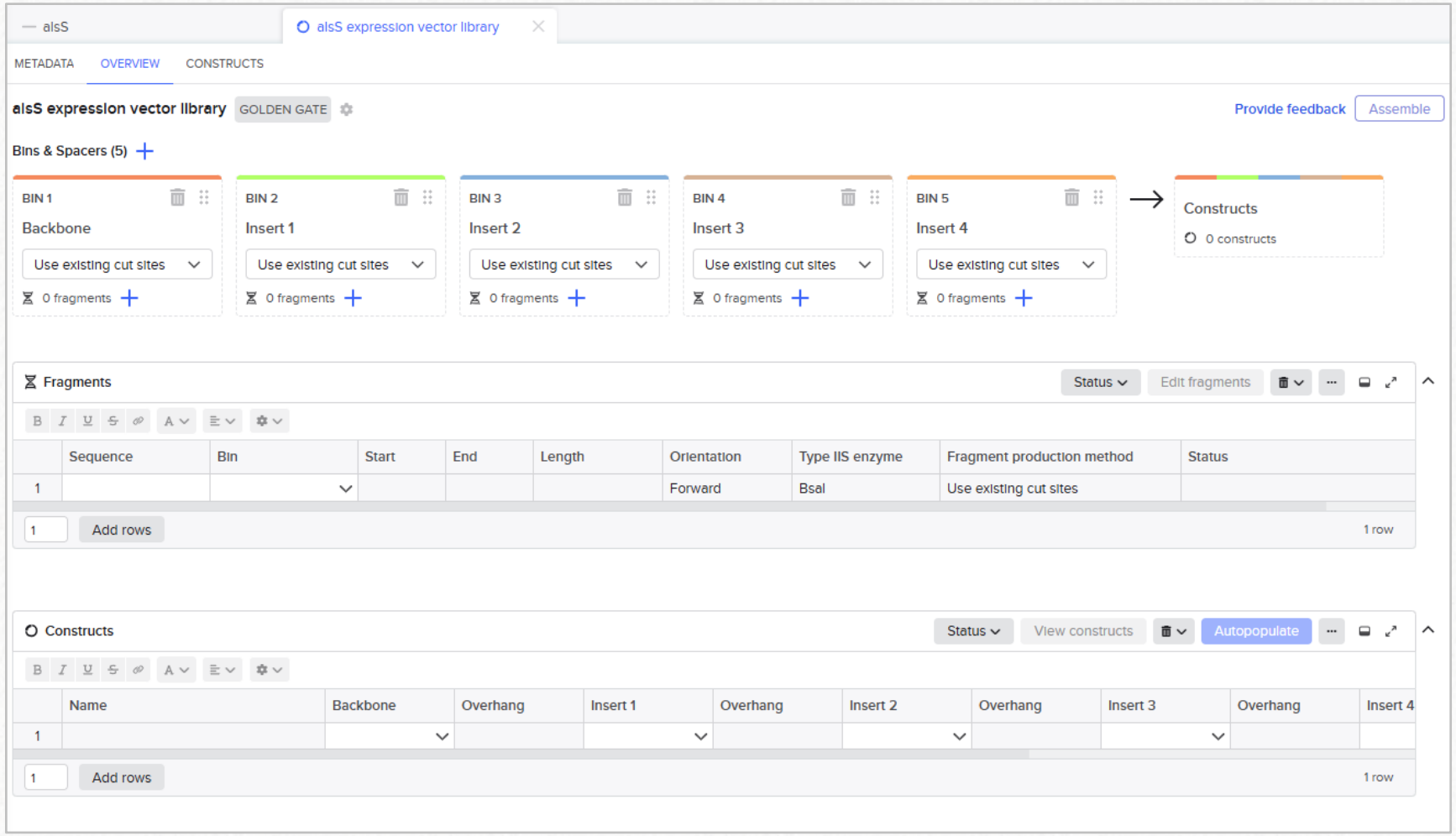


You can modify these parameters later
(before finalizing the assembly)

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

Construct Assembly

Combinatorial Cloning Tool: Full view



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

All added fragments will show up here

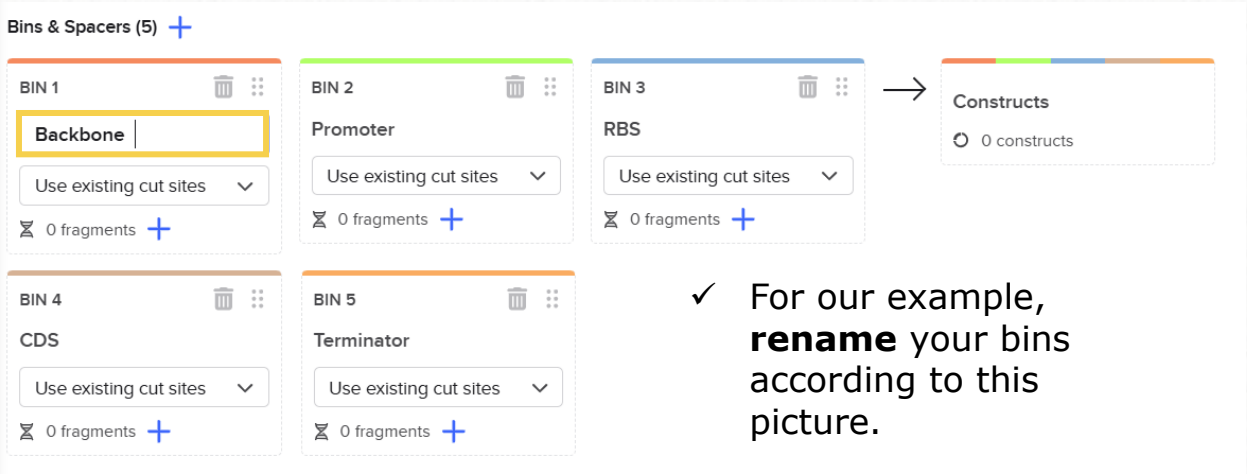
(You can change some configurations)

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

Construct Assembly

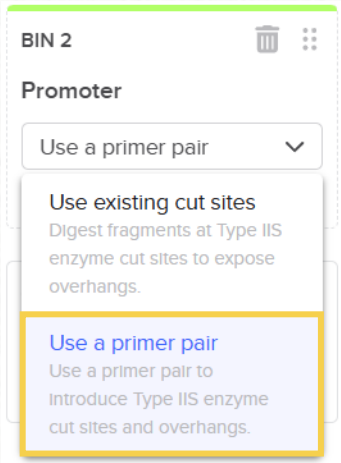
Combinatorial Cloning Tool: Bins and spacers

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



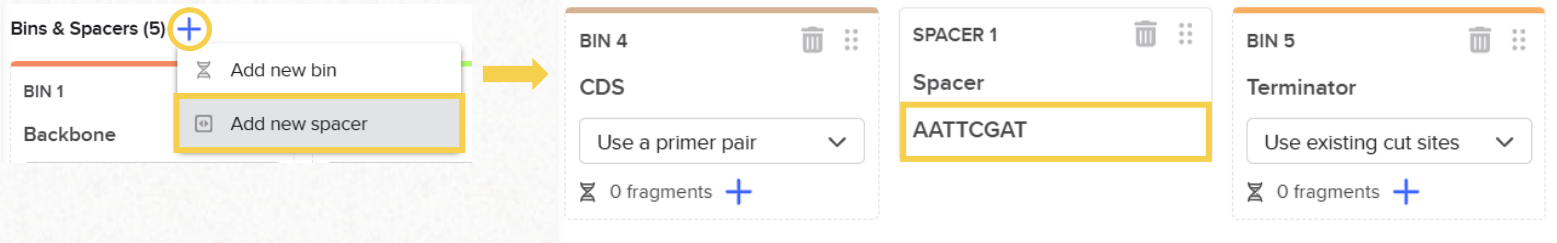
✓ For our example, **rename** your bins according to this picture.

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



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

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.



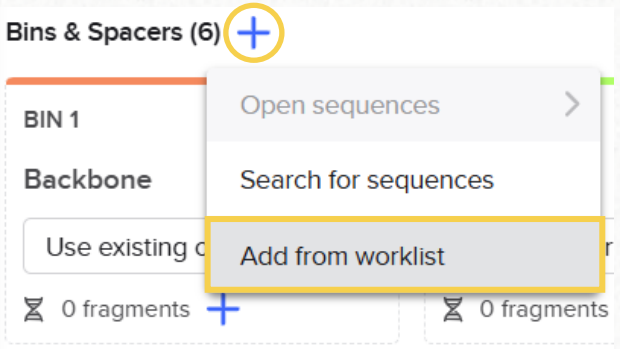
✓ Spacers will not be used in our example.

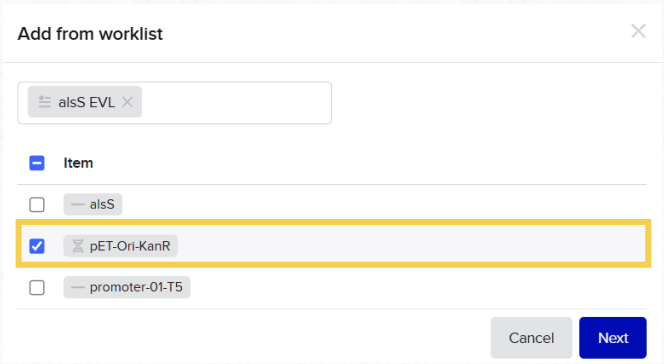
Construct Assembly

Golden Gate: *Set fragments in corresponding bins*

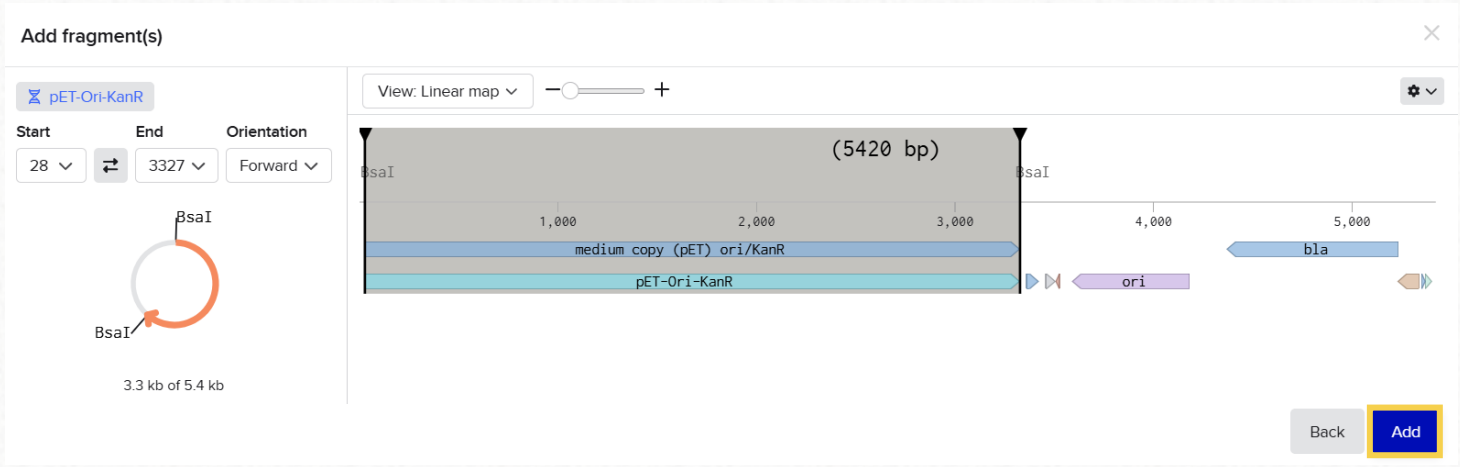
1. Backbone

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





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



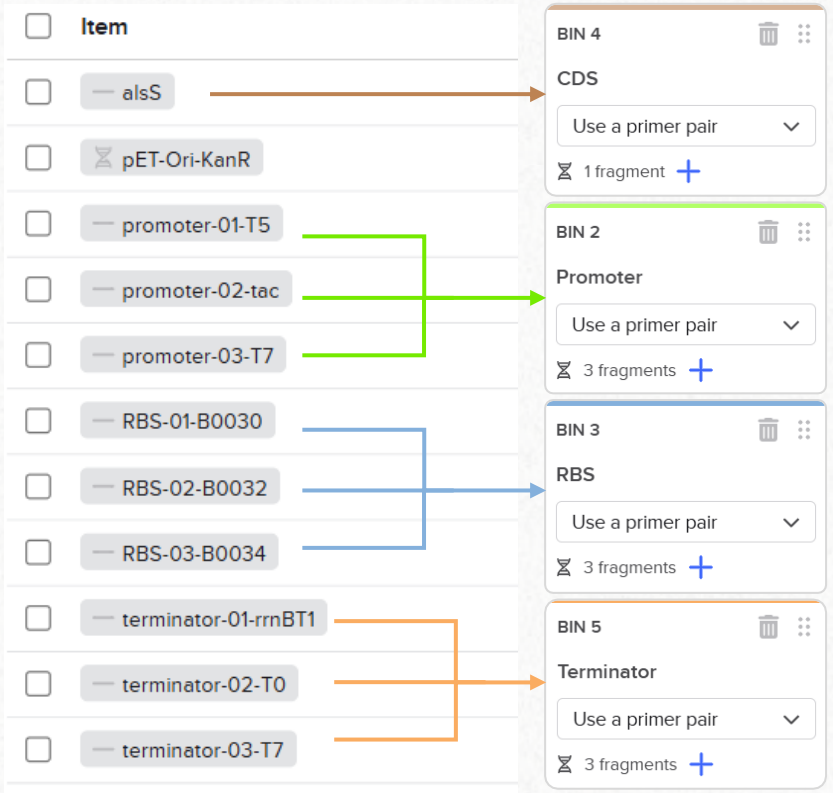
- ✓ Since this bin was configured to use **existing cut sites**, Benchling has detected the **BsaI** sites in the sequence and automatically selected the region between them.
- ✓ If you choose the option to **create a primer pair** for a sequence, you will be able to freely select the region you'd like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

Construct Assembly

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.

Construct Assembly

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.

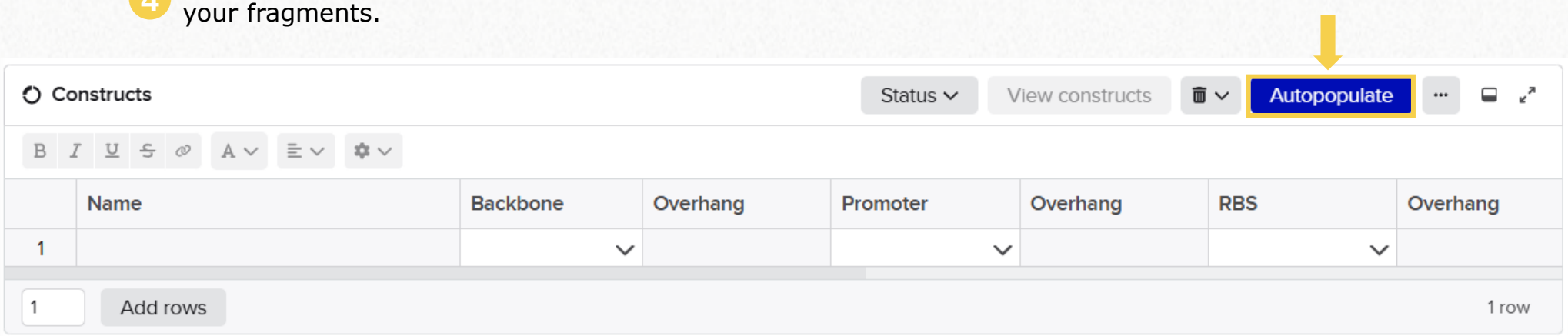
Fragments — Status — Edit fragments — Close

	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	BsaI	Use existing cut sites			Looks good
2	promoter-01-T5	Promoter	1	45	45	Forward	BsaI	Use a primer pair			Looks good
3	promoter-02-tac	Promoter	1	46	46	Forward	BsaI	Use a primer pair			Looks good
4	promoter-03-T7	Promoter	1	36	36	Forward	BsaI	Use a primer pair			Looks good
5	RBS-01-B0030	RBS	1	52	52	Forward	BsaI	Use a primer pair			Looks good
6	RBS-02-B0032	RBS	1	50	50	Forward	BsaI	Use a primer pair			Looks good
7	RBS-03-B0034	RBS	1	49	49	Forward	BsaI	Use a primer pair			Looks good
8	alsS	CDS	1	1713	1713	Forward	BsaI	Use a primer pair			Looks good
9	terminator-01-rrnBT1	Terminator	1	110	110	Forward	BsaI	Use a primer pair			Looks good
10	terminator-02-T0	Terminator	1	126	126	Forward	BsaI	Use a primer pair			Looks good
11	terminator-03-T7	Terminator	1	71	71	Forward	BsaI	Use a primer pair			Looks good

Construct Assembly

Golden Gate: Populate the "constructs" table

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

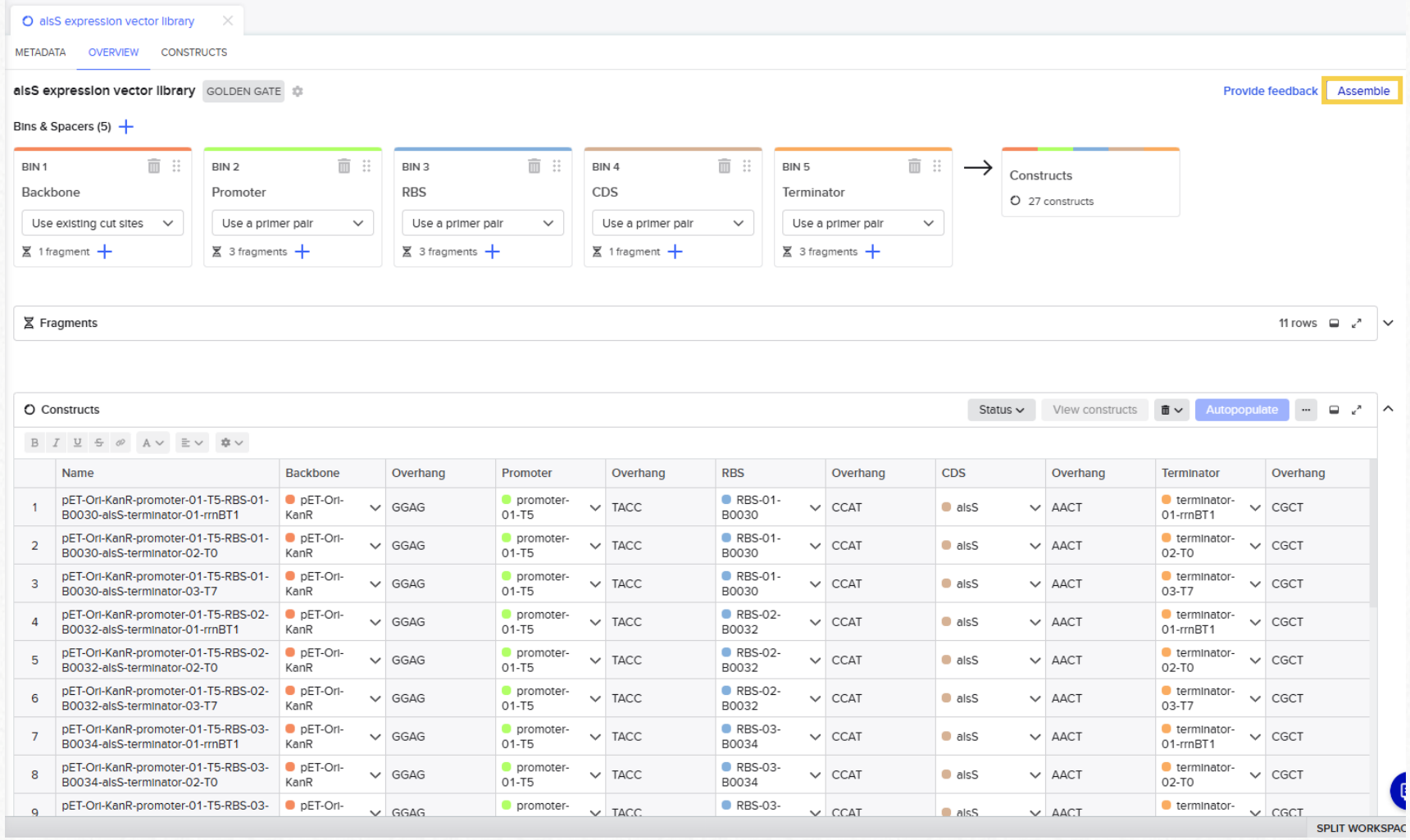


- ✓ You can also create combinations **manually**, with the option of **skipping** bins if you wish to do so
- ✓ It's also possible to **remove** rows that you are not interested in.

