Danmarks Tekniske Universitet

DTU

---

# BioBrick Workshop - Dry Lab exercise

***Benchling – Primer and Plasmid Design***

---

# DTU BioBuilders 2025

Saturday 29/03/2025

# Objective

Participants will learn how to design primers and construct a plasmid for cloning in Benchling, an online molecular biology tool.

## Materials

A. Laptop with an internet connection
B. Benchling account (free version is sufficient)
C. DNA sequences (provided or download manually)
D. Protocol handout (in a digital form)

## Workshop sections

1. Introduction to Benchling

A. Overview of benchling tools
B. Creating a new project and sequence file
C. Understanding Benchling's sequence visualization
D. Annotating features
E. Select a gene of interest, along with a promoter, RBS and terminator

2. Primer design

A. Setting primer design parameters
B. Performing in silico PCR

3. Plasmid design

A. Selecting a plasmid backbone
B. Performing in silico digestion and cloning

4. Finalization and export

A. Checking for errors
B. Exporting the plasmid map

# Step-by-step guide

💡 : This symbol indicates theoretical information that will help you understand the exercise's biological background.

🔧: This symbol highlights exactly what you need to click on and provides all the instructions for the dry lab exercise.

🔎: Other relevant information

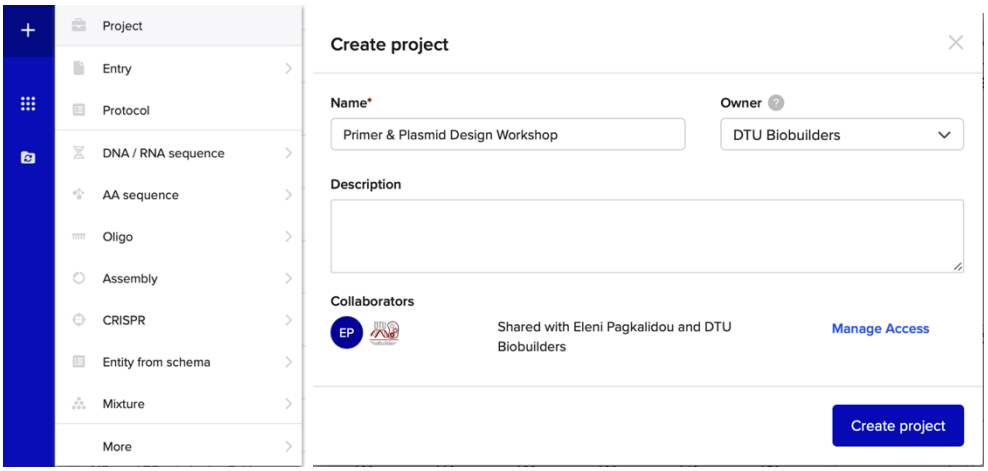
---

**Step 1**: Introduction to Benchling – set up a new project

https://benchling.com/

🔧Log in to your Benchling account and set up your project.

🔧Click on "*Projects*" on the left-side panel. Give the new project a name (e.g., "Primer & Plasmid Design Workshop") and click on "*Create project*".




🔎In the wet lab exercise, the BBa_J04450 construct was provided pre-assembled in the pSB1C3 backbone as part of the iGEM Distribution Kit. We'll simulate the same procedure on Benchling by using Restriction Enzyme digestion & Cloning to mimic how the construct was probably inserted into pSB1C3.

Choose the desirable genetic construct (BBa_J04450).

💡 The BBa_J04450 construct is a well-characterized composite BioBrick device that consists of the following four standardized parts:

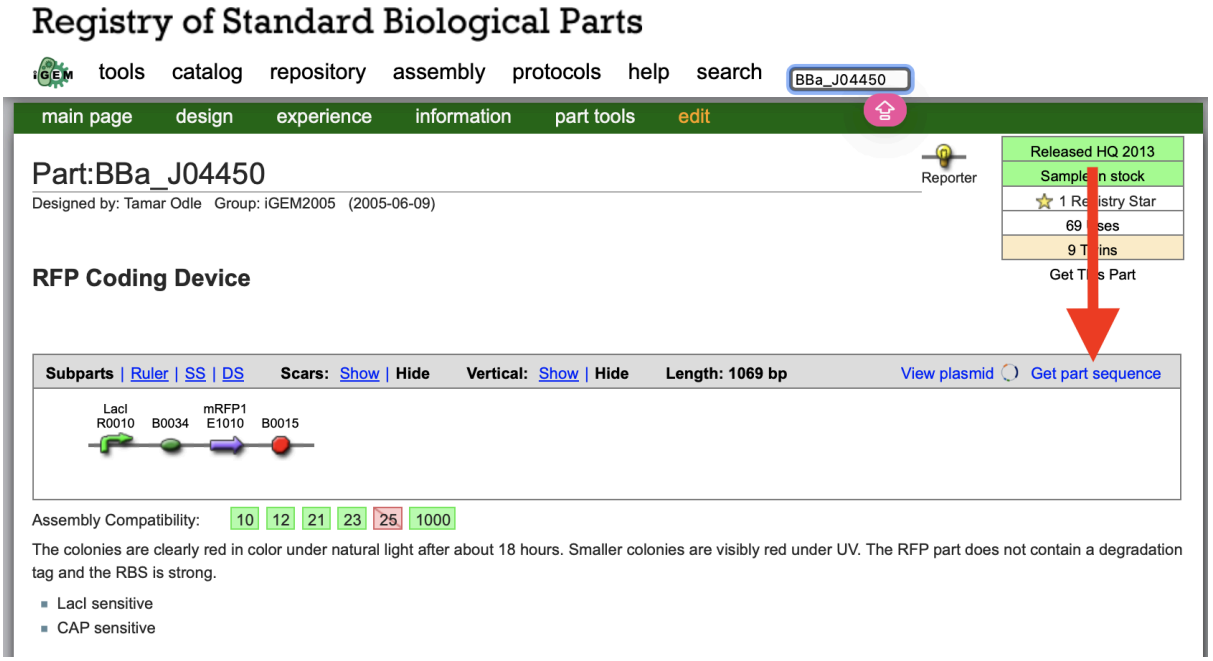
**Promoter (BBa_R0010)** – A lacI-regulated promoter (Plac) that allows for inducible expression in the presence of IPTG.

**Ribosome Binding Site (BBa_B0034)** – A strong, commonly used RBS that ensures efficient initiation of translation.

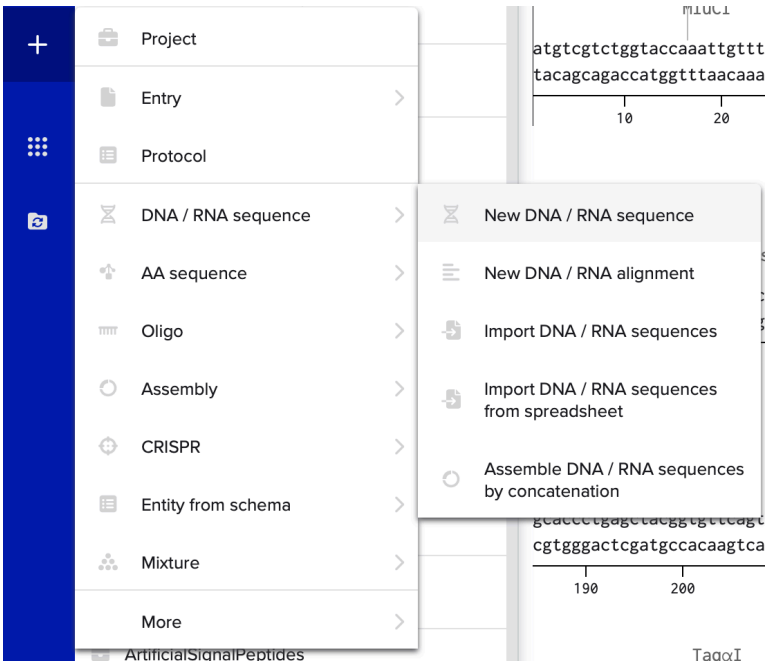
**Reporter Gene (BBa_E1010)** – Encodes the monomeric Red Fluorescent Protein (mRFP1), which allows for easy visual detection of gene expression.

**Terminator (BBa_B0015)** – A double terminator that effectively stops transcription, increasing the stability of the transcript and preventing read-through.