Construct Assembly

Golden Gate: Finalize the assembly

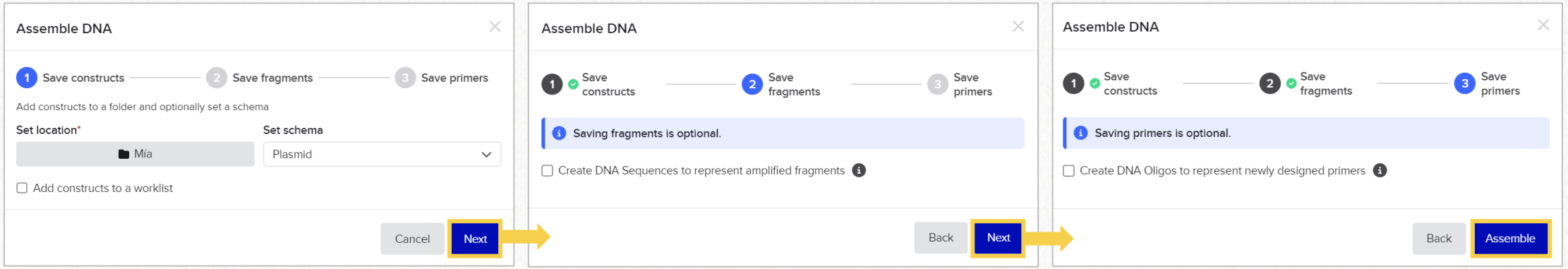
5 Click the "**Assemble**" button to create **primer** (optional), **fragment** (optional) and **plasmid** files for all of your constructs.



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

Construct Assembly

Golden Gate: *Save the constructs and related files*



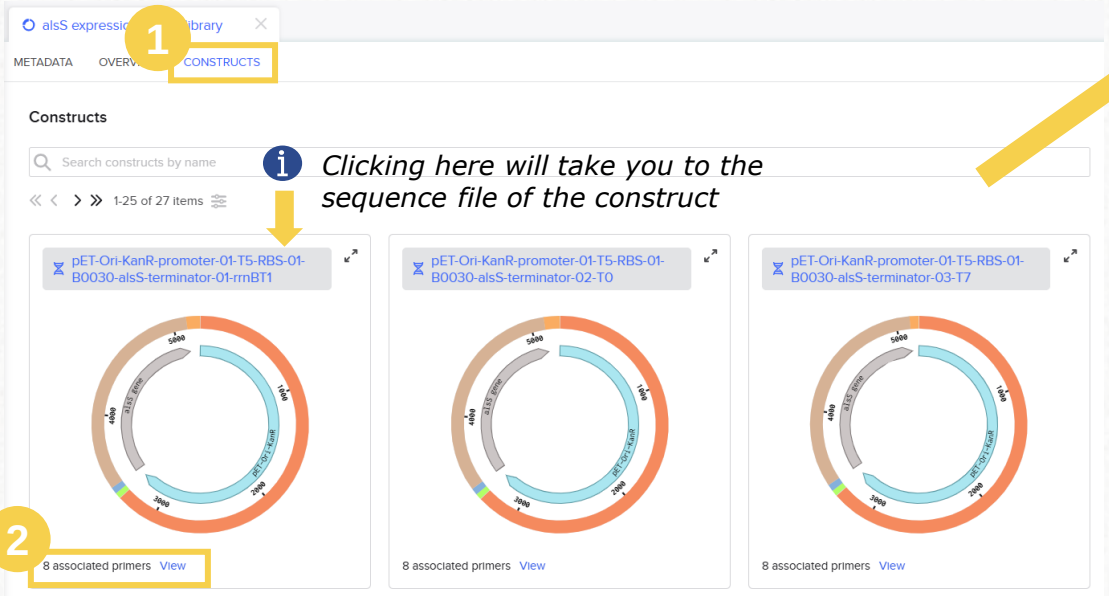
You can choose whether to create files for every primer and related amplicon.

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

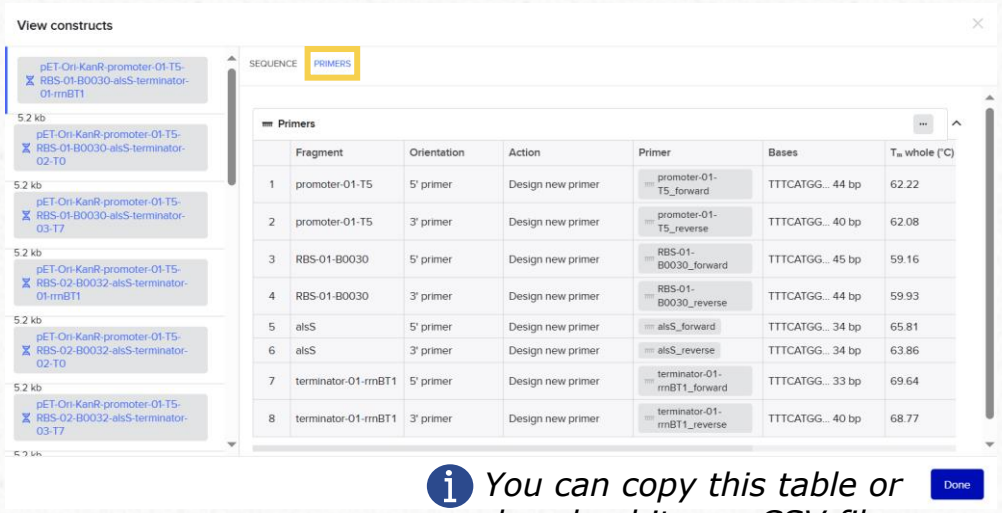
Construct Assembly

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.

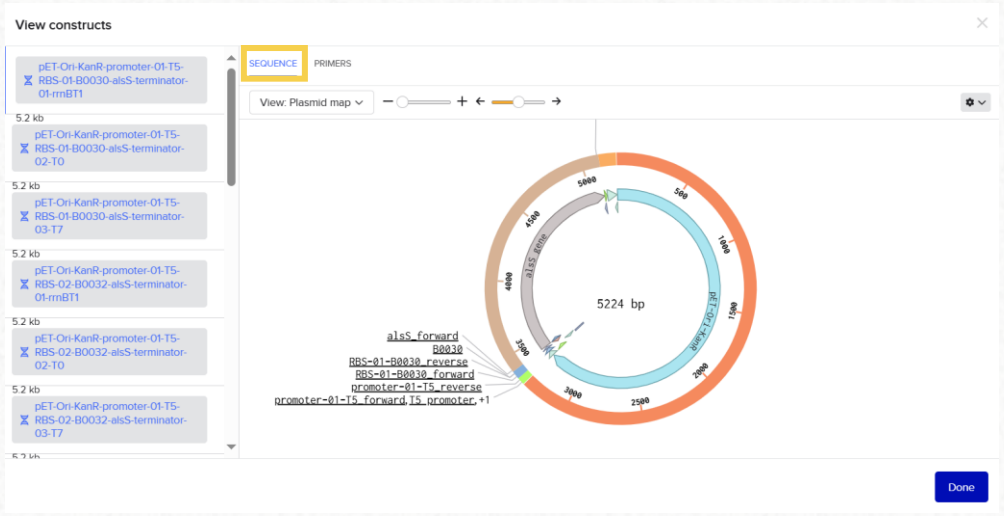


Primer view



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

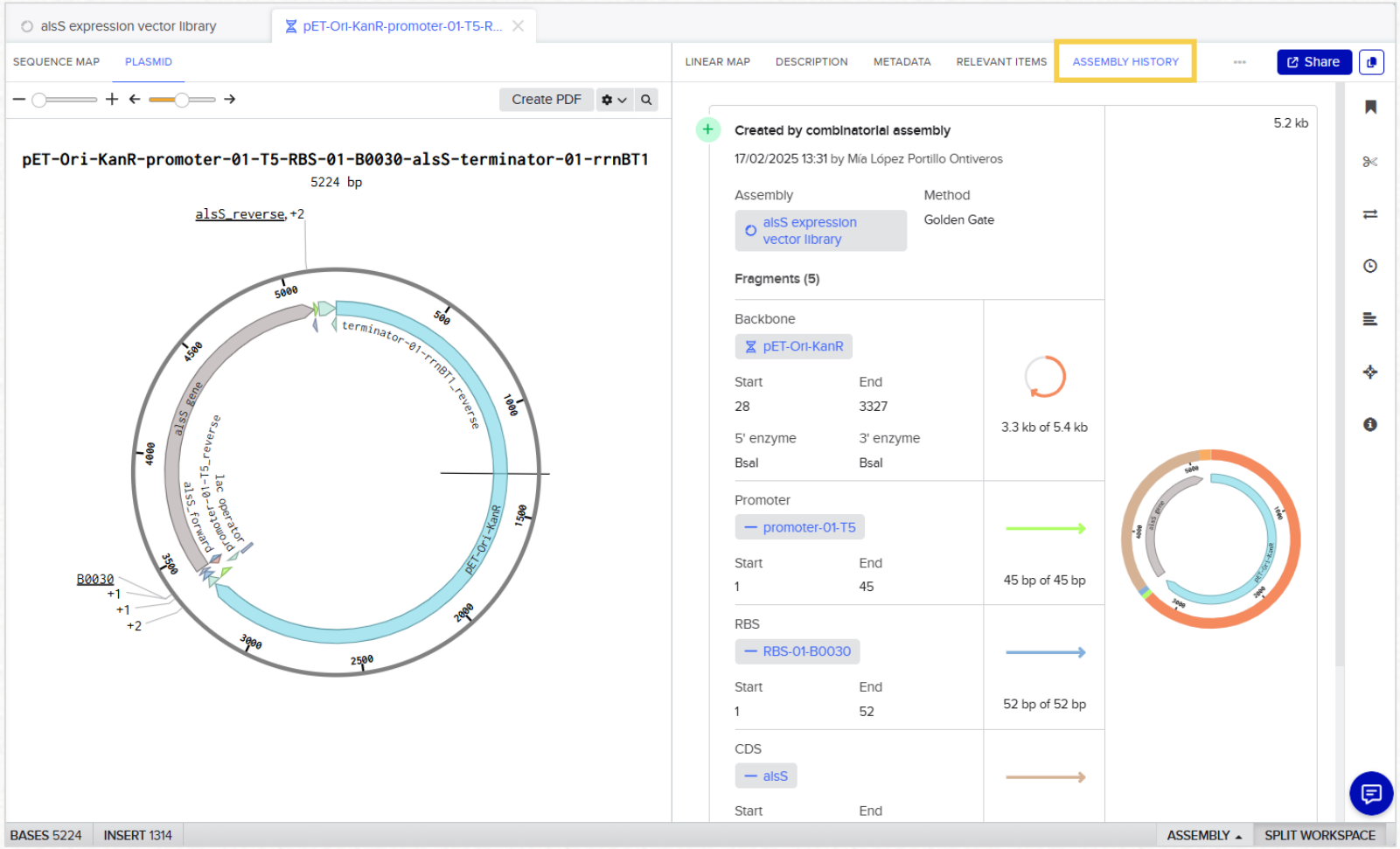
Sequence view



Construct Assembly

Golden Gate: Results

✓ You will also be able to find a file with the resulting construct. By going to the "Assembly History" tab, you will see the fragments that were used to create it, and you can also find a link to the Combinatorial Cloning file.



7. CRISPR tools

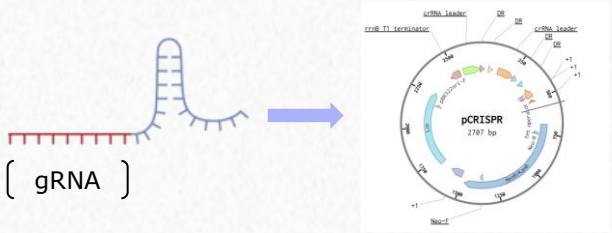
This is the third part of the *hands-on* example.

1. Cloning vector design
2. Creation of expression vector library
3. Gene knockout using CRISPR/Cas9
4. Sequence alignment

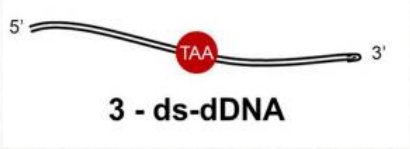
Target: *pta* in *E. coli*

pta

gRNA design + assembly into pCRISPR



HR template design to KO *pta*



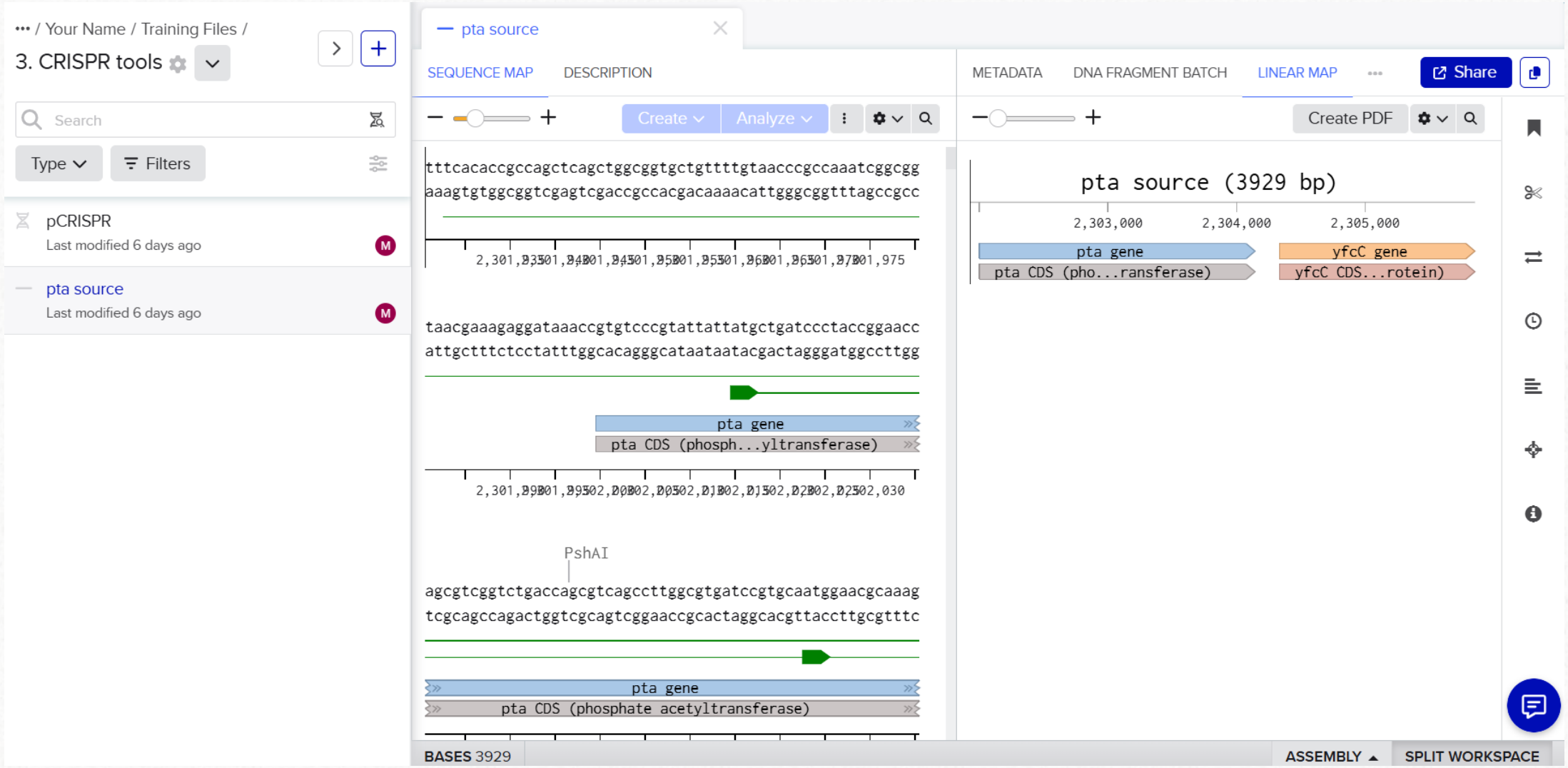
- ✓ gRNA design
- ✓ HR template design

Expected output:

- Selected gRNA for the *pta* gene
- Forward and reverse primers to clone the gRNA into pCRISPR via BsaI
- 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

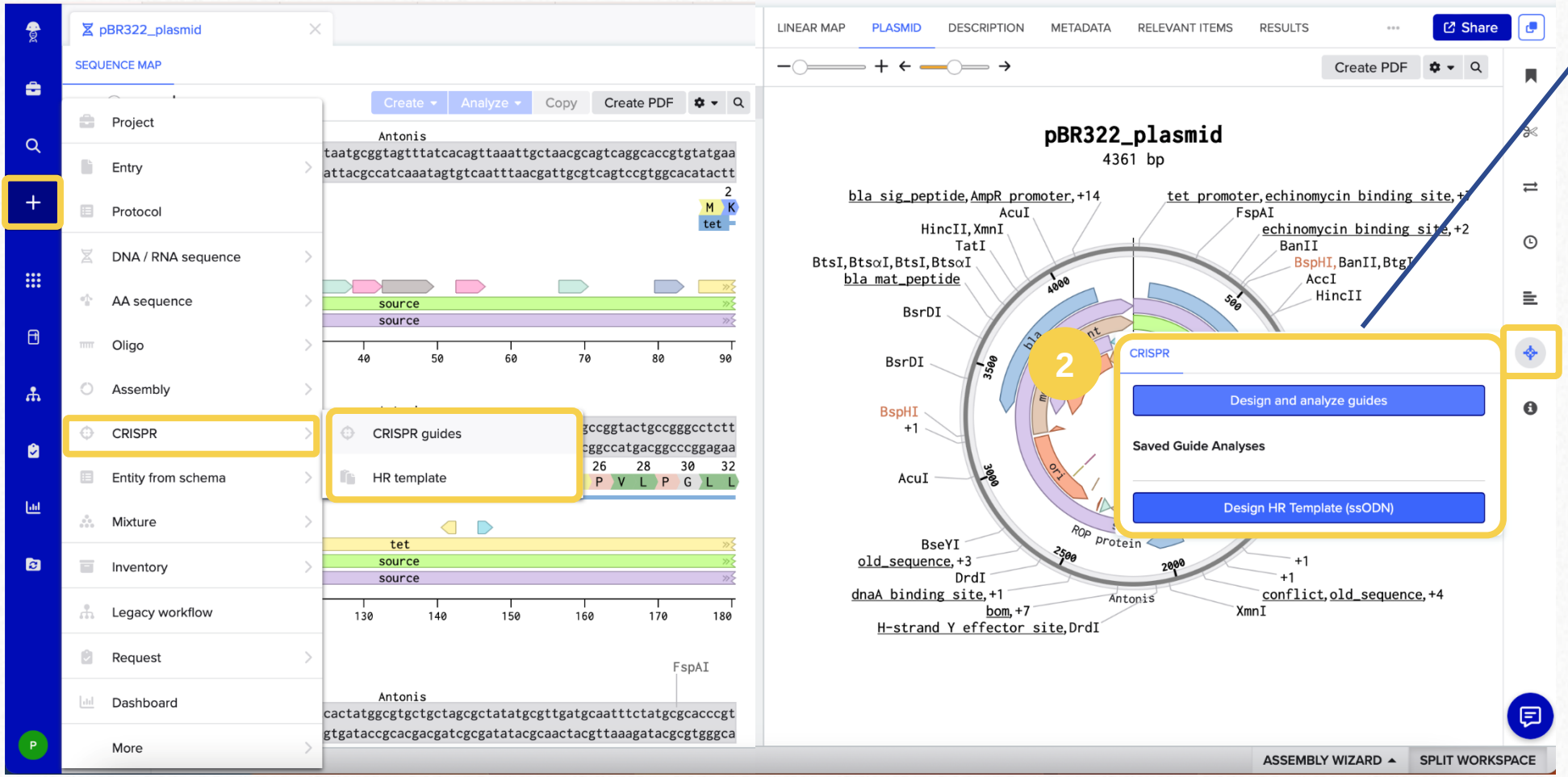
You will need the files in the ***CRISPR tools*** subfolder.



CRISPR tools

Tool overview

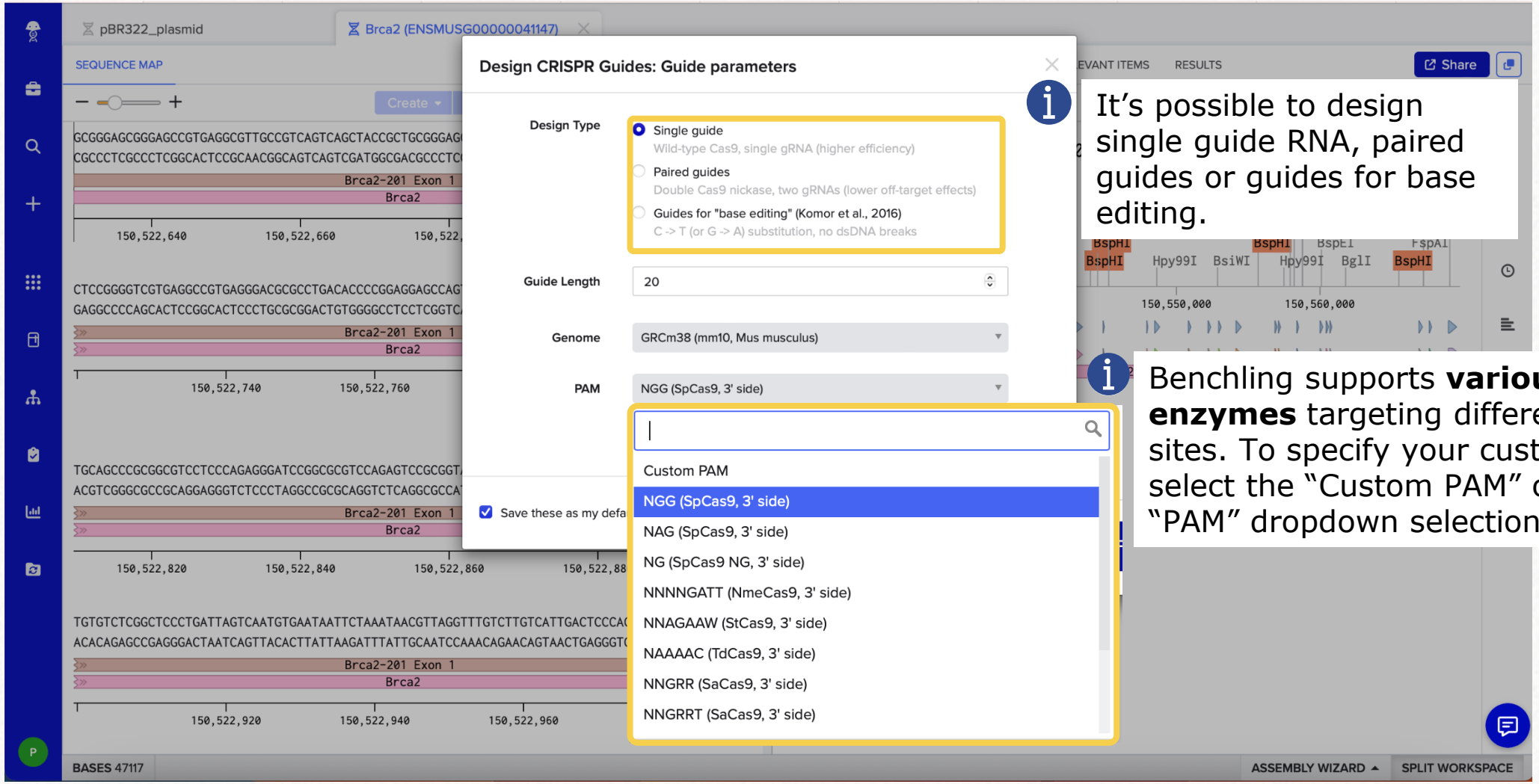
It is possible to create guide RNA sequences and Homologous recombination templates using the CRISPR tool. There are 2 ways to access it:



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

CRISPR tools

Tool overview



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

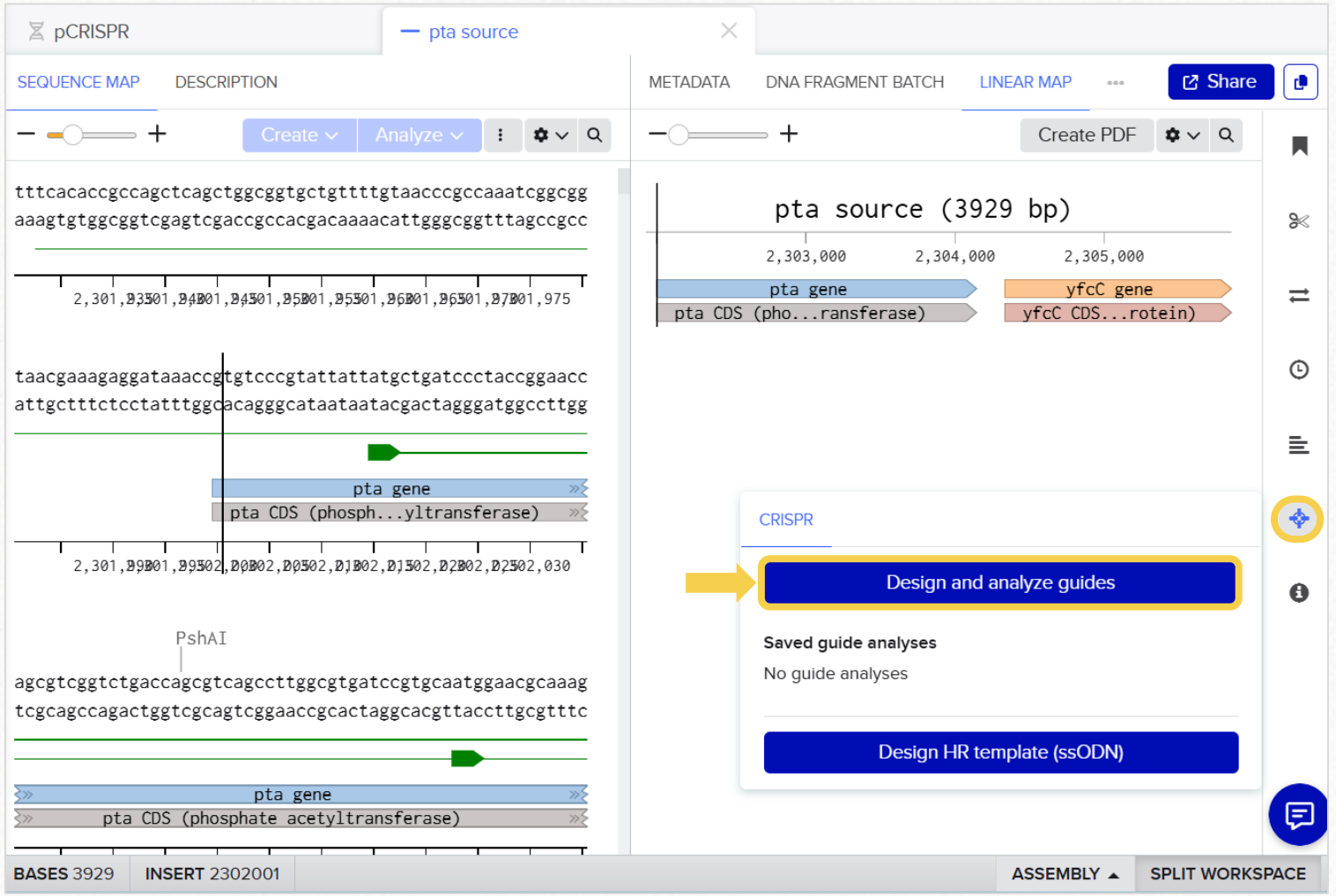
7.1 gRNA design

CRISPR tools

gRNA design

1 Open the **pta source** file.

2 Access the **gRNA design** menu.

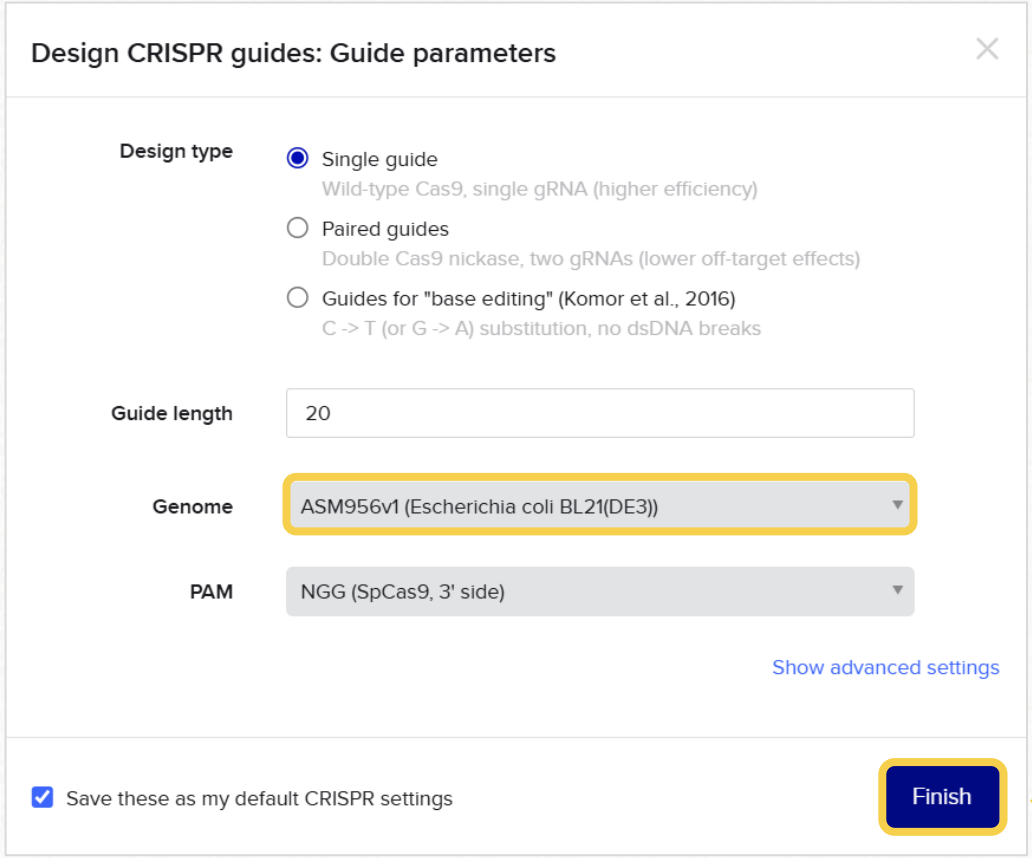


CRISPR tools

gRNA design

3 Change the genome to ***E. coli* BL21(DE3).**

4 Click **Finish** and continue.



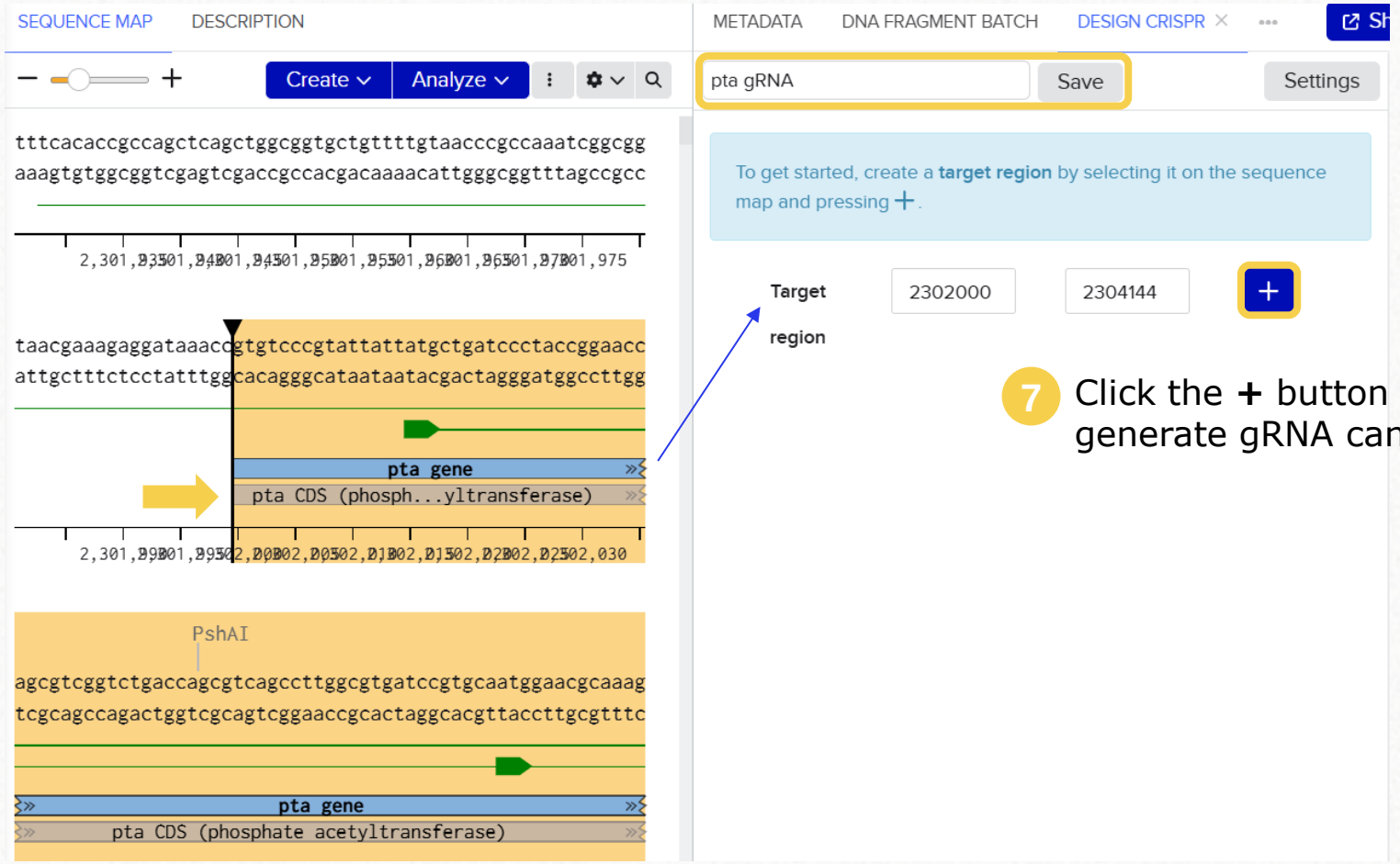
Setting the genome is important for **off-target** calculations.

CRISPR tools

gRNA design

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.

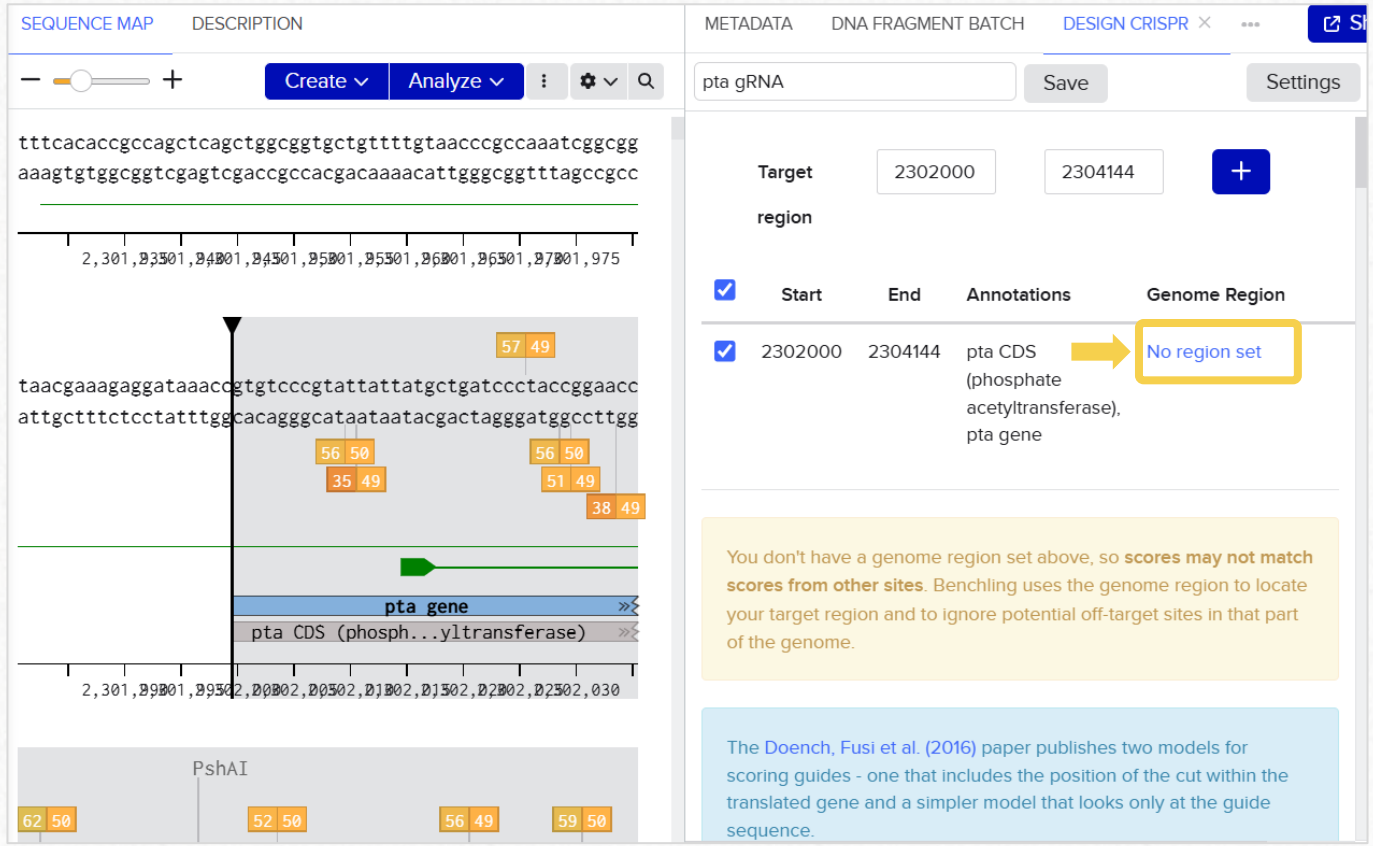


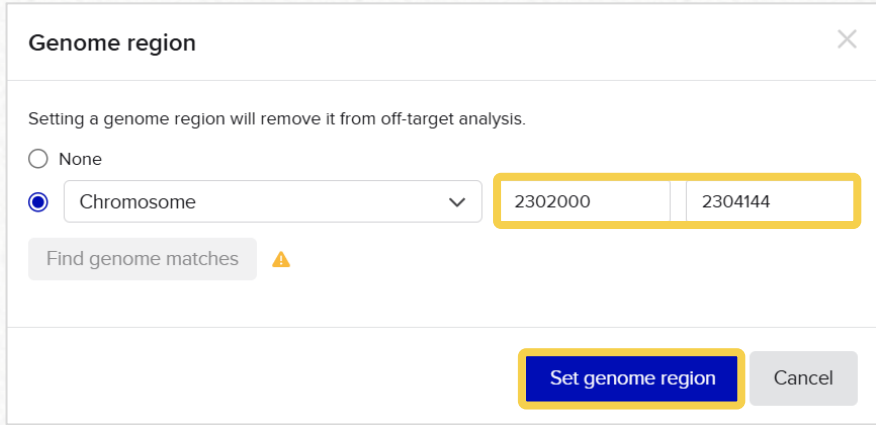
7 Click the **+** button to generate gRNA candidates.