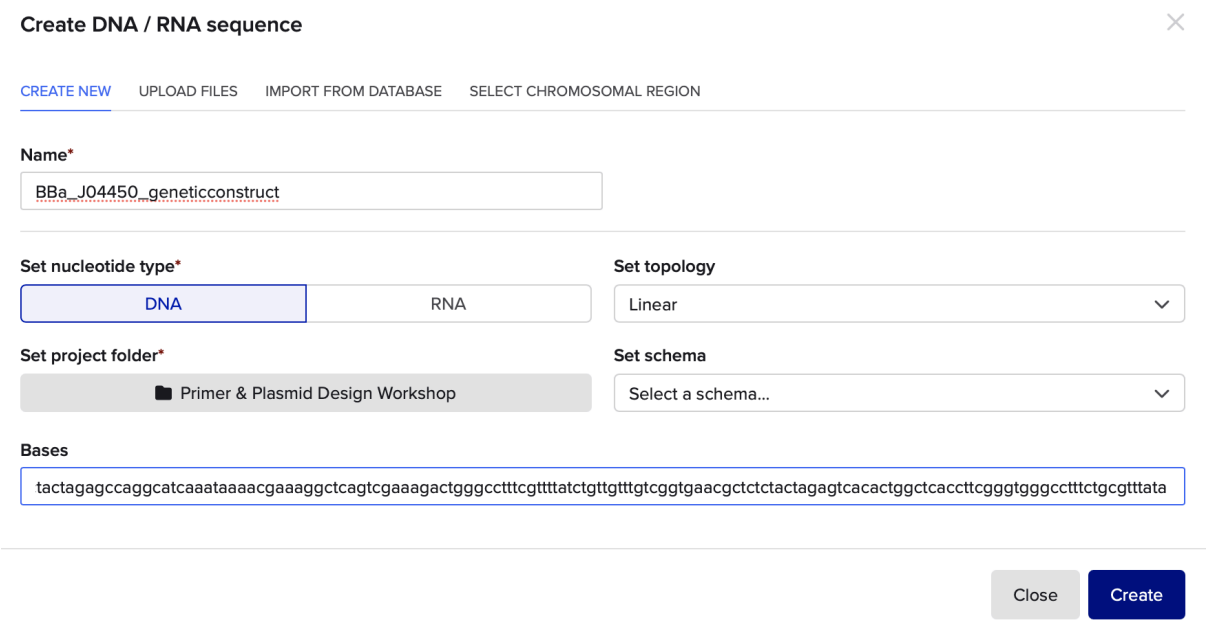
🔧To get the desired part, go to the iGEM registry (https://parts.igem.org/Main_Page), search for the name and then click at "*Get part sequence*". Copy the sequence.




🔧Click "*Create*" → "*DNA/RNA Sequence*" → "*New DNA/RNA sequence*", since we are pasting a DNA sequence from the registry.



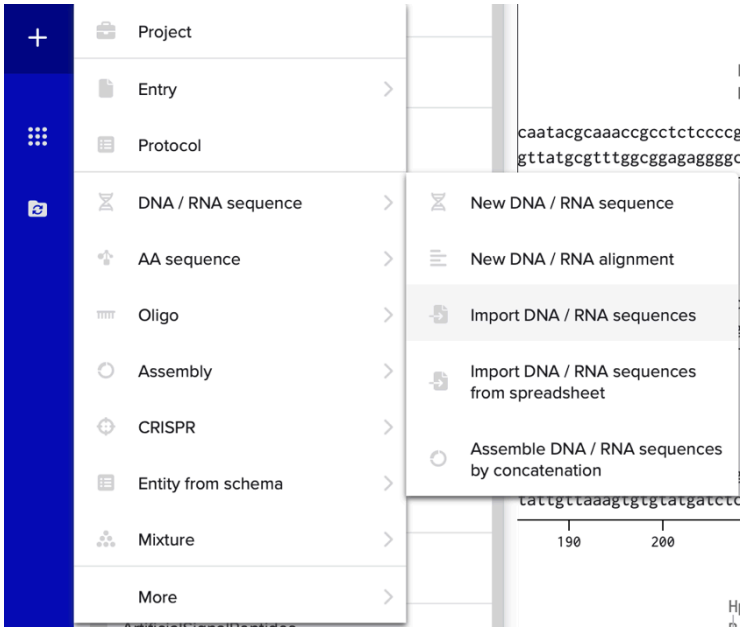

🔧Name your new sequence with an appropriate name that will help you distinguish it from other sequences, set the nucleotide type and project folder, and paste the sequence you copied from the registry and then "*Create*".

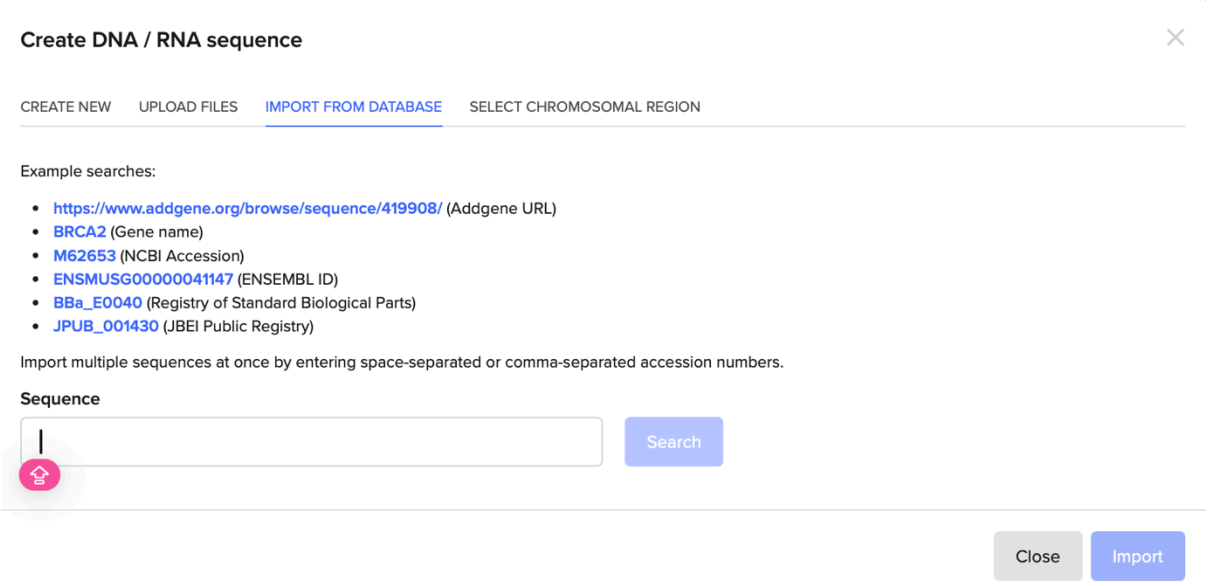



🔎 For other genes, go to NCBI Genbank and get the sequence in a fasta or gb. format. By downloading the file you can click on "*Import DNA / RNA sequences*" > "*Upload file*".

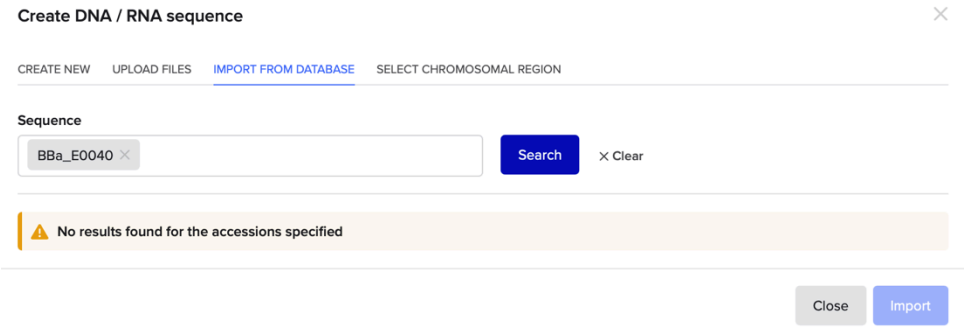
🔎 For all iGEM registered parts, there is the option to import directly the sequence by:

"*Create*" > "*Import DNA / RNA sequences*" > "*Import from database*" > write the part's code



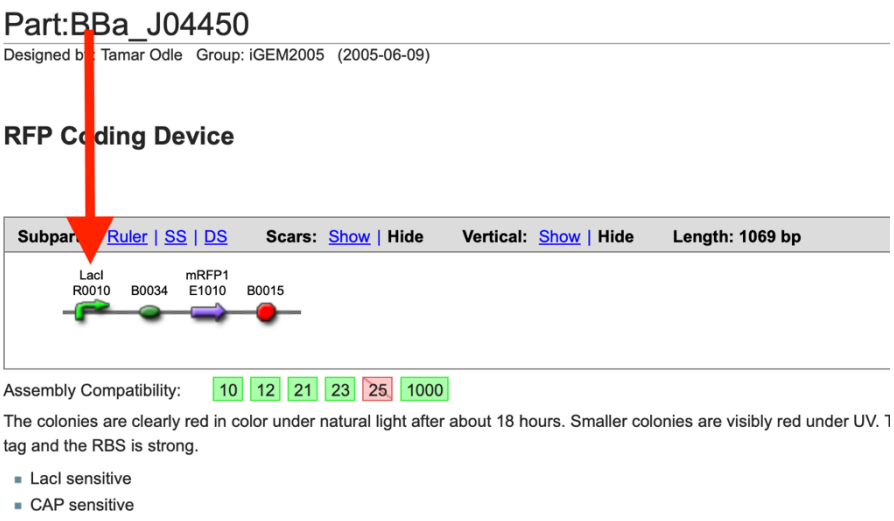




It looks like Benchling faces some technical problems and currently this option doesn't lead to anywhere, so we'll annotate the sequence manually.

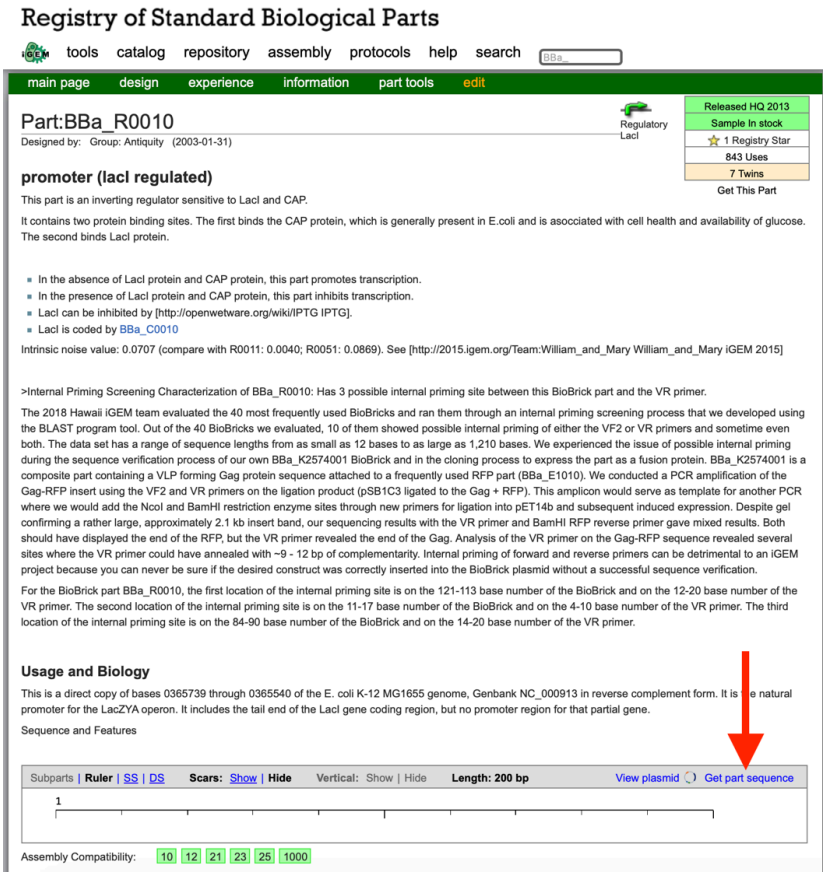



🔧Since we have added our BBa_J04450 sequence, going back to the registry we can get the sequence for each subpart and manually annotate them in the sequence on Benchling. The genetic construct consists of the 4 different elements: LacI promoter (R0010), RBS (B0034), mRFP1 (E1010) and Terminator (B0015). We'll follow the same procedure to annotate each one.

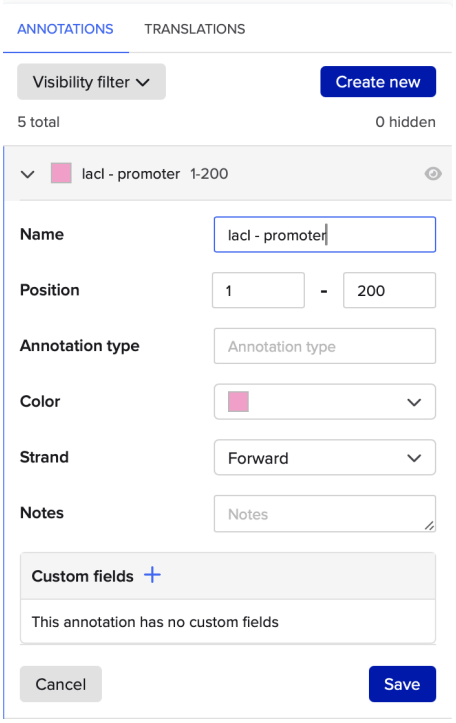
First, click on each subpart's icon.




Scroll a bit further down, until you see the "*Get part sequence*" again.




🔧Copy the sequence and search for it in Benchling by Ctrl+ F, paste the sequence and see the highlighted bases. Select them, right click > “Create annotation". Fill out the info as shown:



Follow the same procedure to annotate the rest of the parts.

💡The mRFP1 sequence provided in the iGEM Registry does not include a stop codon, because it is designed to be part of a modular BioBrick system, allowing for flexible assembly with other genetic parts.

Choose a plasmid backbone (pSB1C3)

🔧Download the backbone's sequence from Snapgene's database and upload the file:

https://www.snapgene.com/plasmids/basic_cloning_vectors/pSB1C3

"Create" > "DNA / RNA sequence" > " New DNA / RNA sequence" >" Upload from files"

## **Step 2**: Primer design

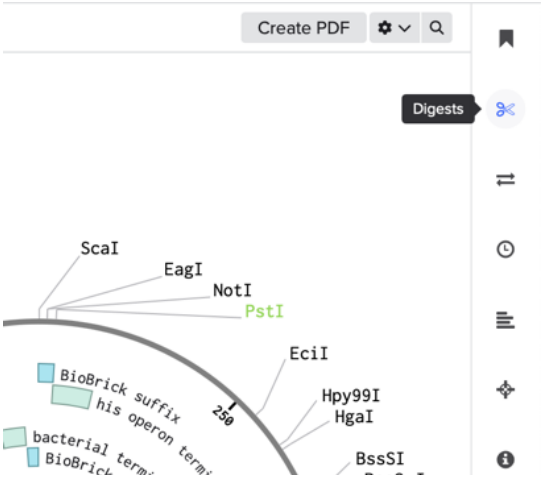
### Restriction Enzyme digestion & cloning

For restriction enzyme (RE) cloning, the backbone will be digested with two different enzymes to generate sticky ends, and the corresponding RE recognition sites will be introduced at the ends of the insert using PCR primers.

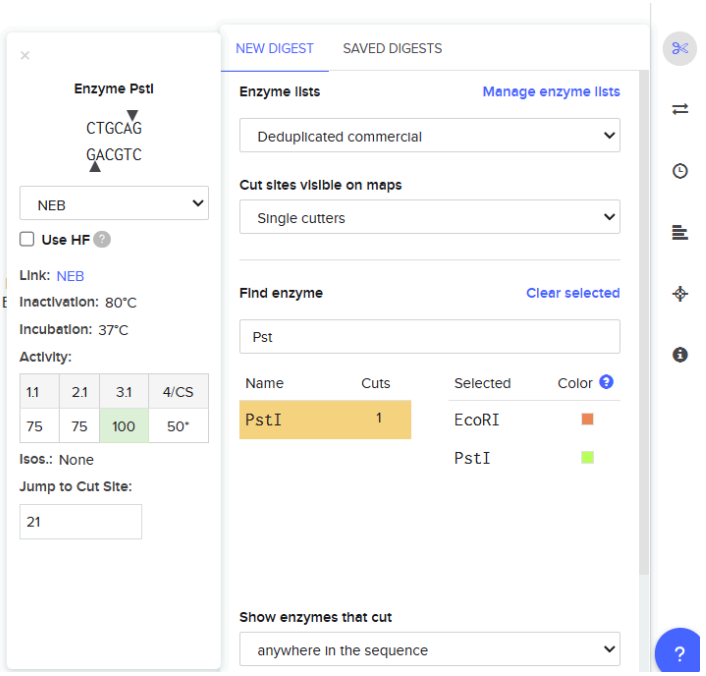
Specifically, the backbone will be cut with EcoRI and PstI.

💡These enzymes were chosen because neither the pSB1C3 backbone nor the BBa_J04450 insert contains internal EcoRI or PstI recognition sites, avoiding unwanted fragmentation. Additionally, these sites are not located within any essential regions of pSB1C3, such as the origin of replication (ori) or the chloramphenicol resistance (CmR) gene. This careful selection of restriction enzymes ensures that BBa_J04450 can be accurately and efficiently inserted into pSB1C3.