CRISPR tools

gRNA design

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

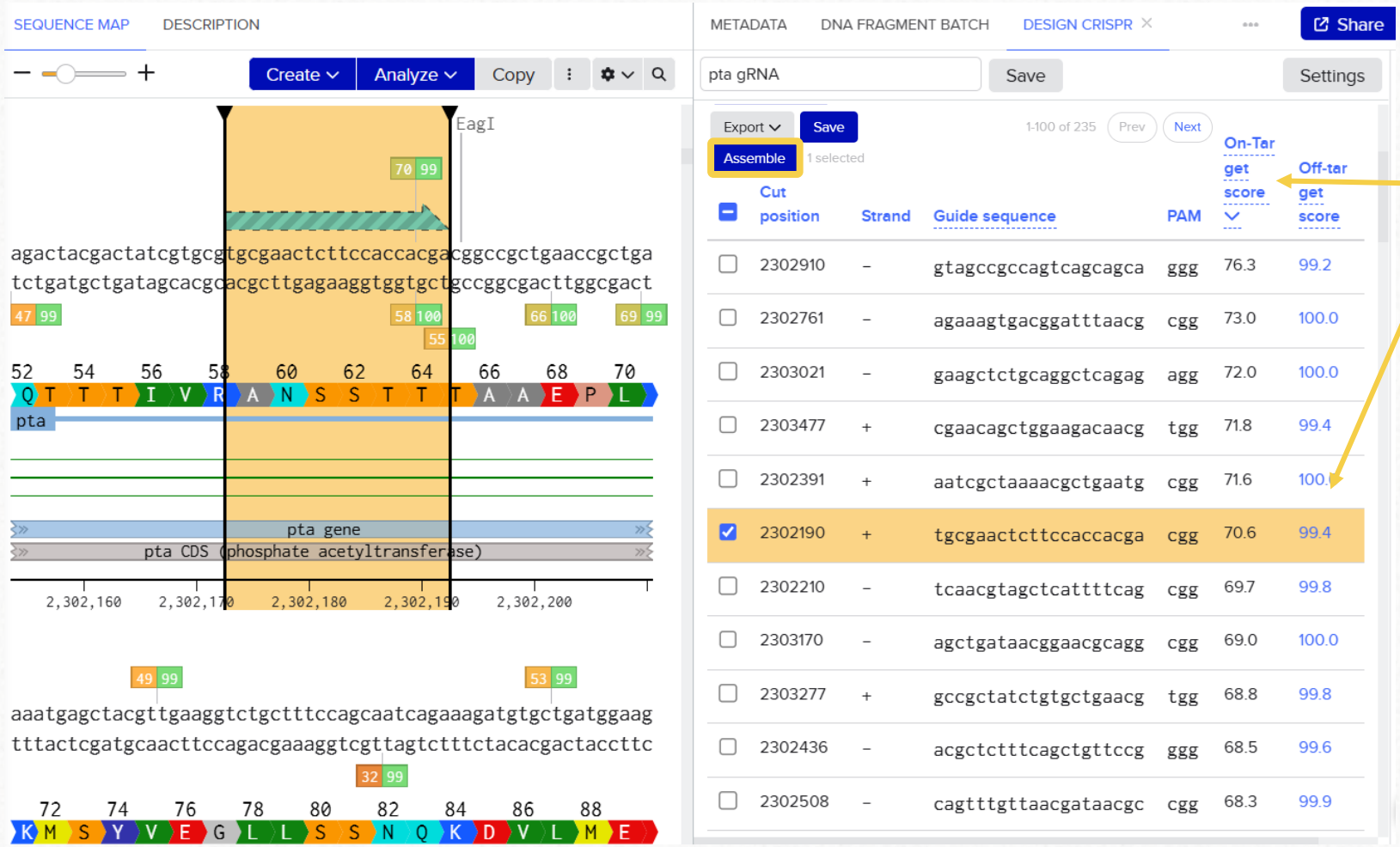




CRISPR tools

gRNA design

Benchling 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.



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

10 Select the gRNA as shown.

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

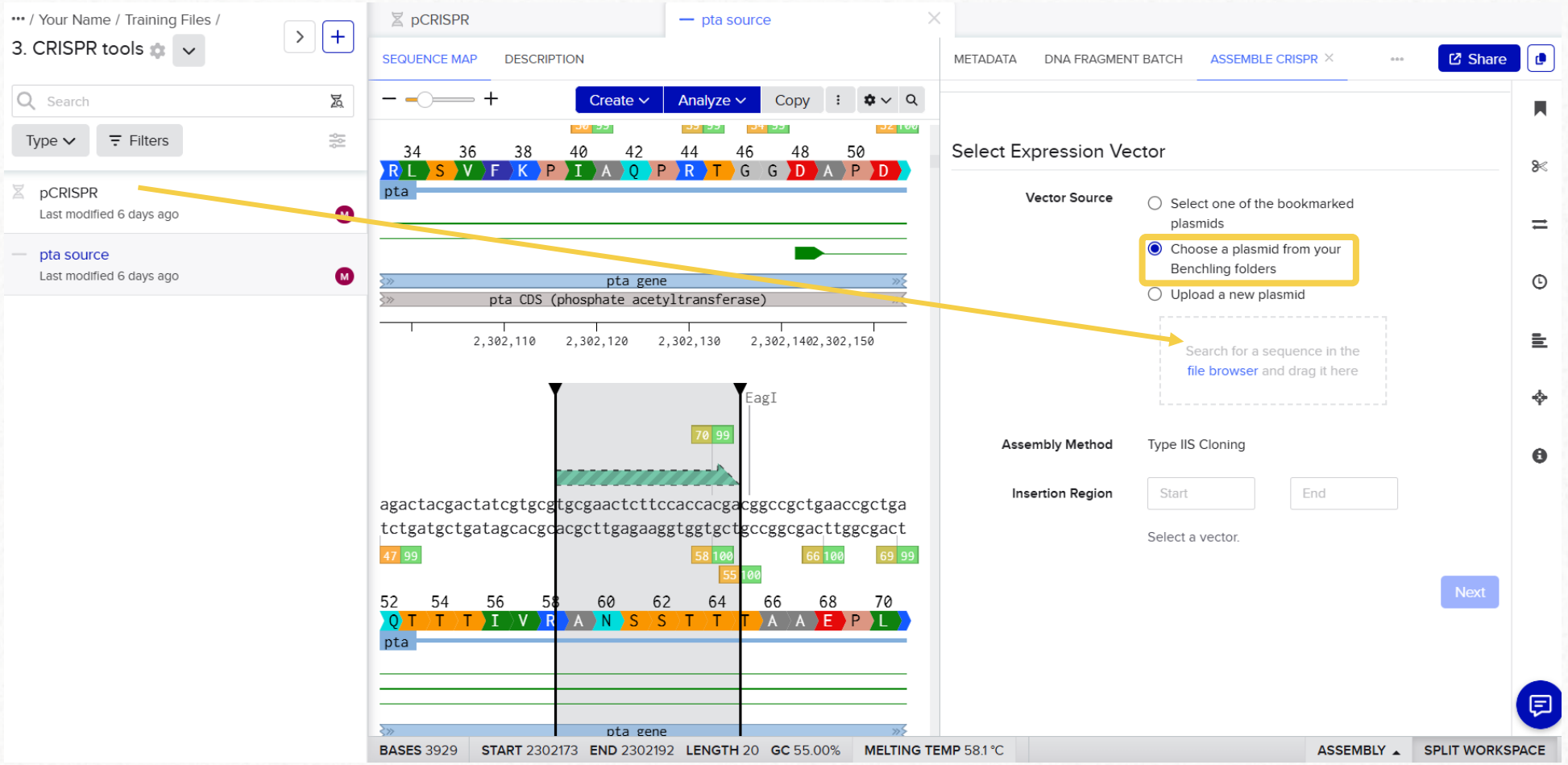
11 Click **Assemble**.

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

CRISPR tools

gRNA design

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



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

CRISPR tools

gRNA design

13 Set the **insertion region** as shown.

14 Click **Next**.

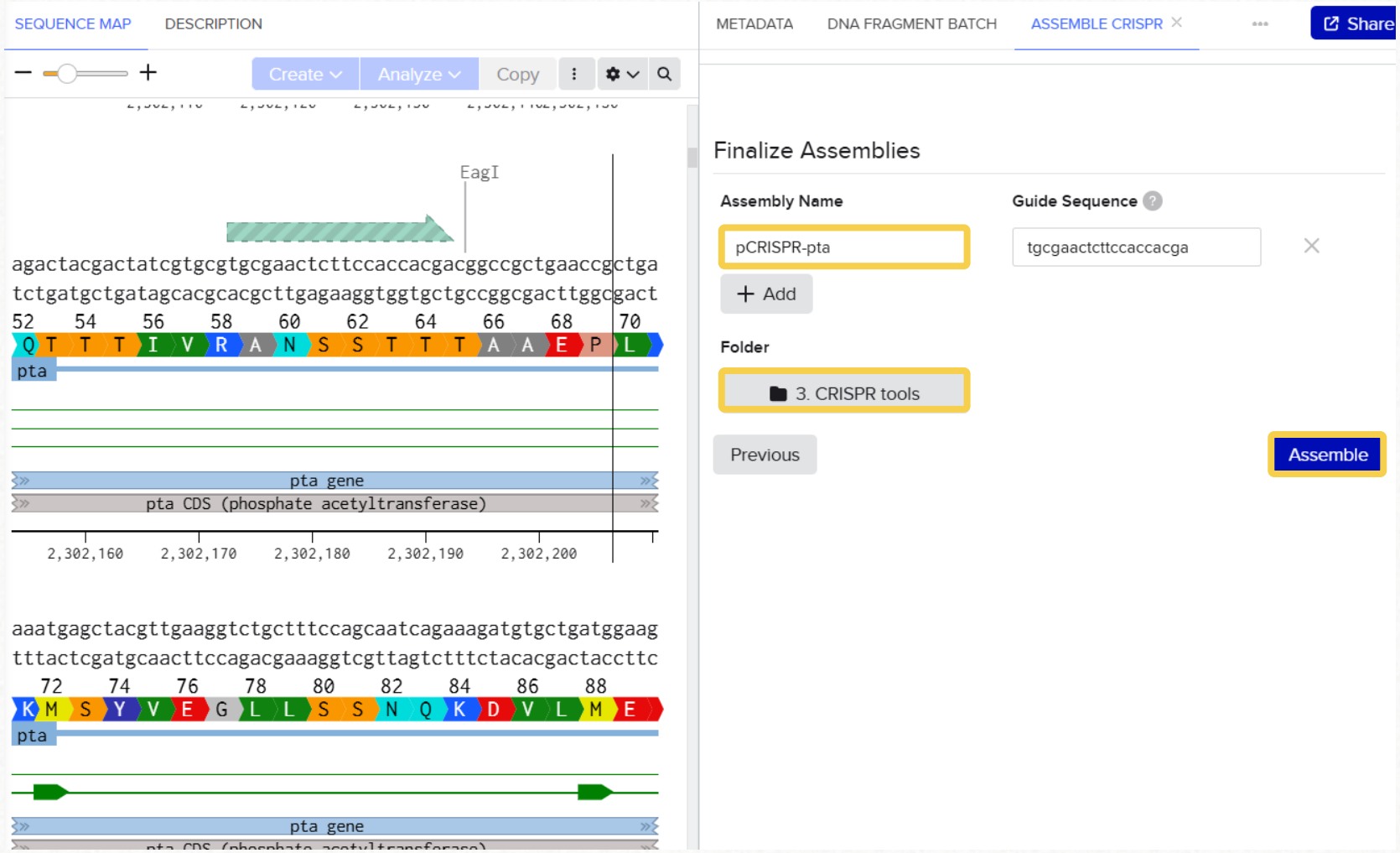
Benchling will look for Type IIS restriction sites in the region.

Sometimes it may not work as expected; in this case, refer to this article.

CRISPR tools

gRNA design

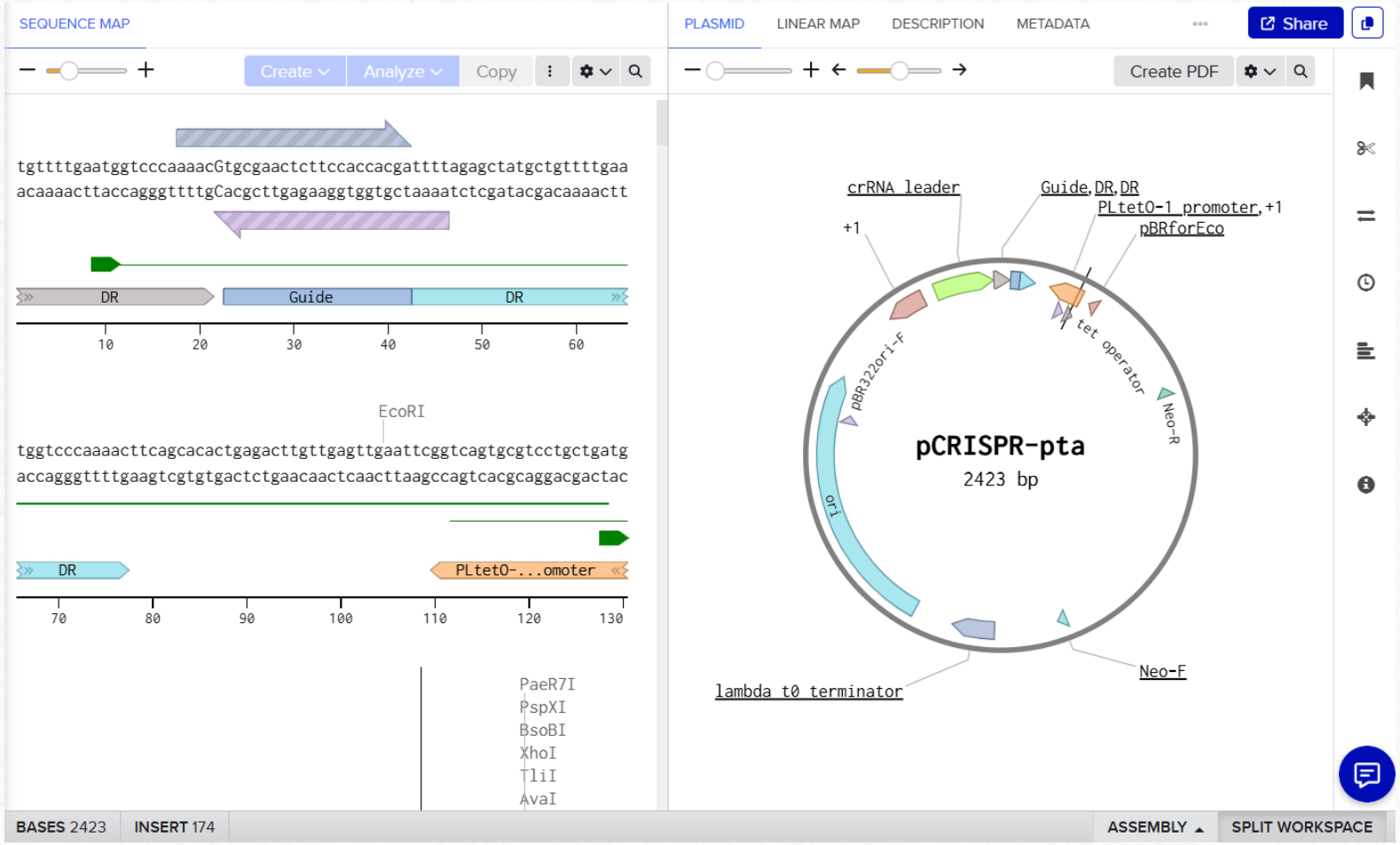
15 Name your assembly, choose a location to save it and click **Assemble**.



CRISPR tools

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.



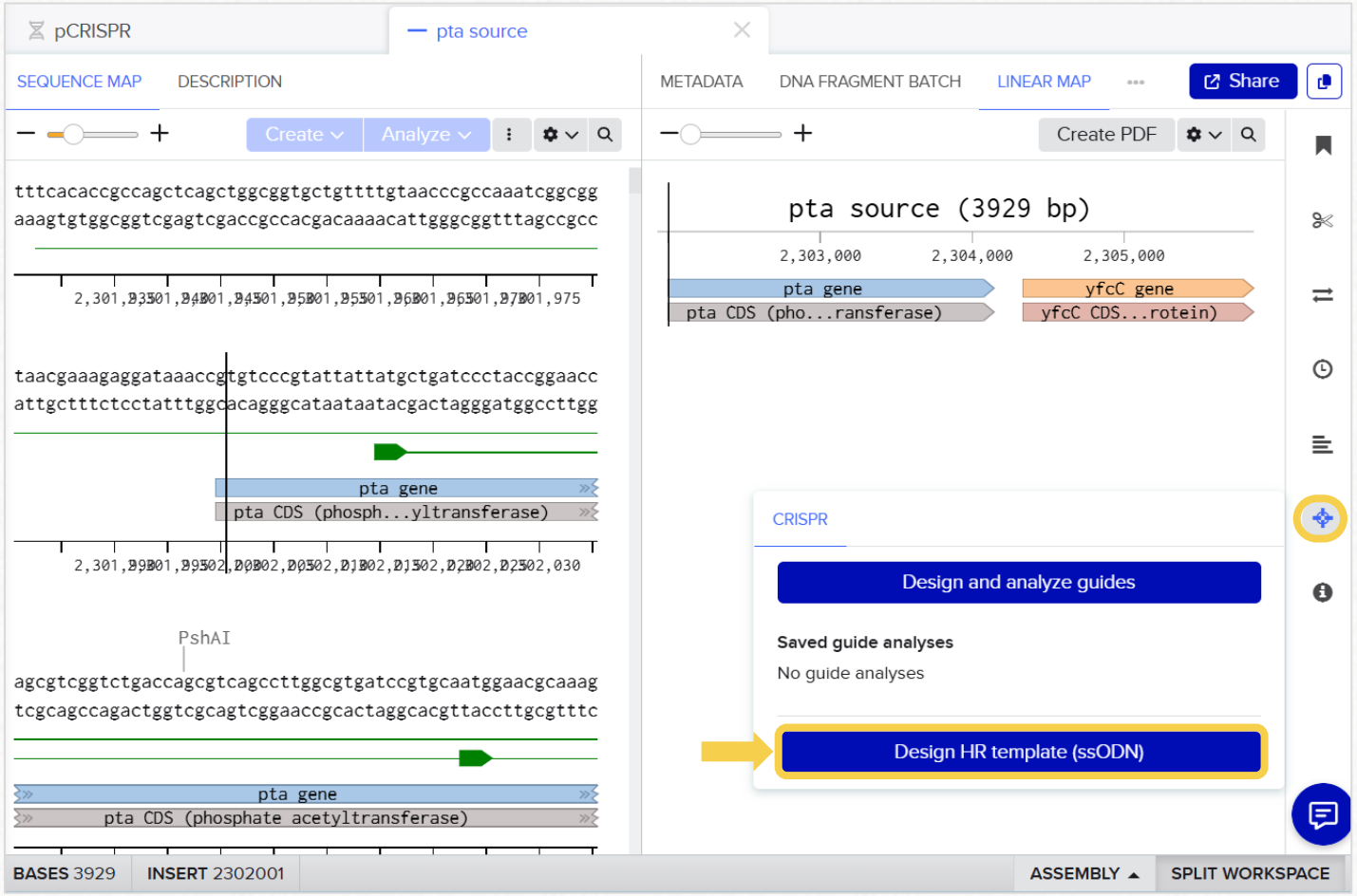
7. CRISPR tools

7.2 HR template design

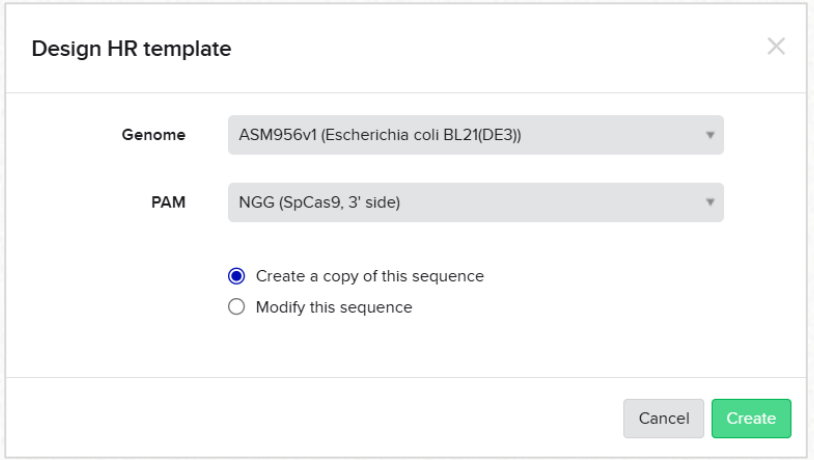
CRISPR tools

HR template design

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



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

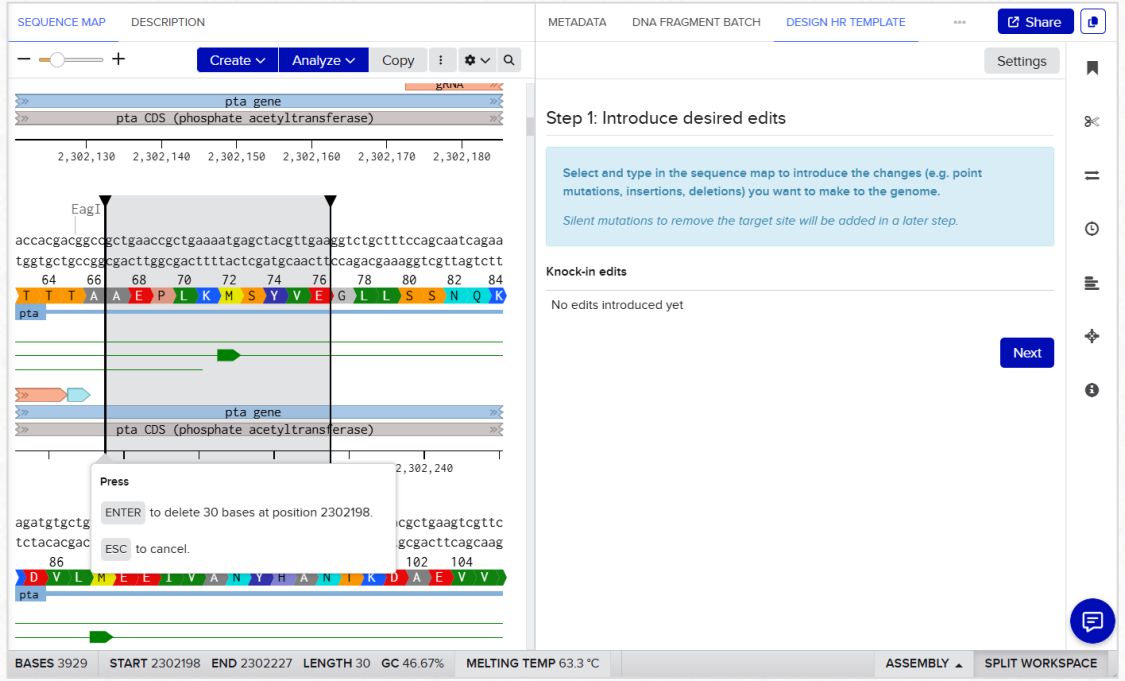


CRISPR tools

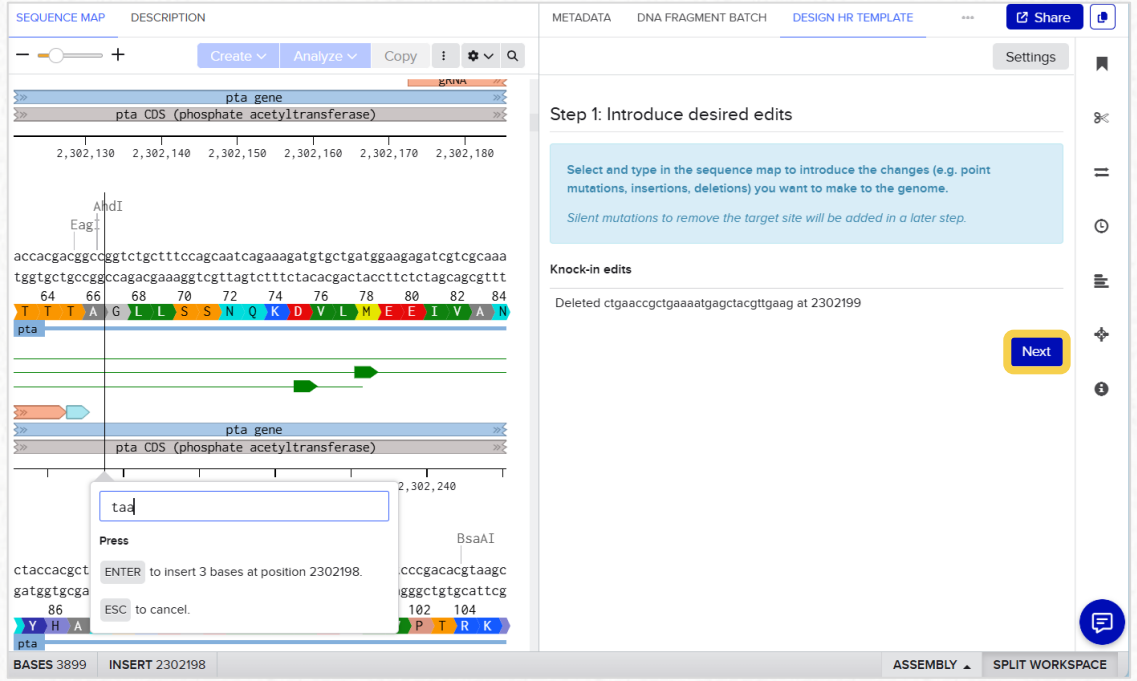
HR template design

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.



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

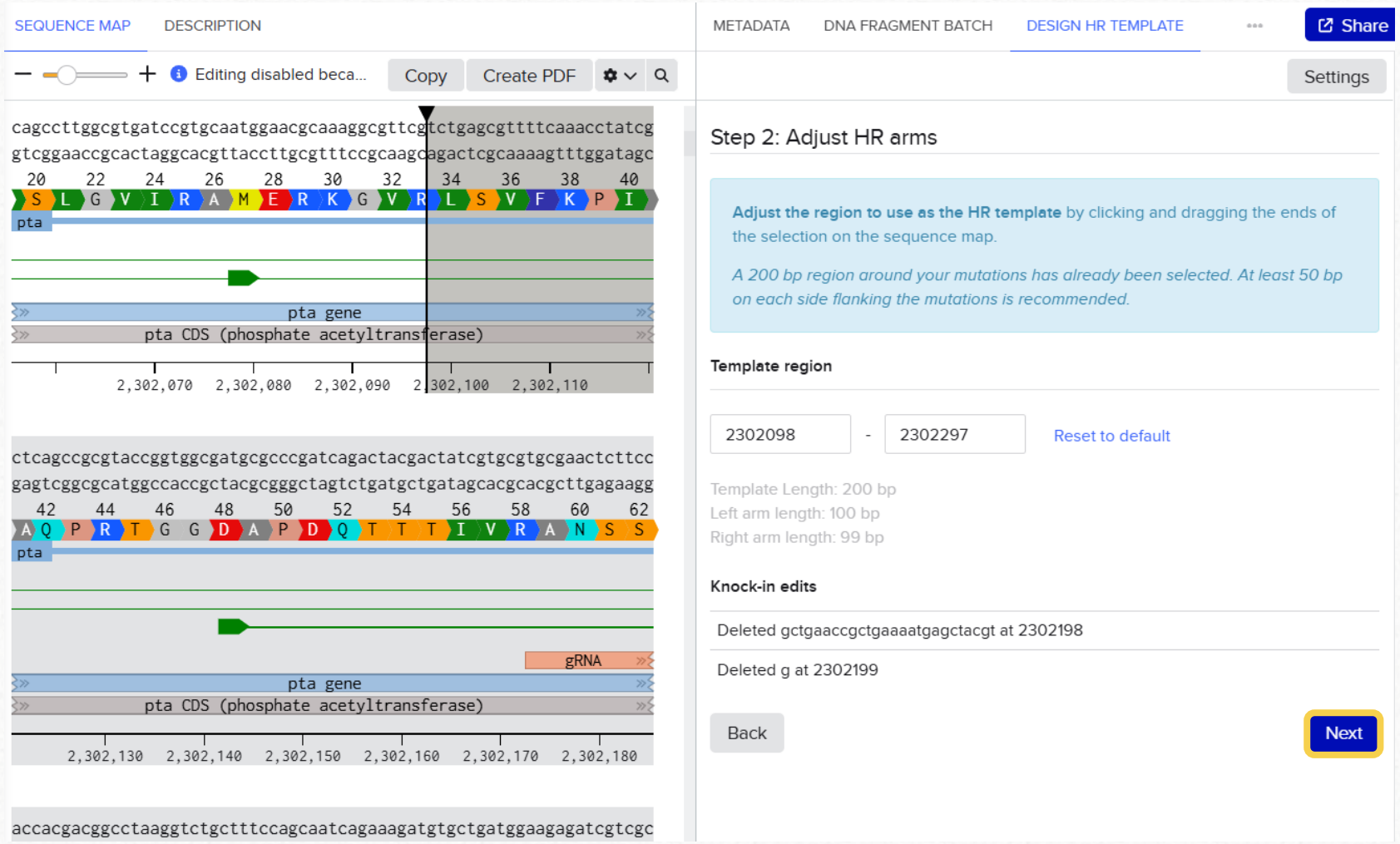


6 Click **Next**.

CRISPR tools

HR template design

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

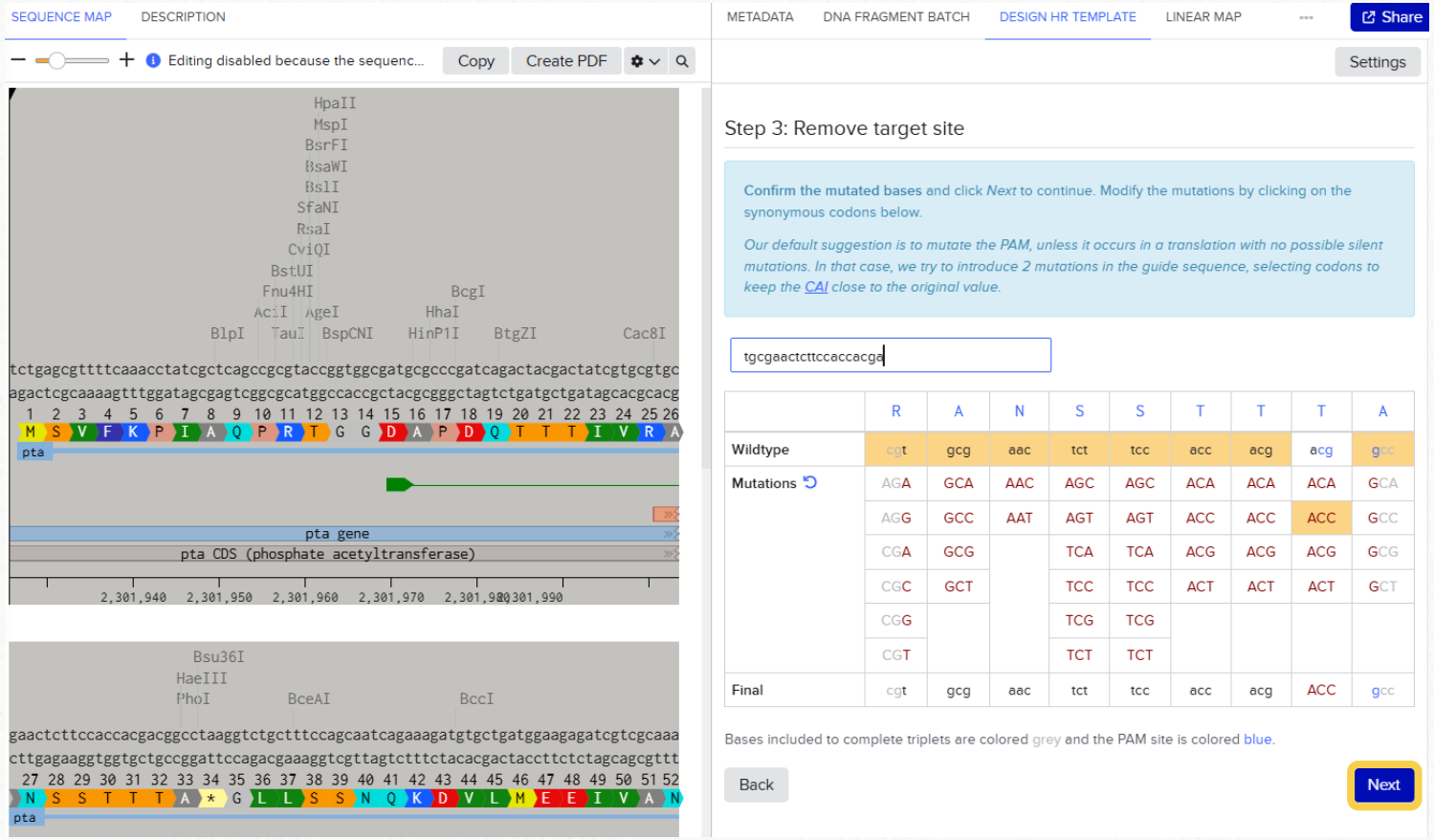


7 Click **Next**.

CRISPR tools

HR template design

8 Paste the gRNA sequence: tgcgaactcttccaccacga



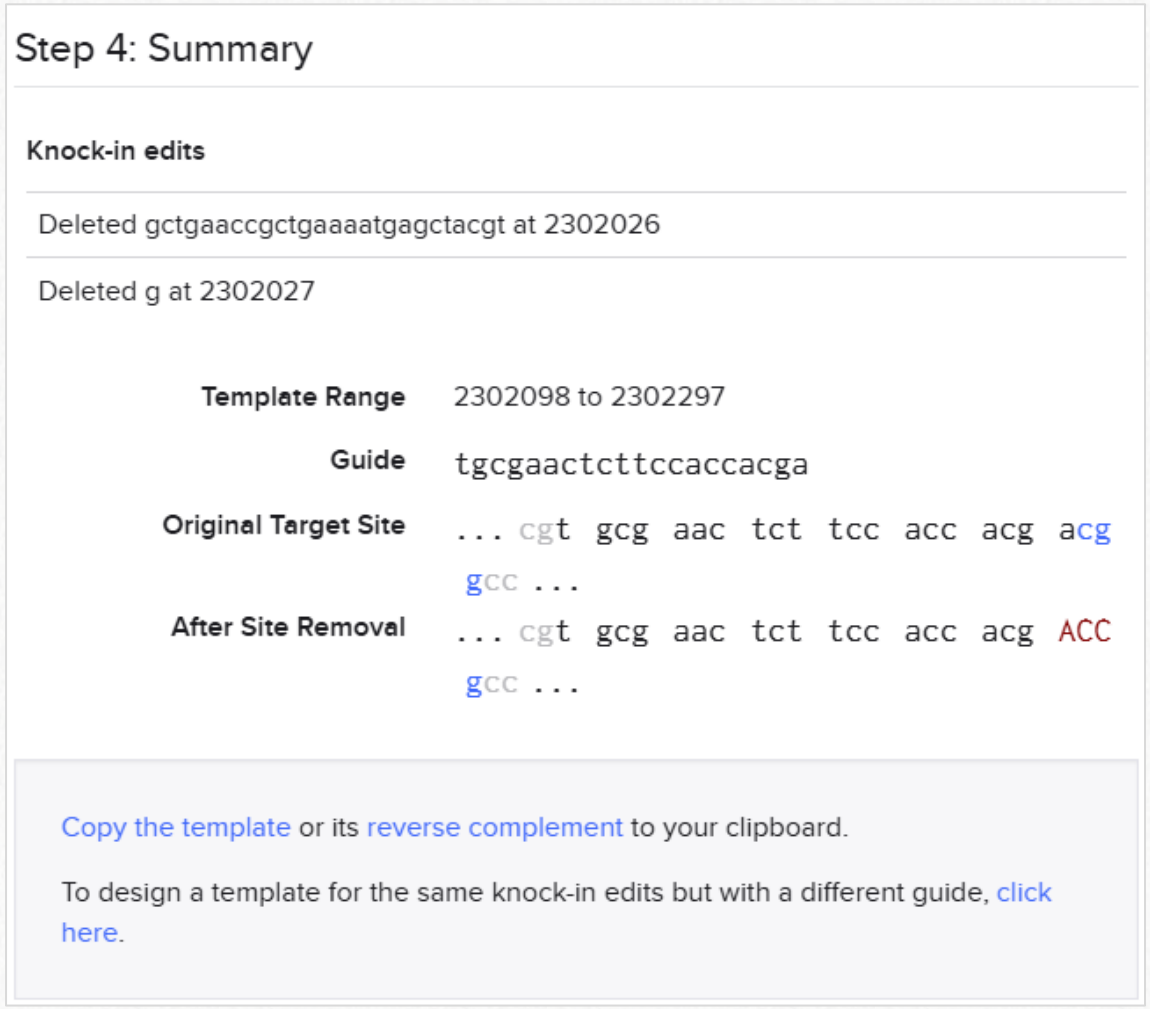
The PAM will be removed from the HR template to prevent the degradation of the ssODN.

You can choose from several alternatives, as shown in the table.

9 Click **Next**.

CRISPR tools

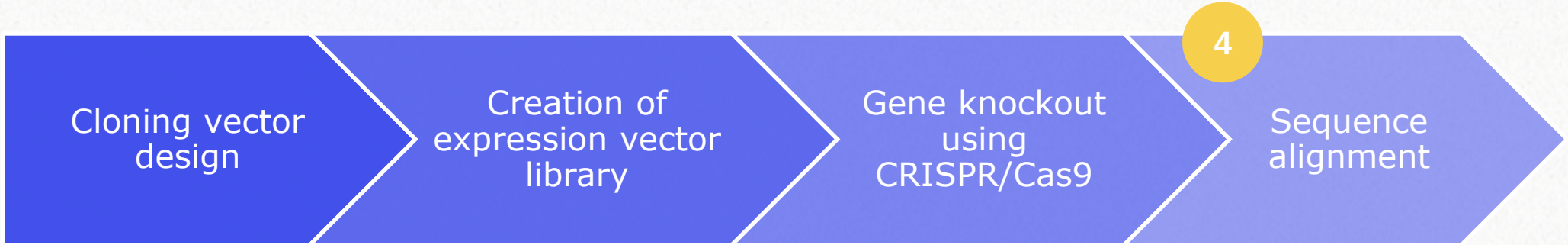
HR template design

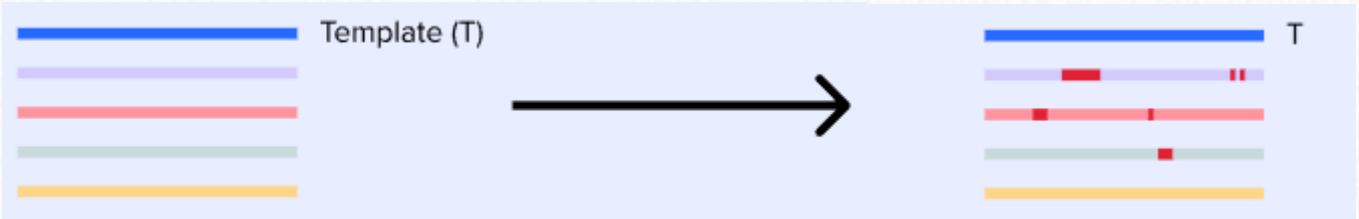


- ✓ After the design process, you can copy the resulting HR template and paste it onto a new DNA sequence file to save it.

8. Sequence alignments

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





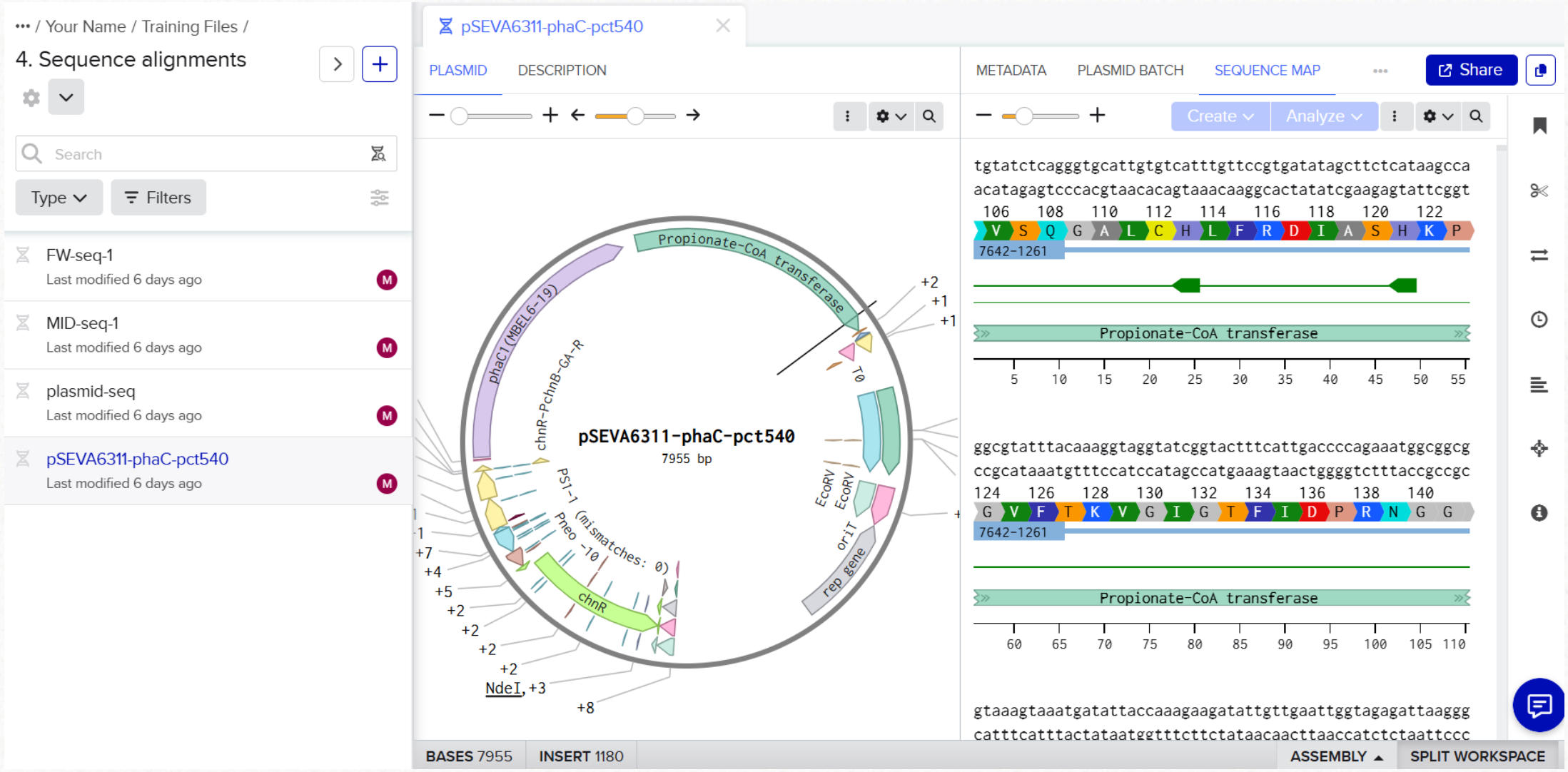
✓ Multisequence alignment

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.