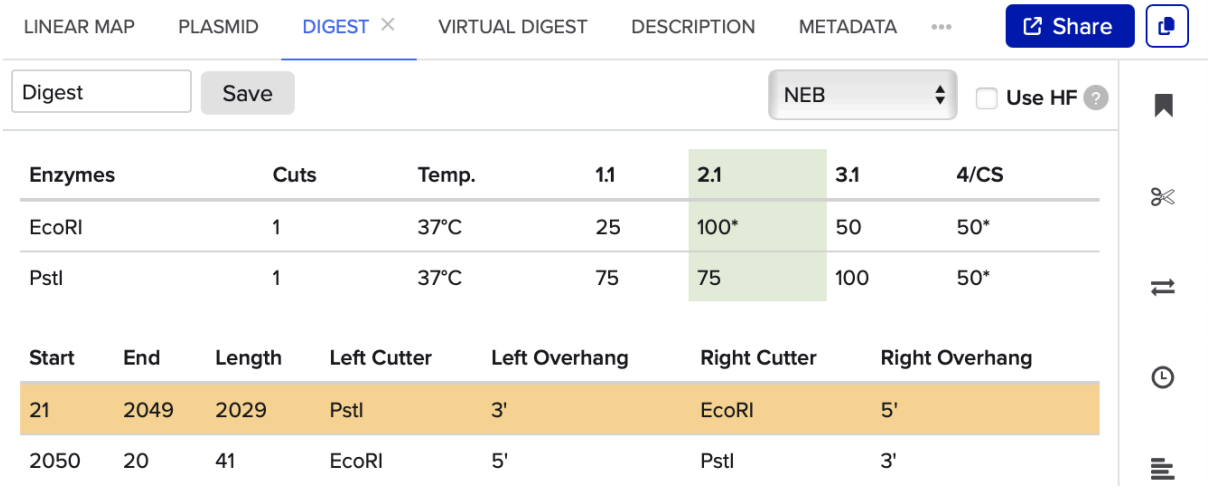
🔧In **pSB1C3**, click on "*Digests*" on the tool bar on the right side.



🔧Choose **EcoRI** & **PstI**.

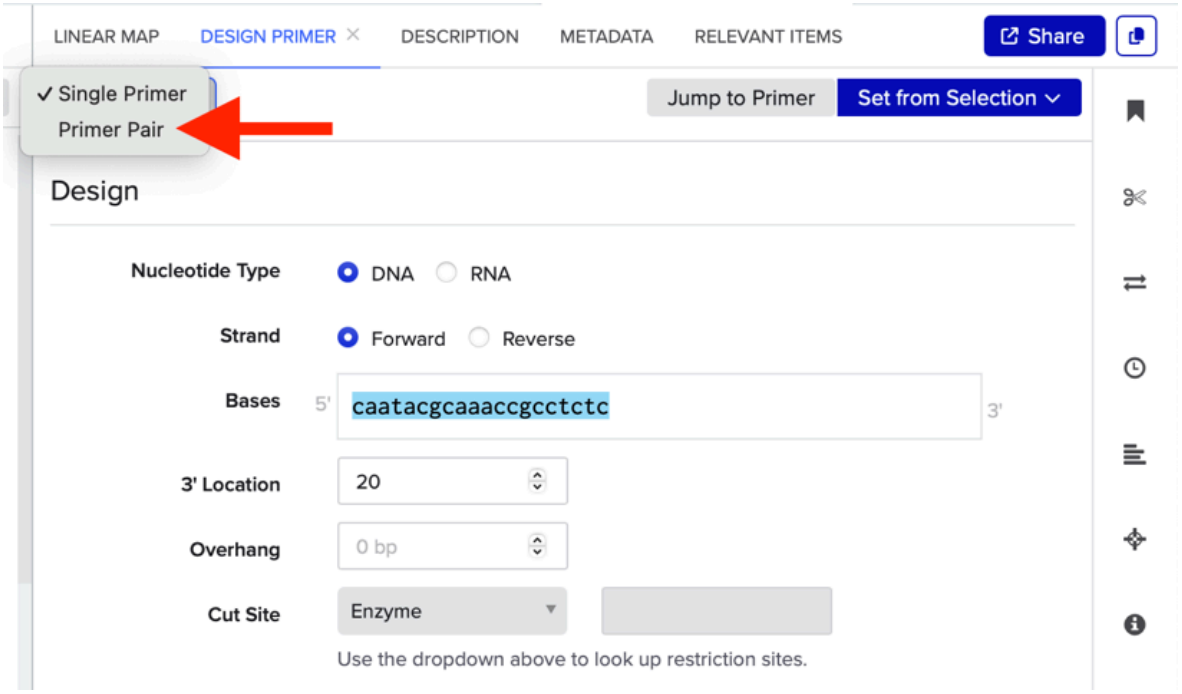



🔧 Click "*Run digest*" to simulate cutting the vector.
🔧 Click on the part you want to keep and "*Save*".




To add the specific sticky ends to our BBa_J04450 construct, the restriction enzyme sequences have to be inserted by creating PCR primers, and then digested with the restriction enzymes.

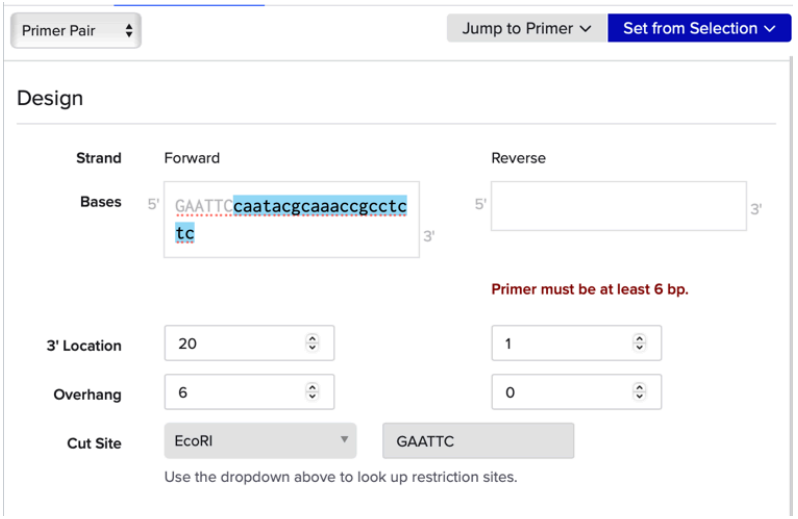
🔧Highlight the 20 first bp of the J04450 sequence, right click > "*Create primer*" > "*Forward*". On the pop-up window, click on "*Single Primer*" on the top left and change it to "*Primer Pair*".



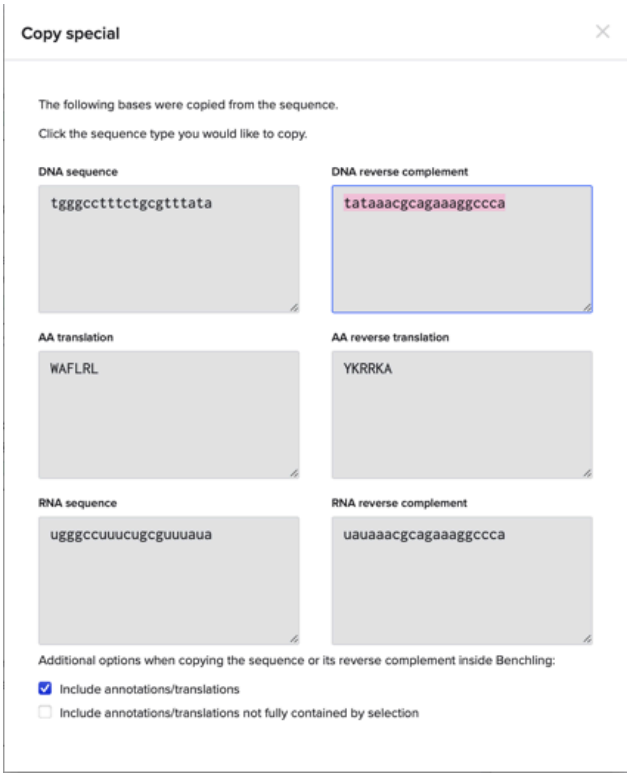

🔧Add the EcoRI recognition site at the start of sequence, as overhang, to create the forward primer.

**EcoRI (GAATTC)** → The primary restriction enzyme for inserting the part.

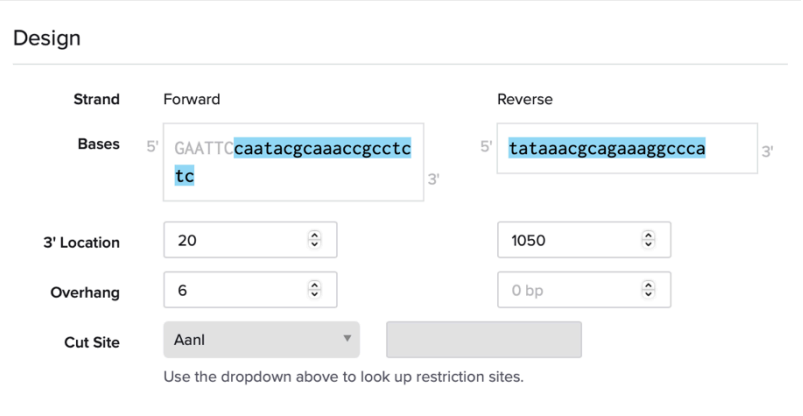
```
Forward primer: 5′- GAATTC – 3′
```




🔧For the reverse primer, highlight the 20 last bp of the J04450 sequence, right click > "*Copy special*" > "*Reverse complement*". On the pop up window, copy the sequence on the right top box "*DNA reverse complement*", to copy the reverse sequence of the one you just selected. This will be the first part of the reverse primer.