8. Sequence alignments

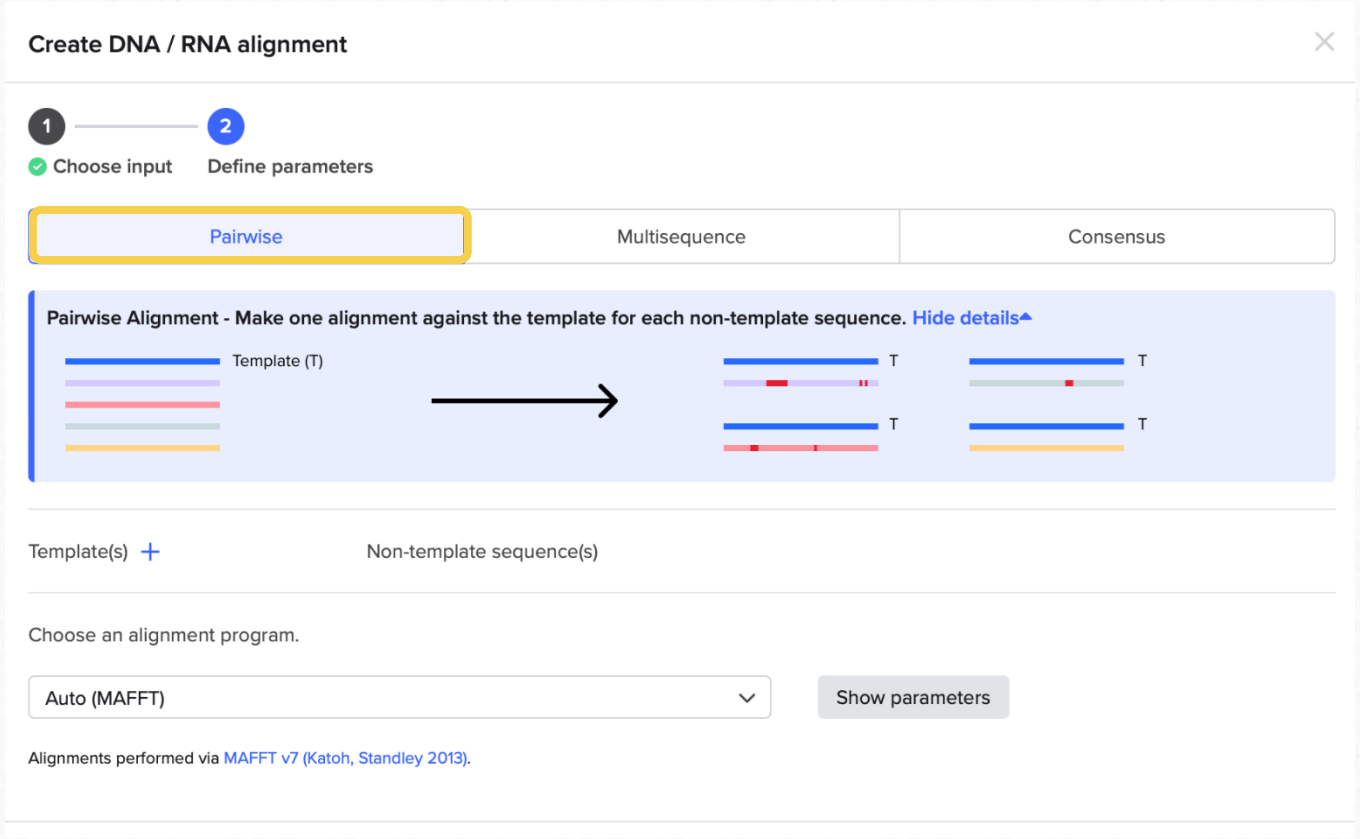
8.1 Alignment tool

Alignment creation

Alignment tool overview

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:



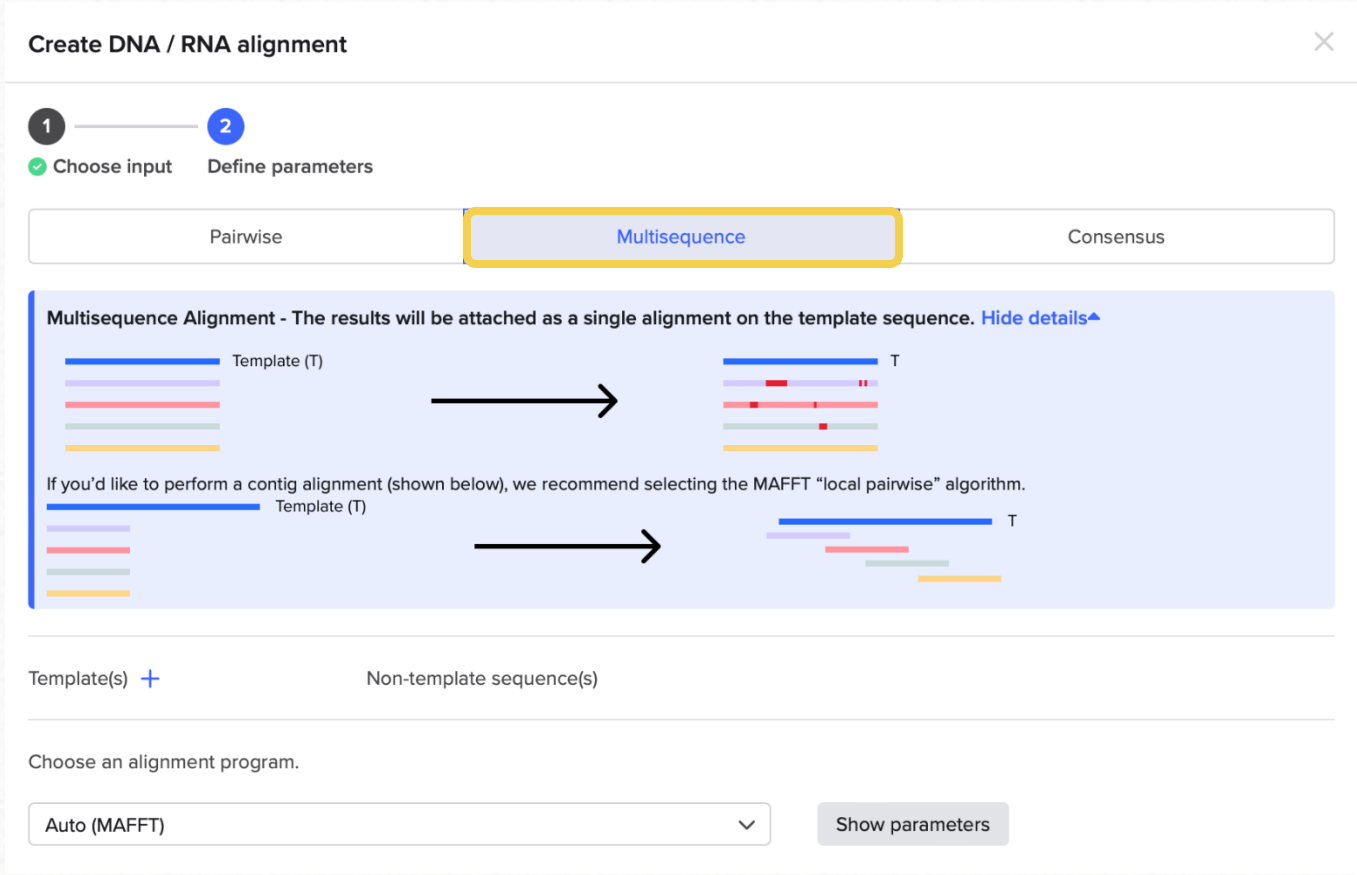
1 **Pairwise alignment:**
Sequences are compared against a template sequence, creating individual alignment files for each non-template seq.

Alignment creation

Alignment tool overview

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:



2 **Multisequence alignment:**

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

Alignment creation

Alignment tool overview

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.

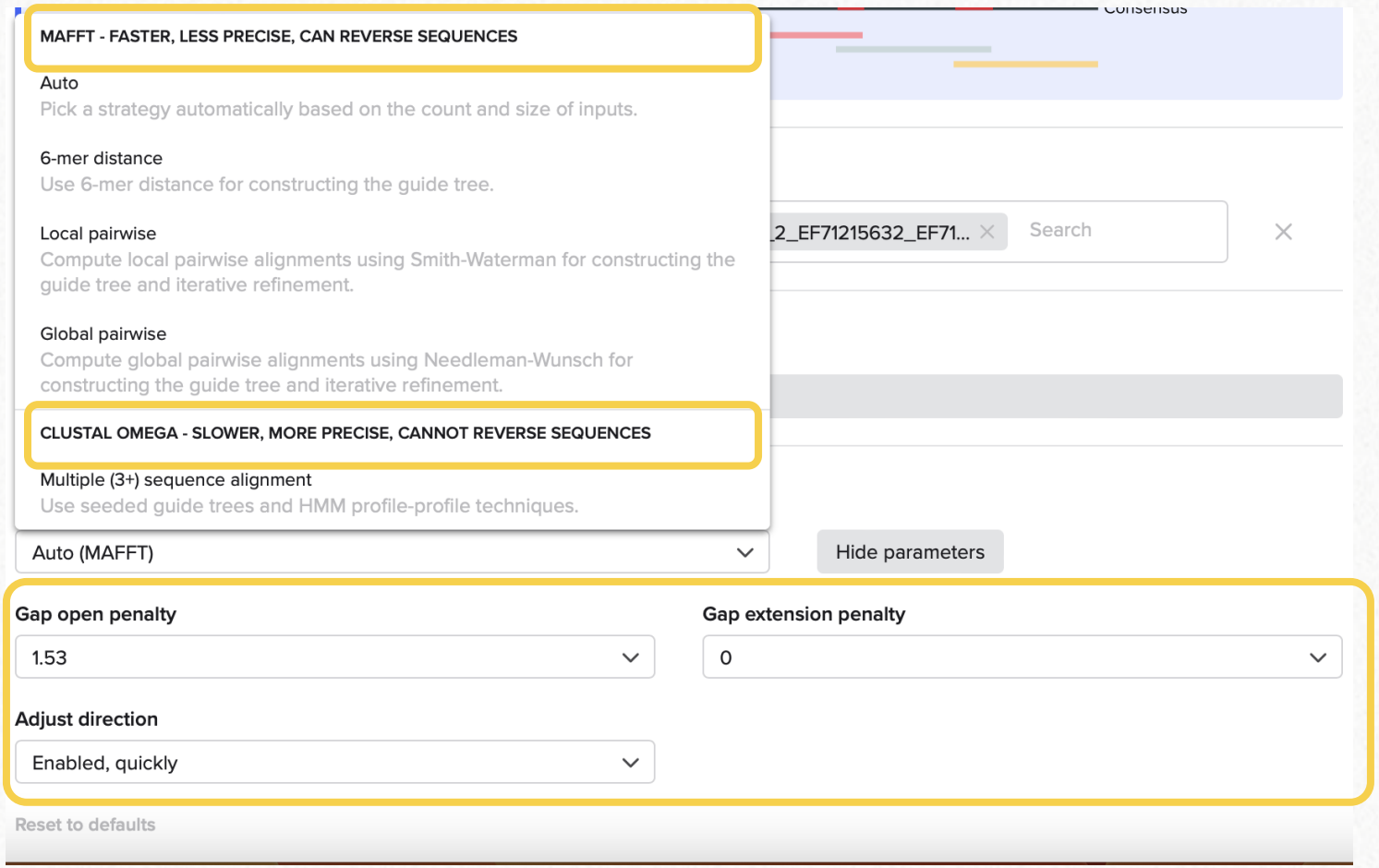
- ✓ There are **three alignment options** and several alignment programs available:

3 **Consensus alignment:**

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

Alignment creation

Alignment tool overview



- ✓ 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

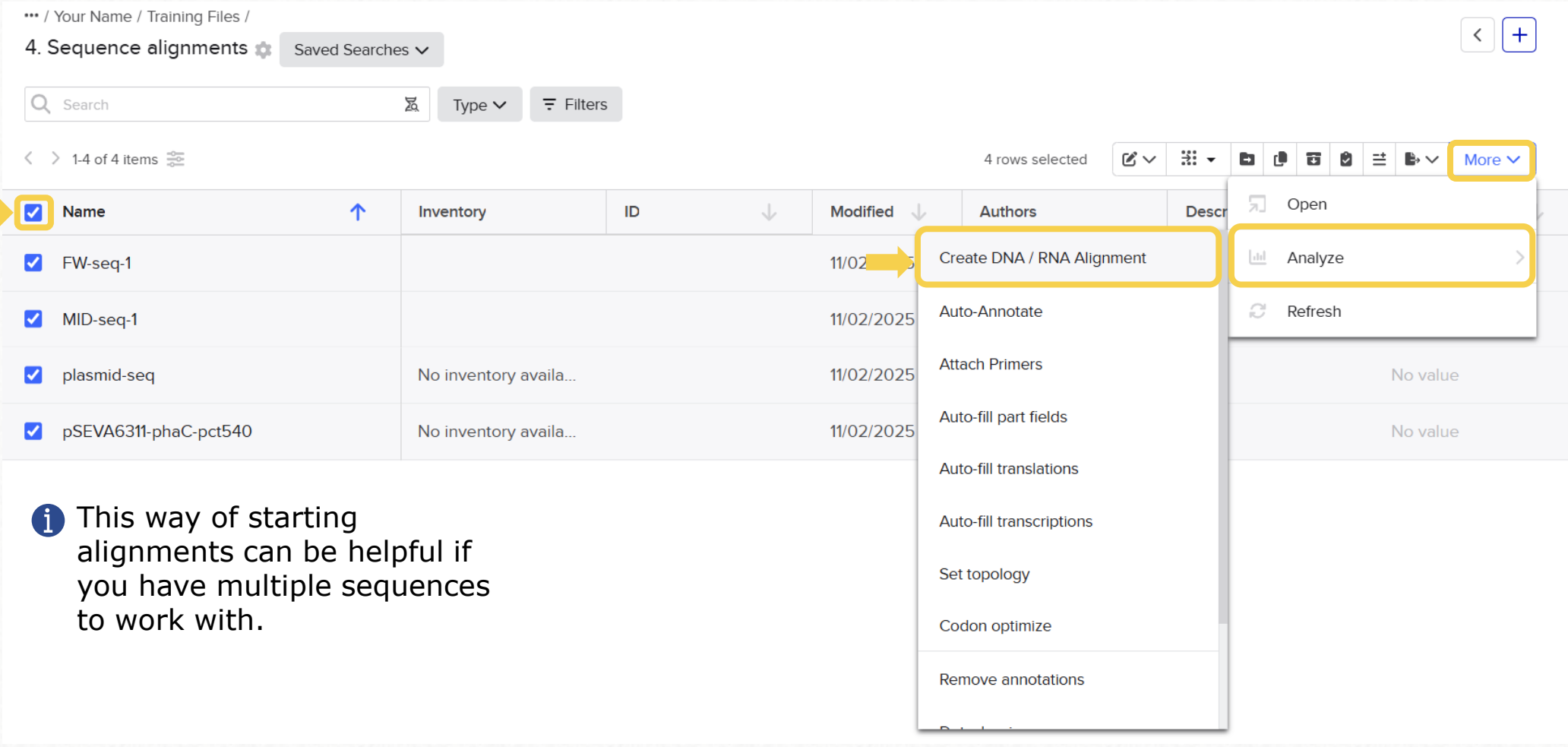
8.2 Multisequence alignment

Alignment creation

Multisequence alignment

1. Open the **Sequence alignments** folder.

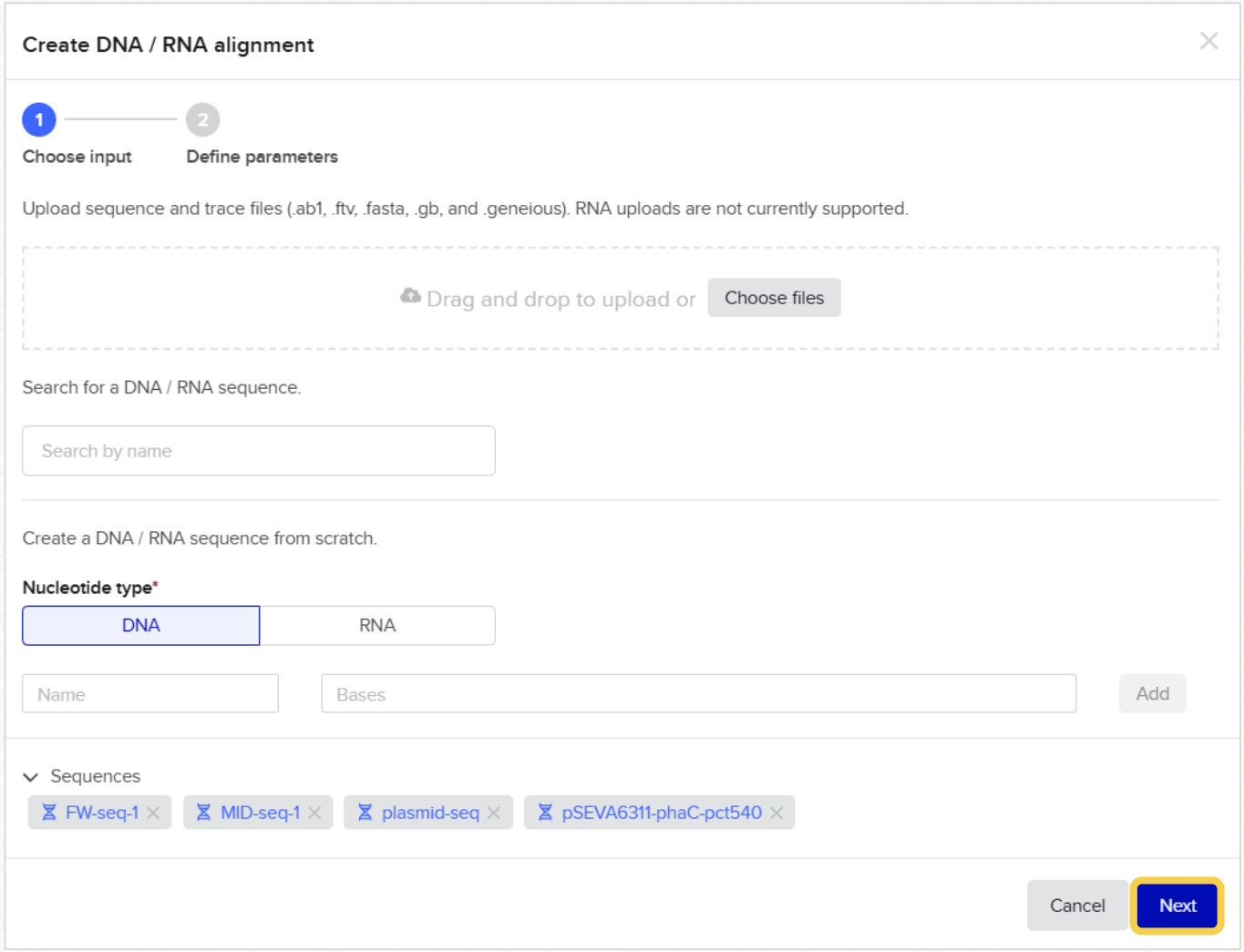
2. Select all the files in the folder. From the **Analyze** menu, select **Create DNA/RNA Alignment**.