🔧Paste the sequence in the Design Primer window, and add the reverse primer's location.

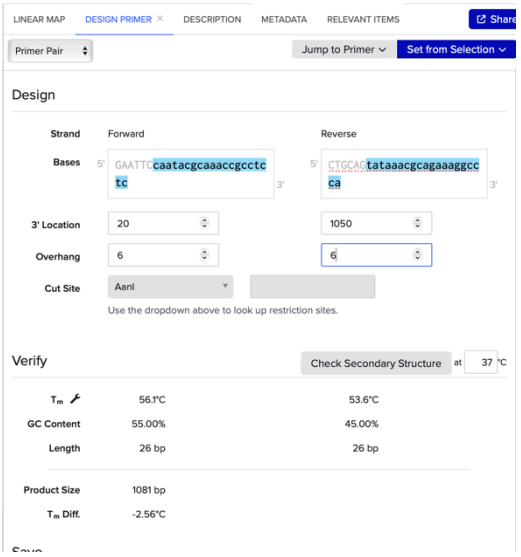



🔧Add the PstI recognition site at the 5′ end of the reverse primer as an overhang, to create the reverse primer.

**PstI (CTGCAG)** → The second restriction enzyme for inserting the part.

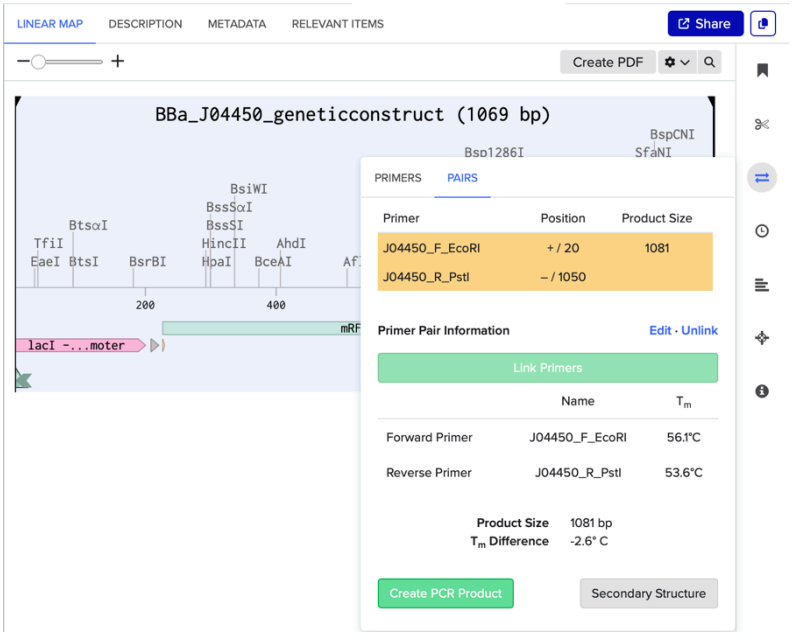
💡Since PstI recognizes the palindromic sequence 5′-**CTGCAG**-3′, its reverse complement is identical to the original sequence. Therefore, when designing the reverse primer, the same sequence (CTGCAG) can be used directly without needing to reverse-complement it.

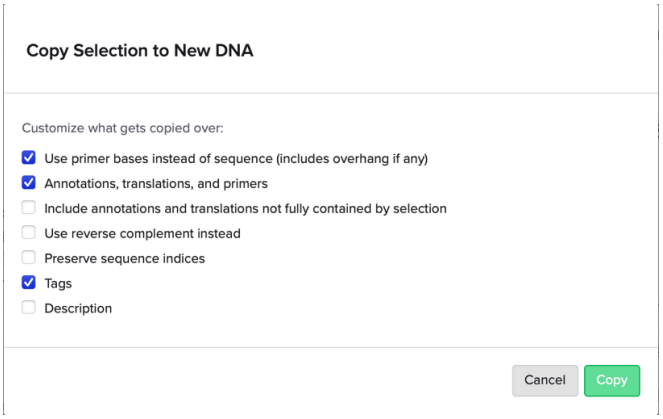
```
Reverse primer: 5′- CTGCAG – 3′
```



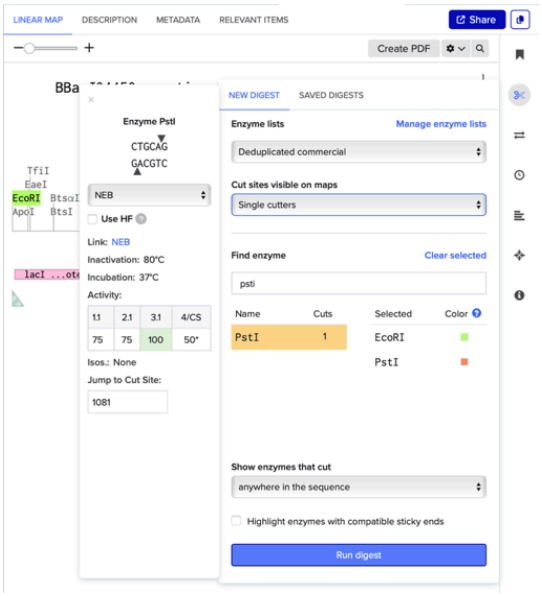

🔧Save the primers with names that clearly state what they are and click on "*Save Primers*".


🔧On the tool bar on the right, if you now click on the "*Primers*", select "*Create PCR Product*". Then, "*Copy*" and save the new DNA to your folder.

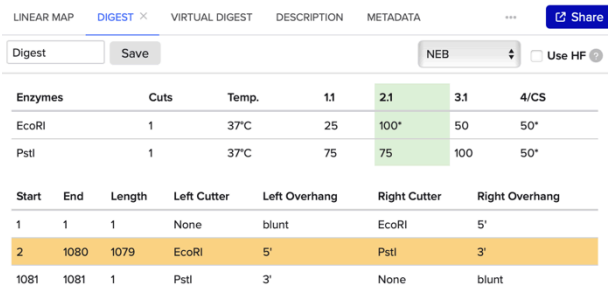




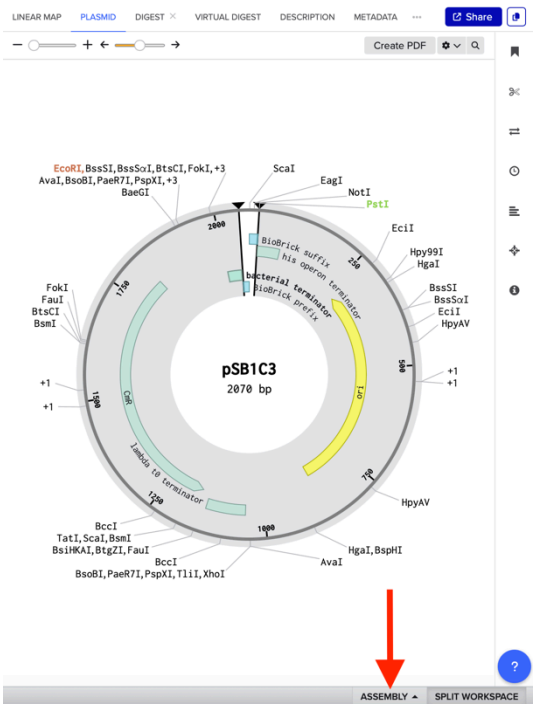

🔧 "*Run Digest*" on the on the new J04450 part too to create sticky ends, compatible to the backbone's.



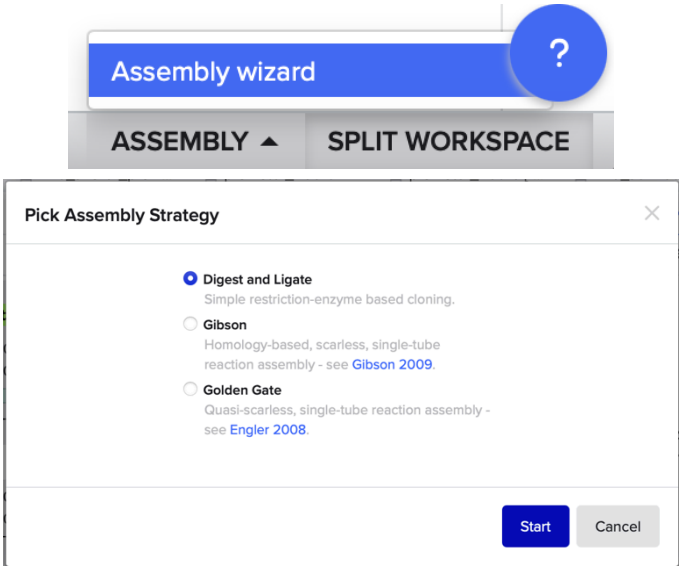

🔧 Click on the part you want to keep and "*Save*".