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

Alignment creation

Multisequence alignment

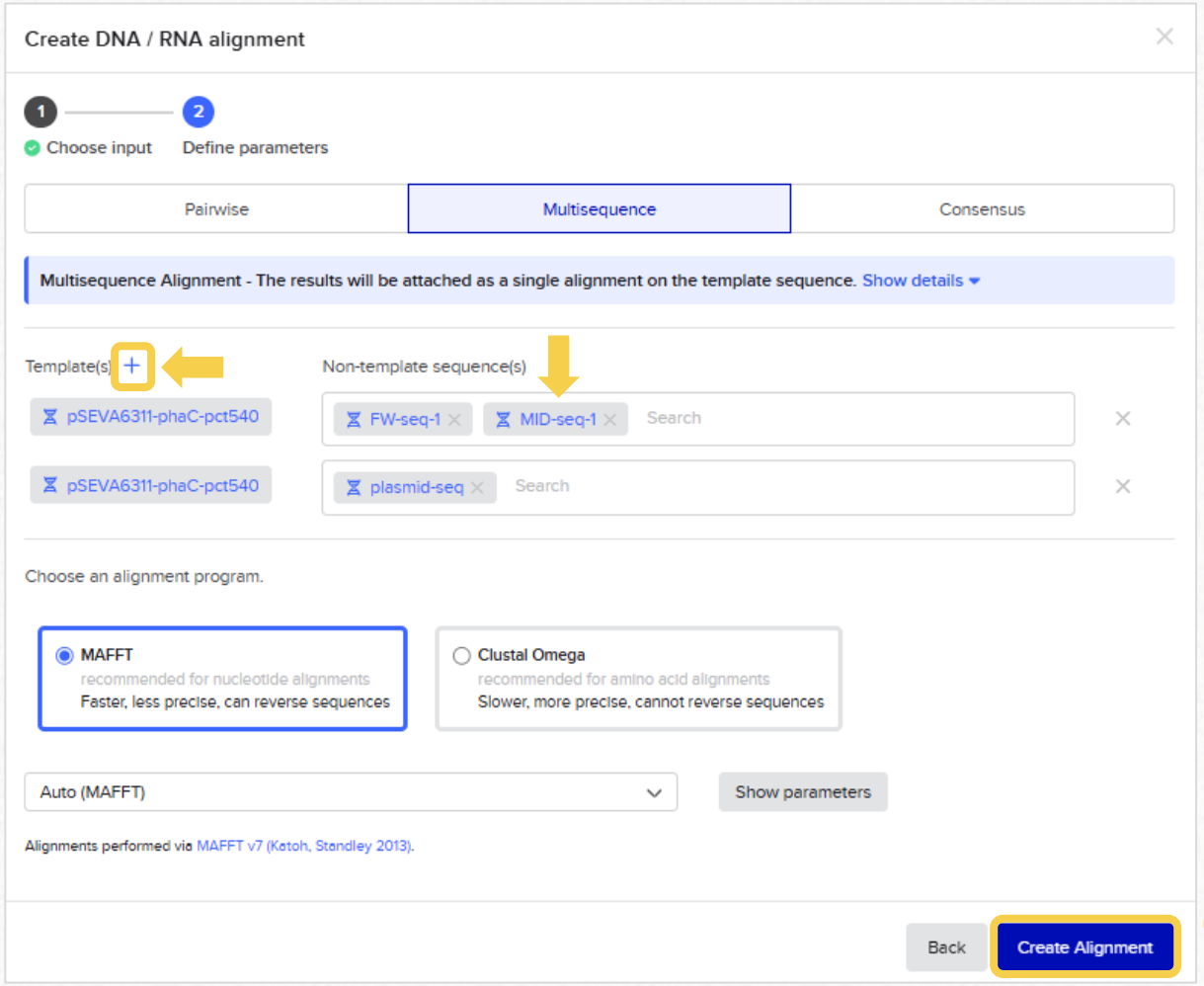


3 Click **Next.**

Alignment creation

Multisequence alignment

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

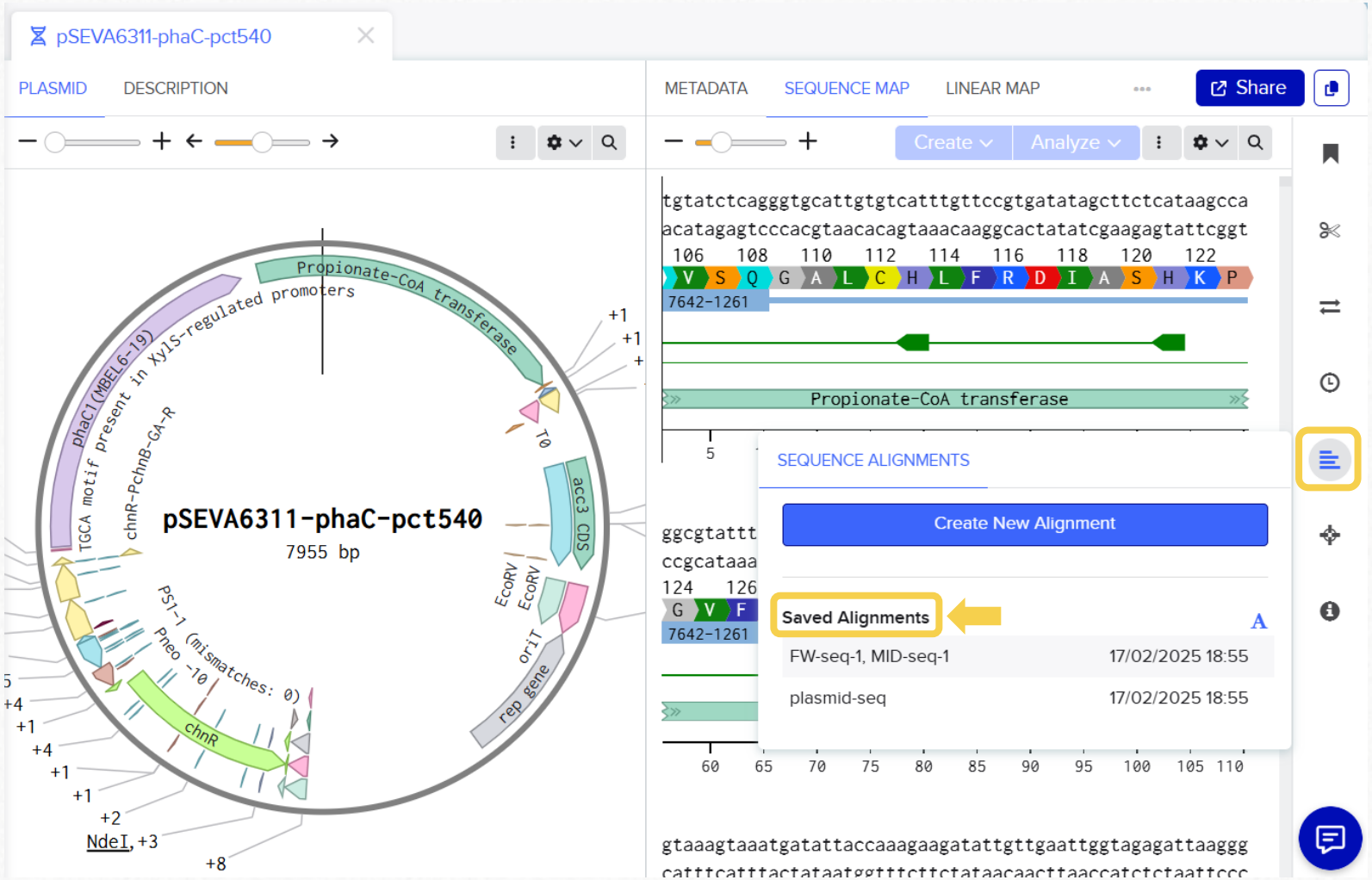


5 Create the alignments.

Alignment creation

Multisequence alignment

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

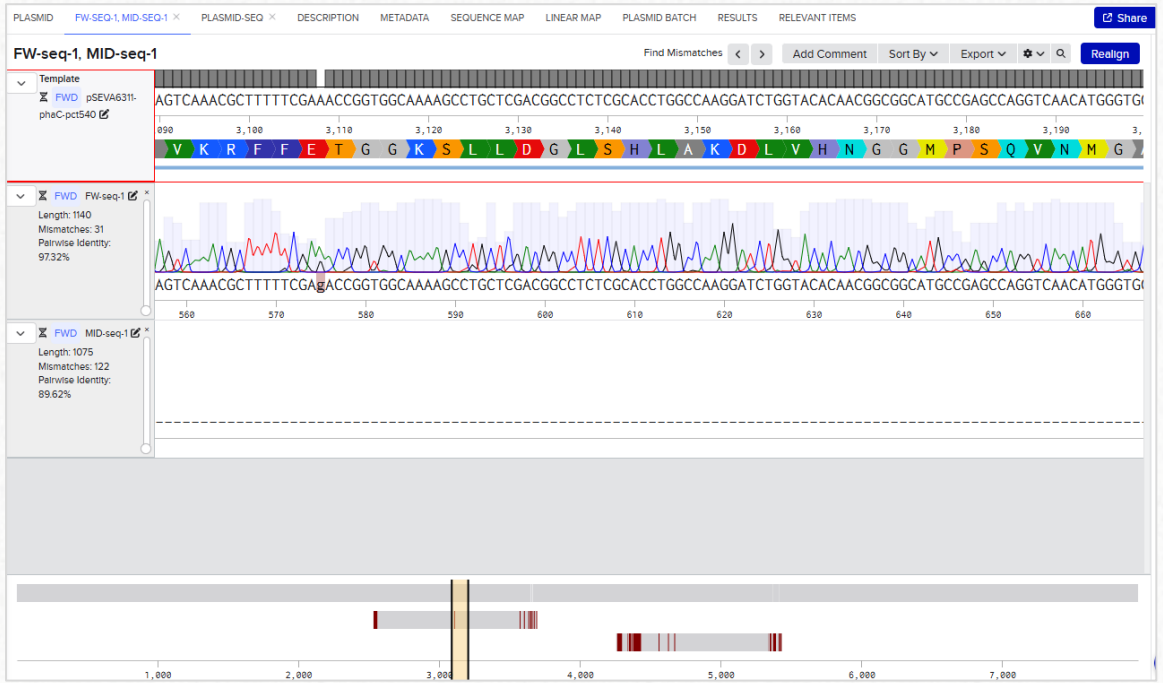


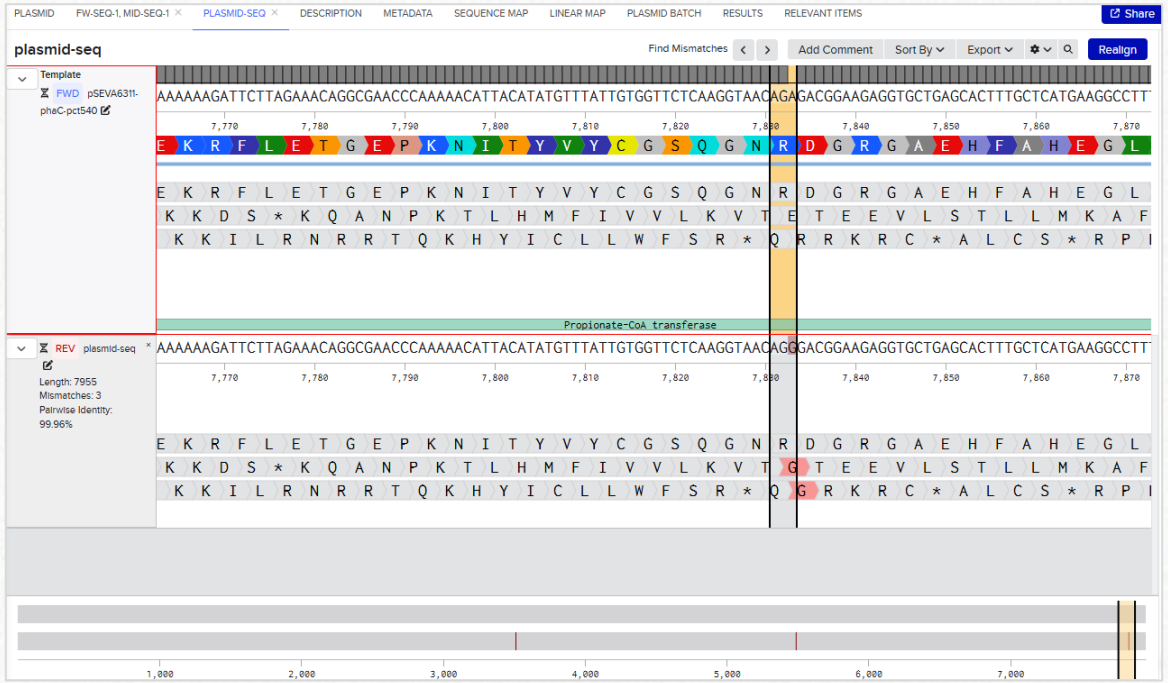
7 Open the alignments.

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.



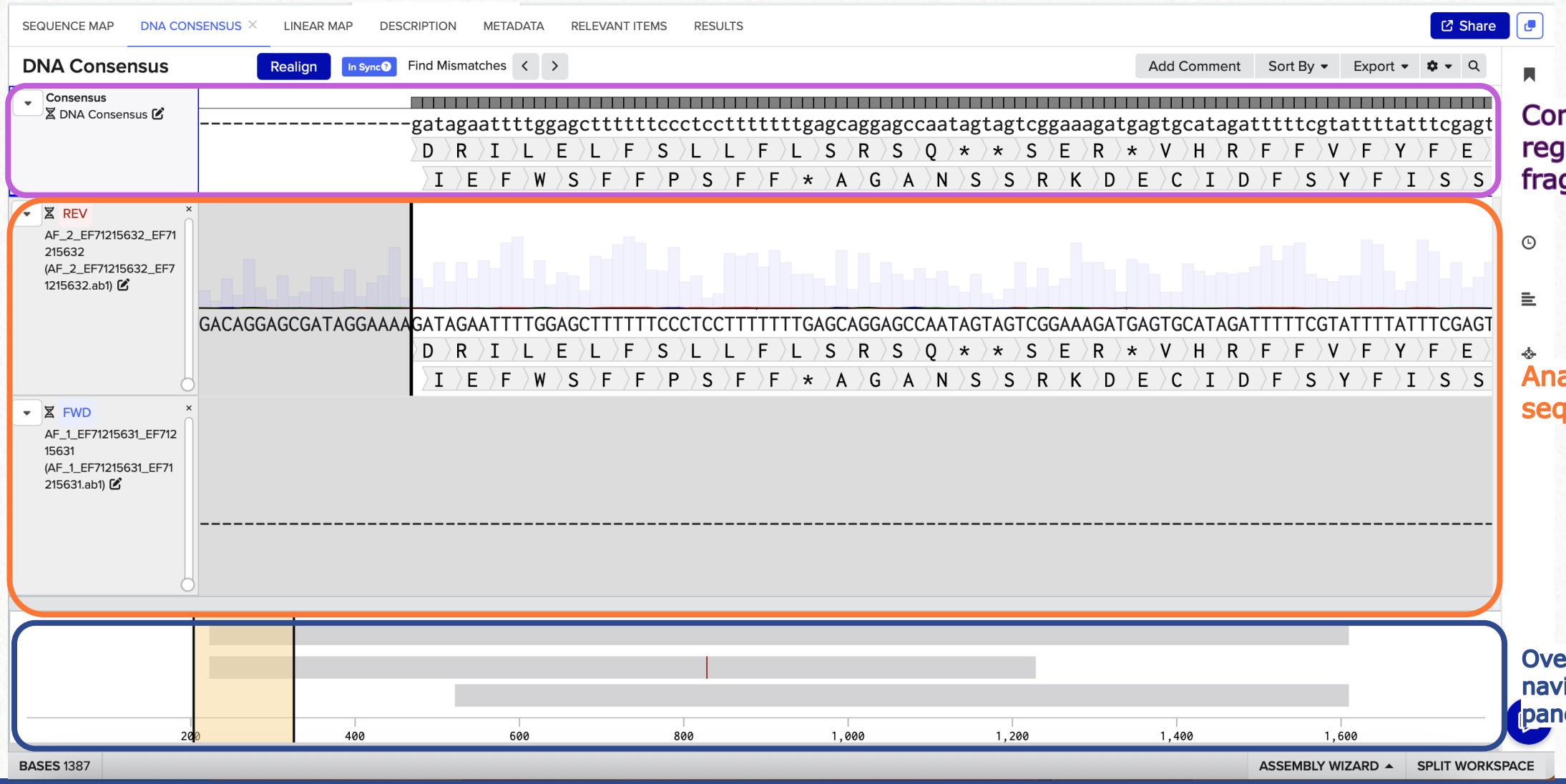


8. Sequence alignments

8.3 Consensus alignment

Alignment creation

Consensus alignment navigation



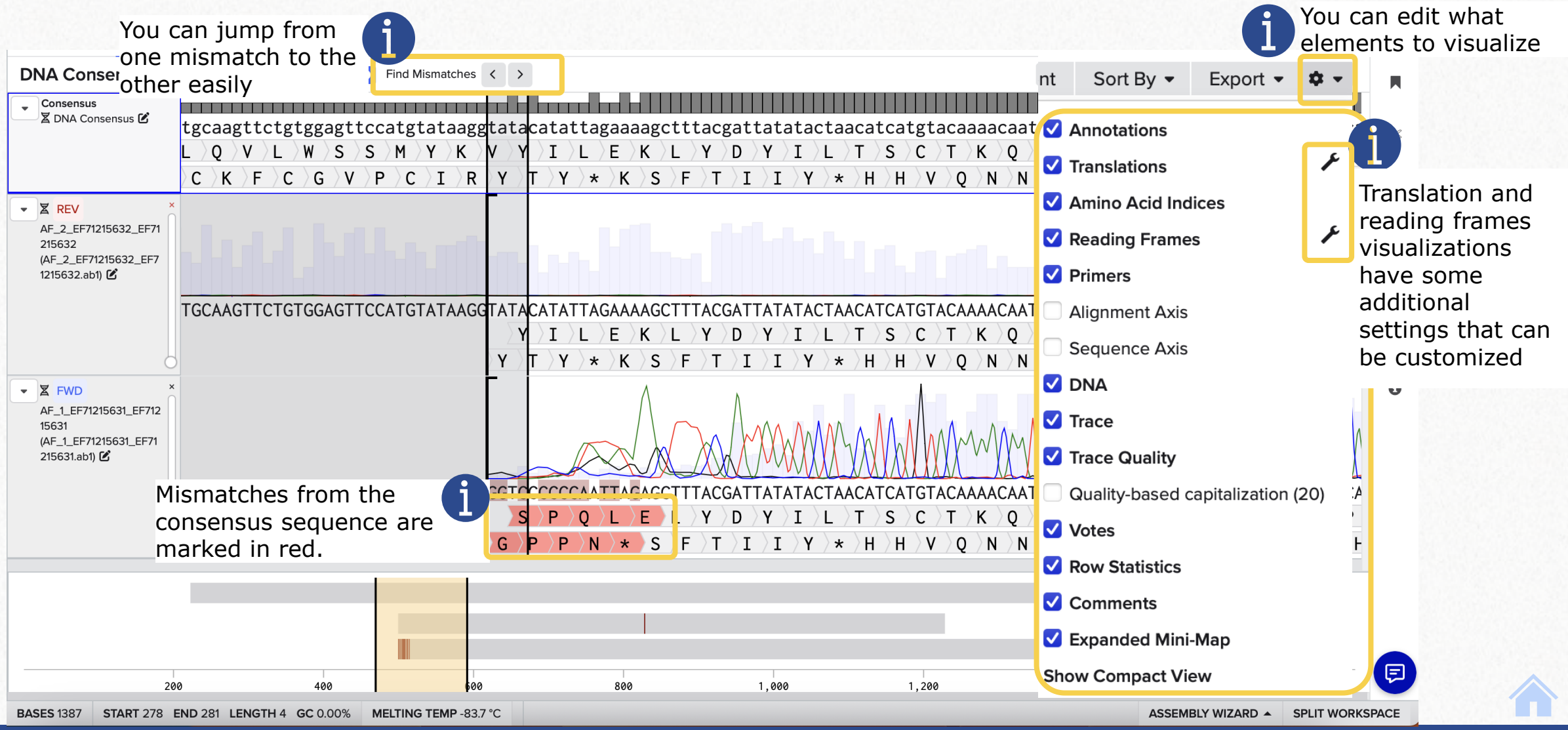
Consensus region fragment

Analysed sequences

Overview navigation panel

Alignment creation

Consensus alignment navigation



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.
- Benchling 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