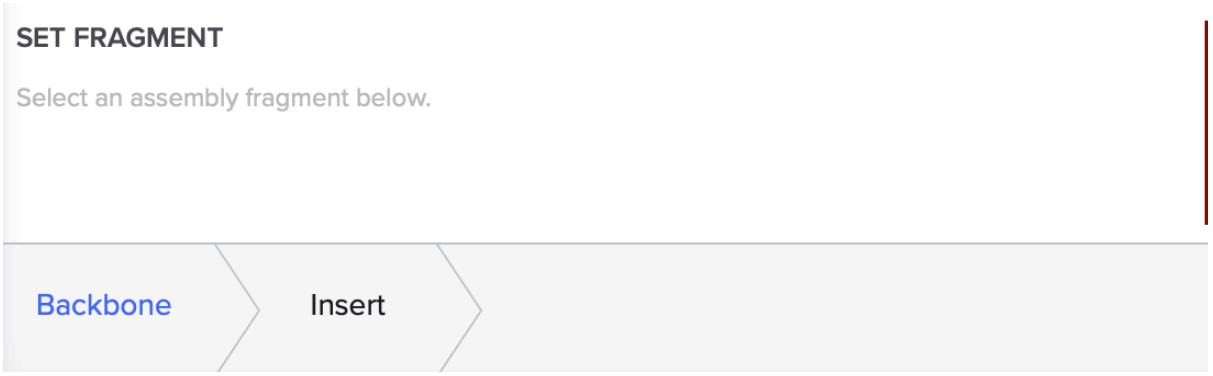

🔧 Click on "ASSEMBLY" on the right bottom of your pSB1C3 plasmid map, to initiate the assembly.



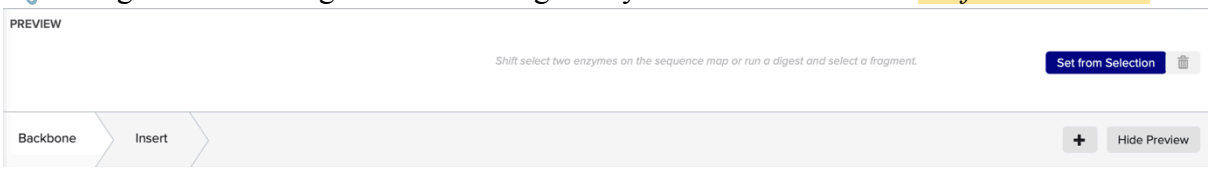

🔧Click on "*Assembly wizard*" and pick the "*Digest and Ligate*" option, then "*Start*".



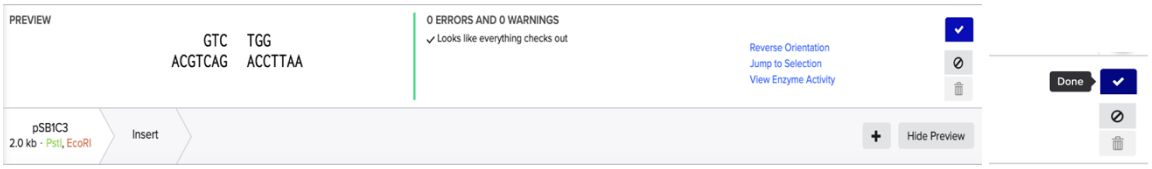
🔧Click on "*Backbone*" to set the backbone (pSB1C3).

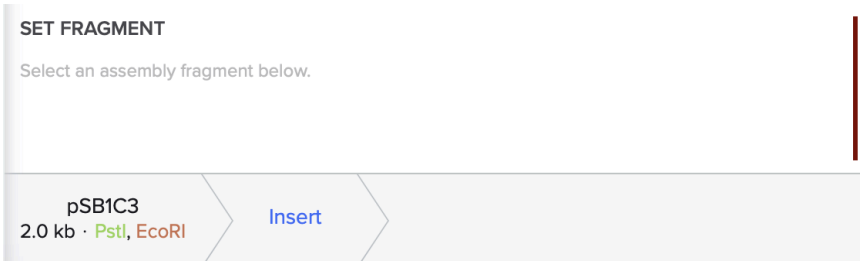



🔧Having selected the right backbone fragment you should be able to "*Set from Selection*".



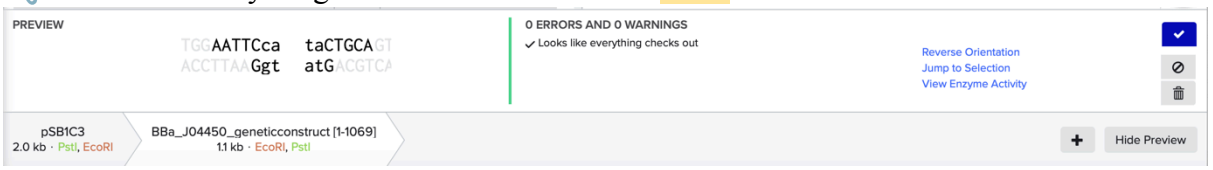

🔧Confirm that everything looks fine and click "*Done*". Then, click on "*Insert*".



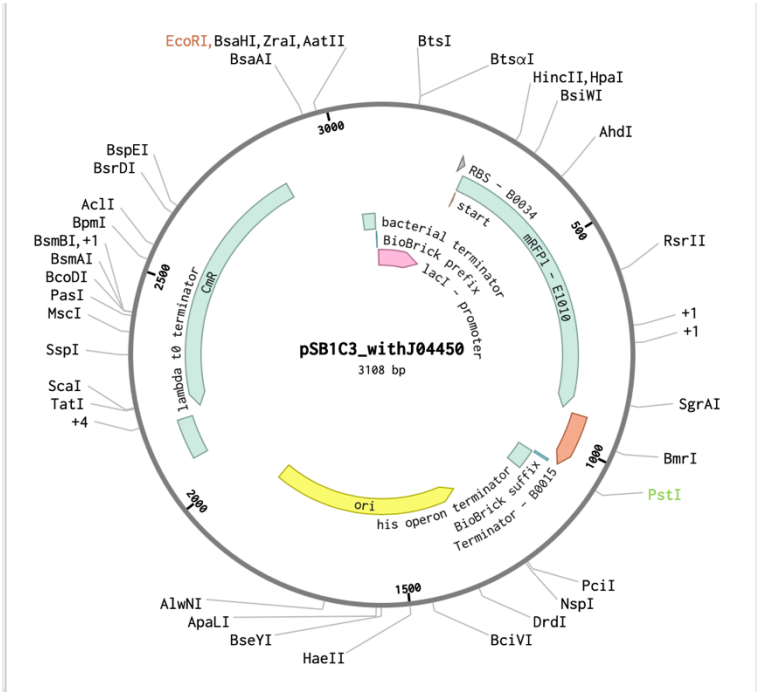

🔧 Change tabs to your insert and click on the part you want to select to enable the "*Set from Selection*" option.

🔧 Check that everything checks out and click on "*Done*".



🔧Name the assembly (e.g., pSB1C3_withJ04450) and "*Assemble*".


Congratulations, this is the plasmid you are using for your wet lab exercise🥳



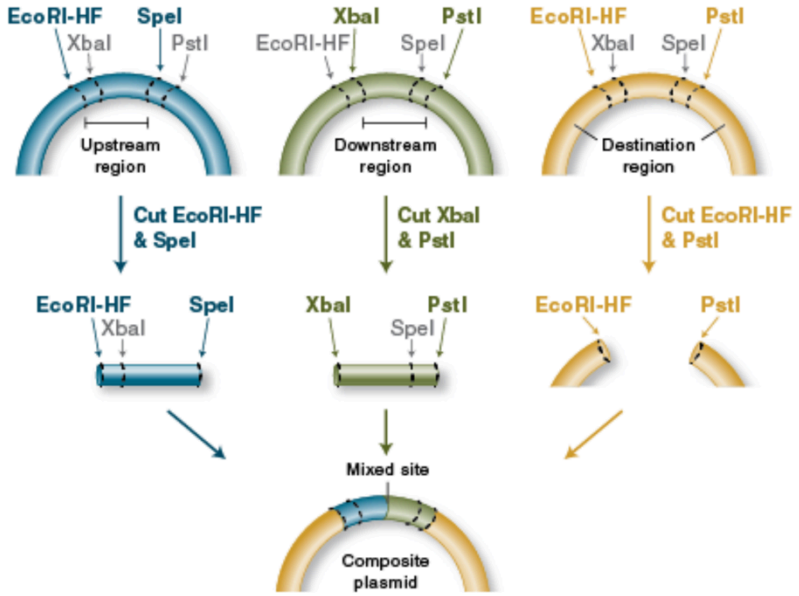

💡In plasmid designs, it's common for the antibiotic resistance cassette to be on the opposite strand, since it's under its own control and not part of the assembled genetic device.