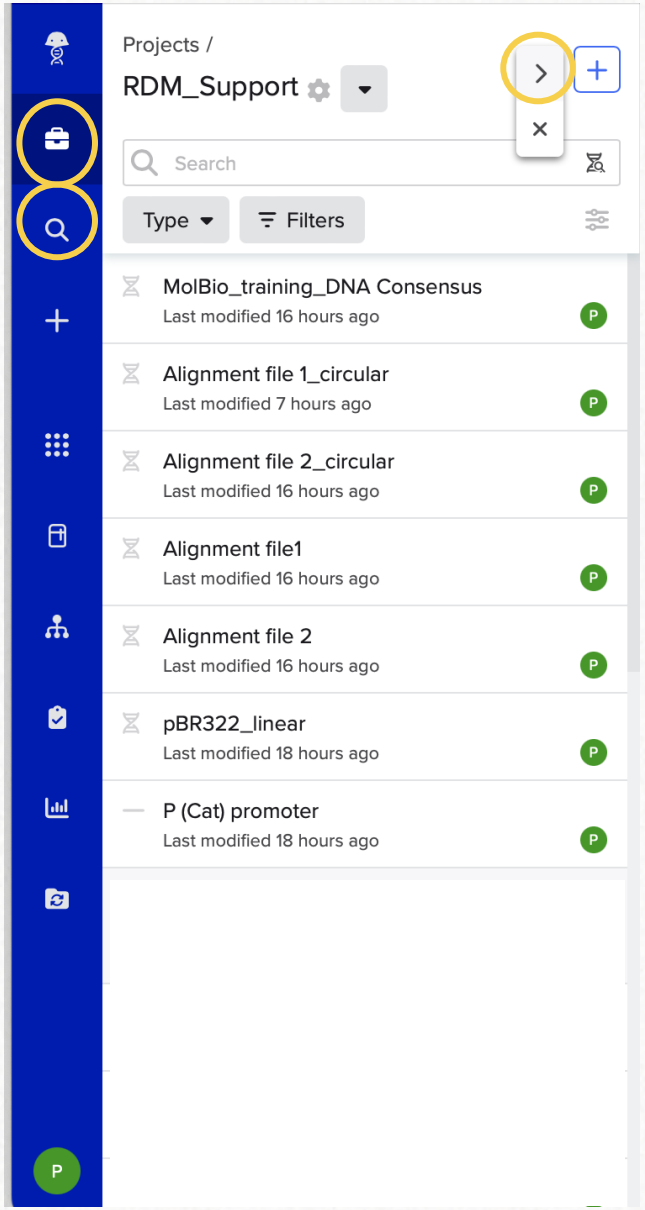
Tips and tricks

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

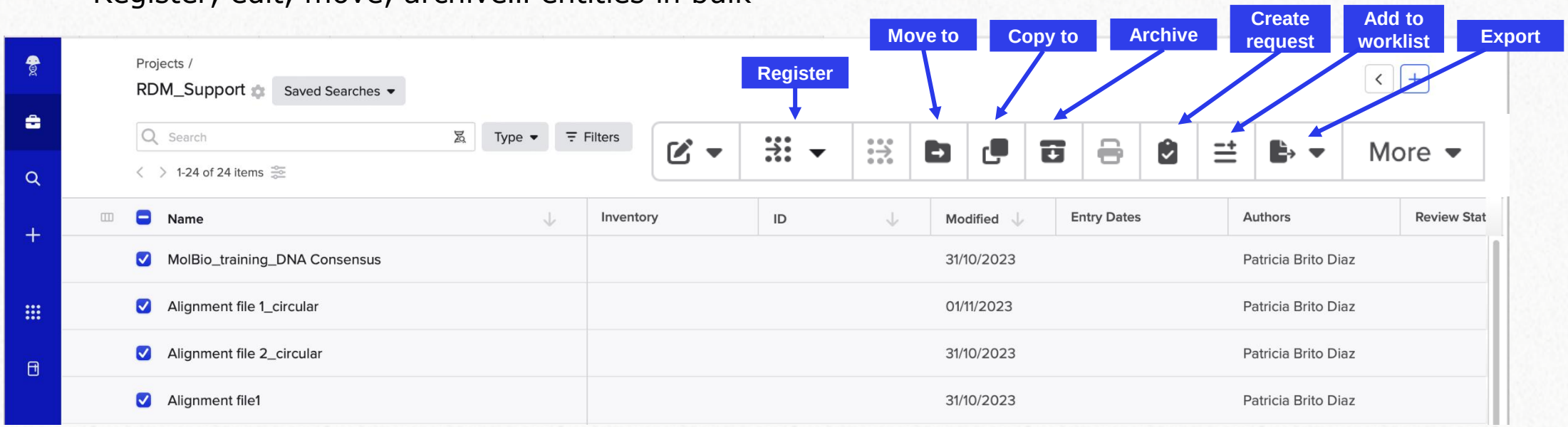


Tips and tricks

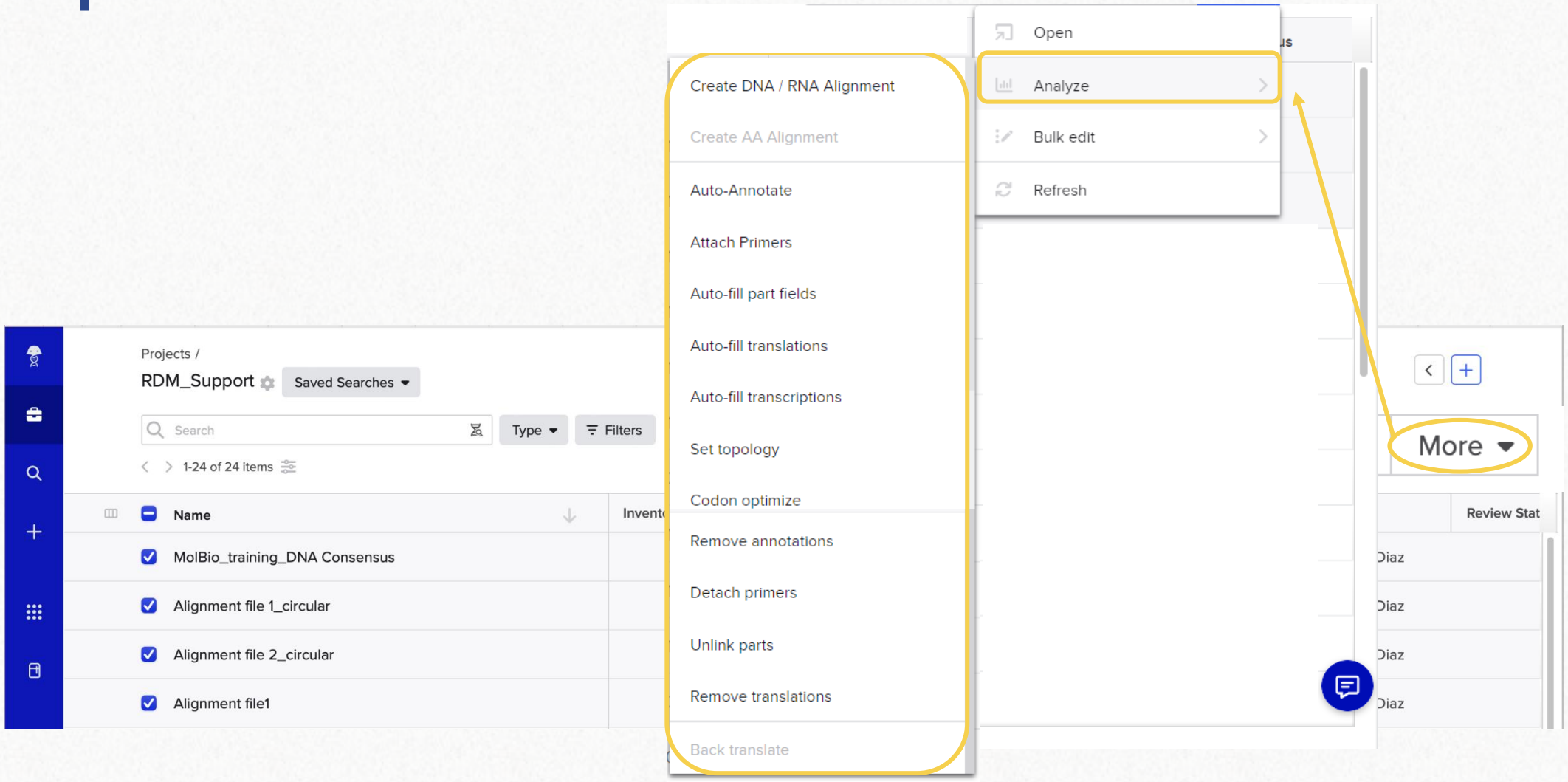
Work in bulk using the expanded view

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

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



Tips and tricks



Tips and tricks

Autoindexing when creating alignments

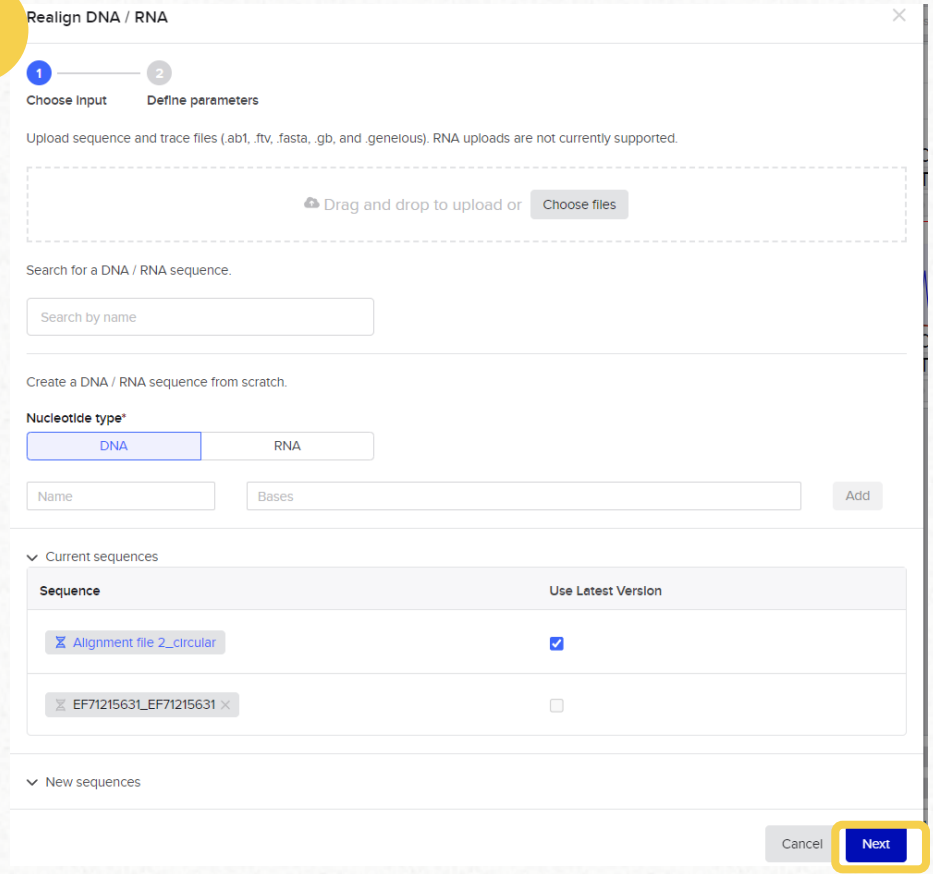
When creating an alignment of circular sequences, Benchling by default performs an **auto indexing** of these sequences.

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

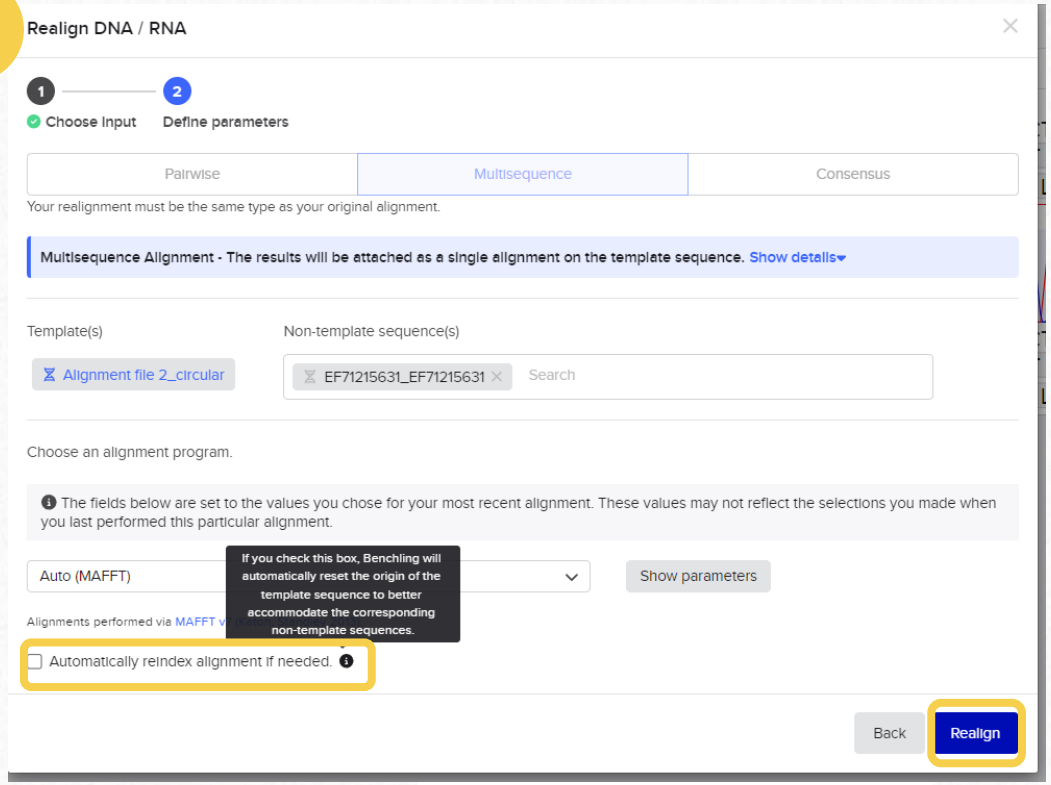
Tips and tricks

Autoindexing when creating alignments

2



3



Tips and tricks

Autoindexing when creating alignments

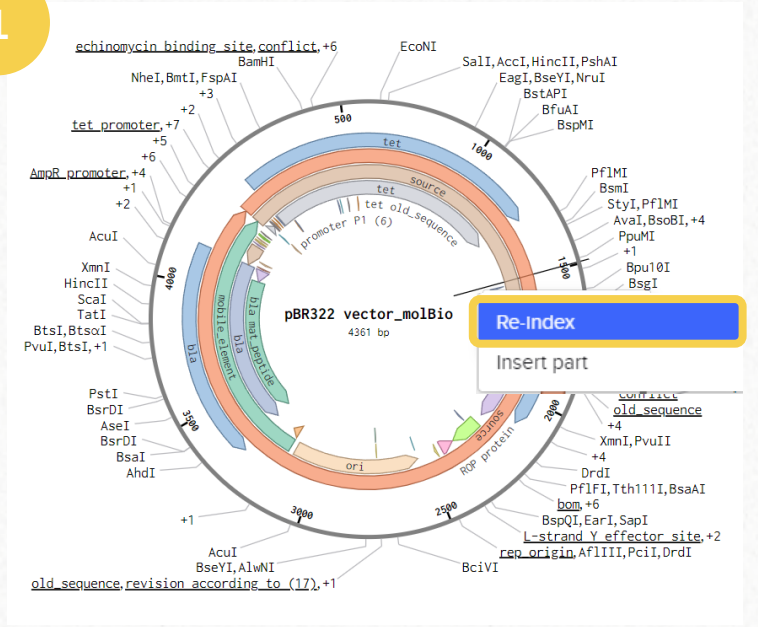
When creating an alignment of circular sequences, Benchling by default performs an **auto indexing** of these sequences.

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

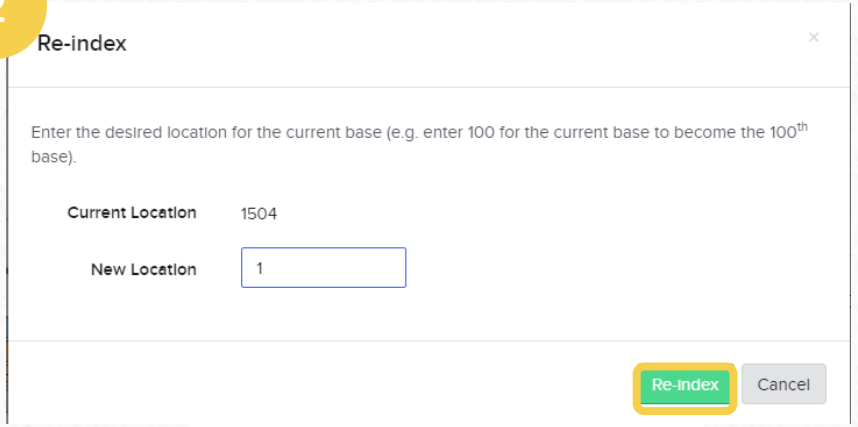
Pro TIP:

- ✓ 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



10. Resources

Questions?

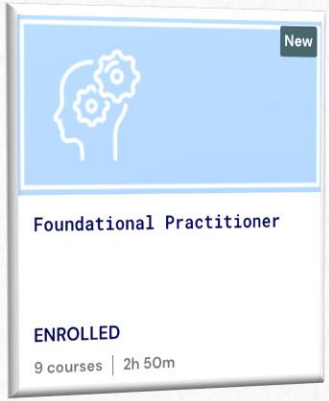
Contact lims_support@biosustain.dtu.dk

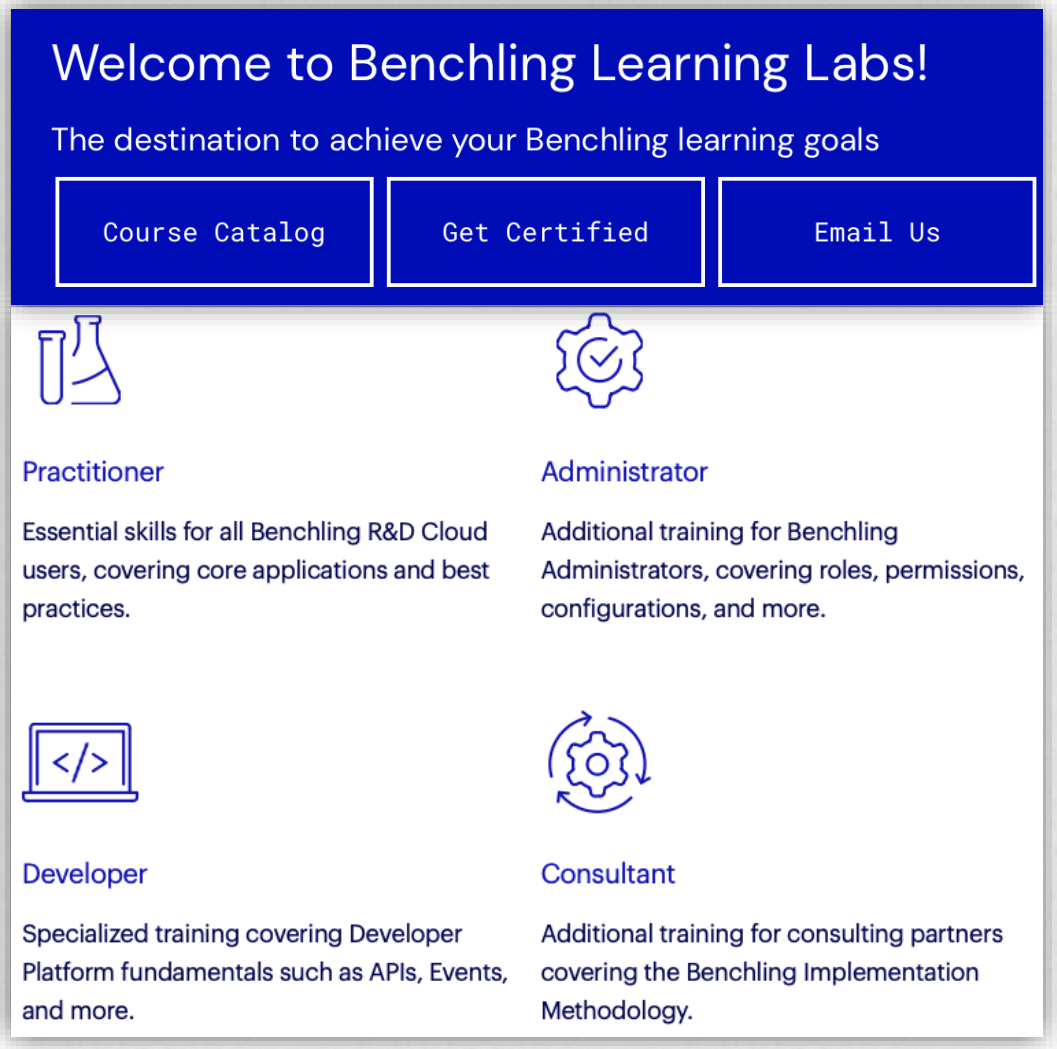
More resources

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





More resources

Benchling Help Center

Benchling provides some short guides on main functionalities

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