## BioBrick Assembly

Another useful technique that could be followed, is Biobrick Assembly to incorporate all different J04450 subparts into the backbone. For this exercise, we divide our construct into 2 subparts instead of 4 (promoter + RBS/mRFP1/dterminator), but a similar procedure can be followed to incorporate more Biobricks into a plasmid.

💡 **BioBrick assembly** is a standardized method used in synthetic biology to combine DNA parts—called BioBricks—into larger genetic constructs. Each BioBrick part is flanked by specific restriction enzyme sites (EcoRI, XbaI, SpeI, and PstI), allowing them to be cut and ligated together in a predictable and modular way. This makes it easy to mix and match promoters, coding sequences, RBSs, and terminators to build custom genetic circuits without needing to redesign the cloning strategy for each combination.

If you are not familiar with the technique, pay attention to the figure below.




New England Biolabs. (n.d.). *BioBrick® assembly*. https://www.neb.com/en/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/biobrick-assembly

💡 In this method, three plasmids/sequences are involved: one containing the upstream BioBrick part, one containing the downstream part, and one serving as the destination vector. Each of these plasmids contains specific restriction sites that allow for precise cutting and ligation.

The upstream part is excised from its plasmid by digesting with EcoRI and SpeI. This releases the DNA fragment with sticky ends compatible with ligation. Simultaneously, the downstream part is cut using XbaI and PstI, generating another fragment with complementary overhangs. SpeI and XbaI produce compatible overhangs that can ligate together, but their ligation forms a mixed site that cannot be cleaved again by either enzyme—this feature ensures stability in the resulting composite part.

The destination plasmid is opened using EcoRI and PstI, creating a site where the upstream and downstream parts can be inserted. After the three fragments are prepared, they are ligated together to form a composite plasmid. The final construct contains both BioBrick parts joined in order, flanked by the original prefix and suffix, and ready for further use or additional rounds of assembly. This standardized method allows for predictable and modular construction of genetic circuits.

---

💡The BBa_J04450 construct is a well-characterized composite BioBrick device that consists of the following four standardized parts:

A. **Promoter (BBa_R0010)**
B. **Ribosome Binding Site (BBa_B0034)**
C. **Reporter Gene (BBa_E1010)**
D. **Terminator (BBa_B0015)**

💡Each of these components is a BioBrick, meaning they can conform to the BioBrick standard assembly rules. This allows to easily replace or combine them with other BioBrick parts using the BioBrick Assembly method or other compatible cloning techniques. For example, you could swap the promoter to test different expression levels or replace mRFP1 with another reporter gene to suit your experimental needs.

💡The BBa_J04450 subparts on the registry don't contain the standard prefix and suffix that are essential to the BioBrick assembly, only the coding region. So, they must be added, again by creating PCR primers.

💡The BioBrick format follows a standardized structure:

<u>BioBrick Prefix (before the gene starts)</u>

This sequence ensures compatibility with **EcoRI** and **XbaI** digestion:

```
5′- GAATTC GCGGCCGC T TCTAGA G XXXXXXXX -3′
```

(EcoRI - NotI - XbaI)

a) **EcoRI (GAATTC)** → The primary restriction enzyme for inserting the part.
b) **NotI (GCGGCCGC)** → Useful for verification but not needed for standard cloning.
c) **XbaI (TCTAGA)** → Used for ligation with a SpeI-cut plasmid.
d) **XXXXXXXX** → The first bases of your gene

The prefix sequence will match the beginning of the forward primer:

```
(1)Forward primer: 5′- GAATTC GCGGCCGC T TCTAGA G XXXXXXXX -3′
```

<u>BioBrick Suffix (after the gene ends)</u>

This sequence ensures compatibility with **SpeI and PstI** digestion:

```
5′ - XXXXXXXX T ACTAGT A GCGGCCG CTGCAG - ‘3
```

(SpeI - NotI - PstI)

a) **SpeI (ACTAGT)** → Compatible with XbaI for assembly.
b) **NotI (GCGGCCGC)** → Again, for verification purposes.
c) **PstI (CTGCAG)** → Used for cutting the plasmid downstream.

💡 The suffix (SpeI and PstI sites) must be included at the 5' end of the reverse primer, but because reverse primers bind to the complementary strand in the opposite direction, you must use the reverse complement of the suffix sequence as part of your reverse primer.

`(2)Reverse primer: 5′ - CTGCA GCGGCCGC T ACTAGT A XXXXXXXX - ‘3`

(PstI - NotI - SpeI)

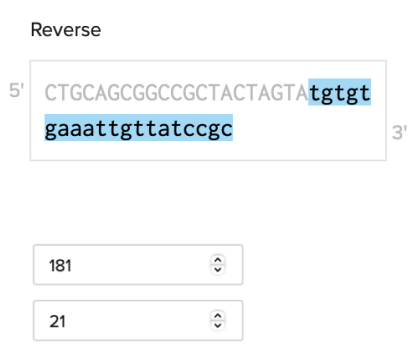
The pSB1C3 backbone already contains the prefix and suffix sequence.
PCR primers need to be created for the promoter (BBa_R0010) and the rest of the J04450 construct (in this case, BBa_B0034+BBa_E1010+BBa_B0015).

🔧Open the first BioBrick - the promoter’s sequence, from the registry.
https://parts.igem.org/Part:BBa_R0010 .
Copy the sequence, and "*Create*" > "*DNA / RNA sequence*" > "*New DNA / RNA sequence*”.
Name it as "*lacI promoter - R0010*“ .

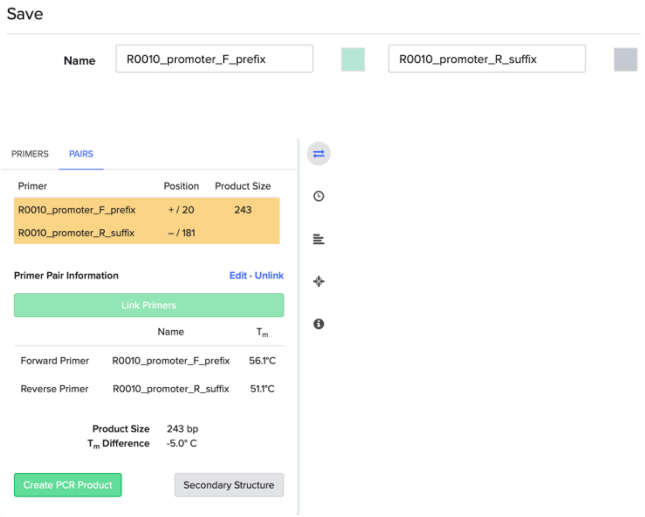
You can annotate it again, if you want.

🔧As before, select the 20 first bp of the sequence, right click > “*Create primer*” > “*Forward*”. Paste the provided forward primer sequence (1) as a 22 bases overhang.


🔧For the reverse primer, select the 20 last bp of your sequence, right click > “*Copy special*” > “*Reverse Complement*”. Copy the reverse complement sequence and paste it at your Reverse primer.
🔧Add the provided 21 bp overhang reverse primer sequence (2) as the 5’end of the reverse primer, at sequence position 181.



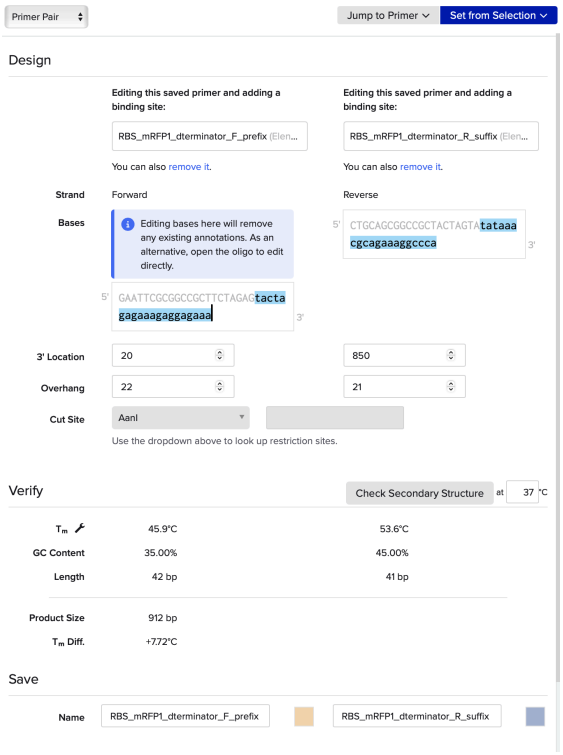
🔧 Save the primers under appropriate names (e.g., R0010_promoter_F_prefix & R0010_promoter_R_suffix) and "*Create PCR product*".




🔧Now, to get hold of the second BioBrick (BBa_B0034 + BBa_E1010 + BBa_B0015), search for the promoter's sequence in the previously annotated J04450 construct sequence, copy and paste the rest of the construct's sequence (promoter's sequence excluded). https://parts.igem.org/Part:BBa_J04450

Once you have the right sequence for the rest of the parts, you can annotate them.

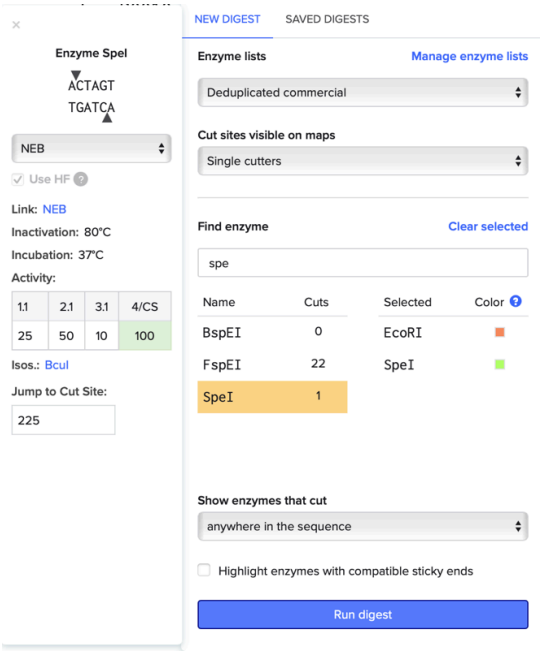
🔧Create primers for this part too. You should follow the same procedure as shown before. The "*Design*" table should look like this:

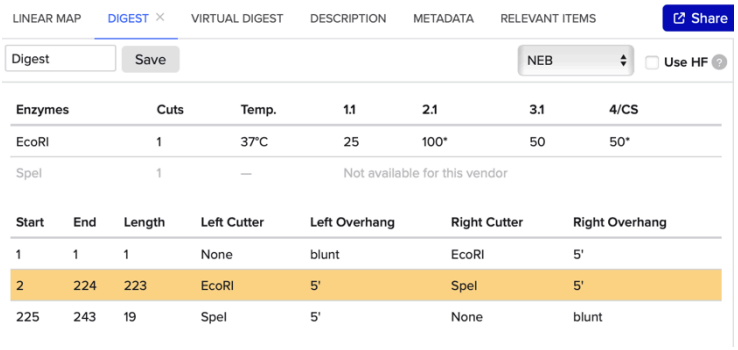


🔧 Save the primer pair and "*Create PCR product*".

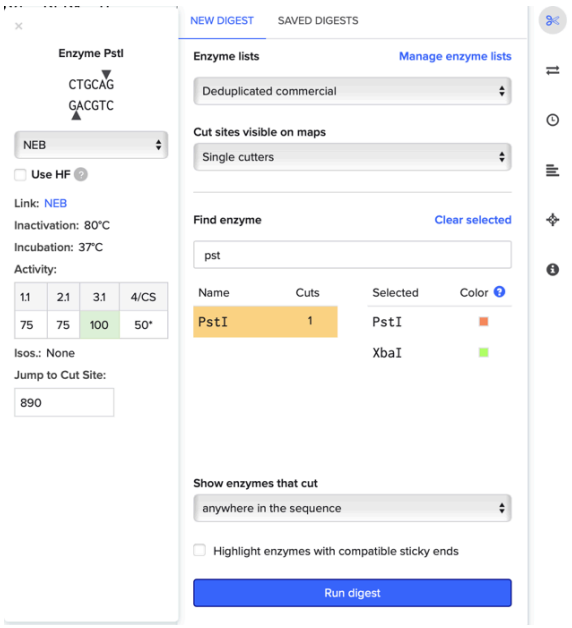
Since Benchling doesn't offer a built-in option for BioBrick assembly, perform "Digest and Ligate" assembly.

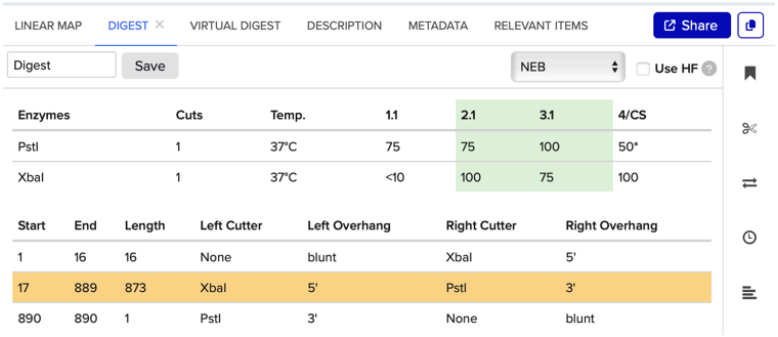
🔧 "*Digest*" the promoter's sequence from the PCR product with EcoRI and SpeI, and save.



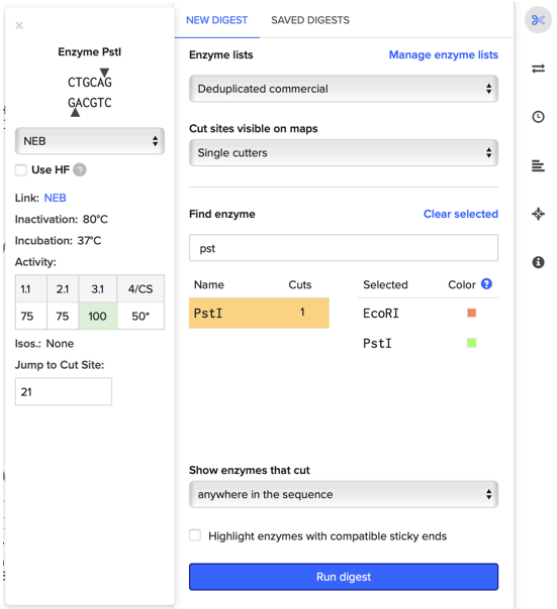



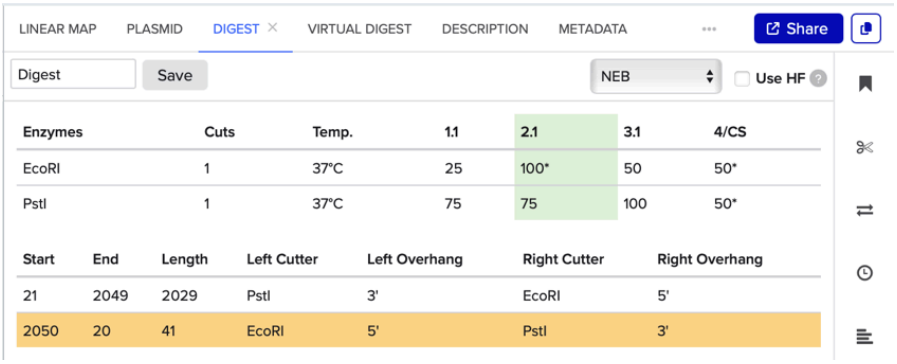

🔧 "*Digest*" the **subpart (RBS_mRFP1_dterminator)** PCR product with **XbaI and PstI**, and save.



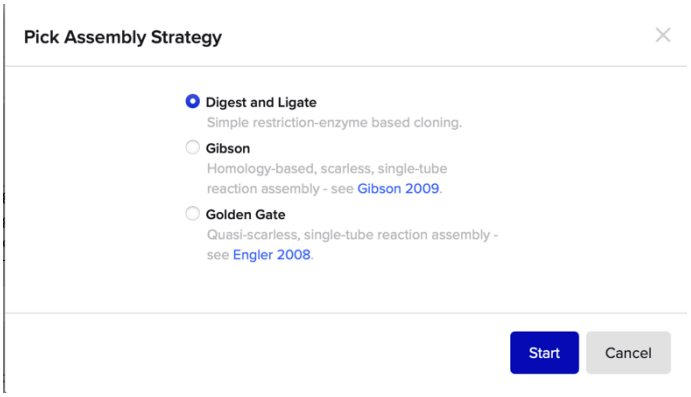




"*Digest*" the the pSB1C3 vector with EcoRI and PstI, and save.





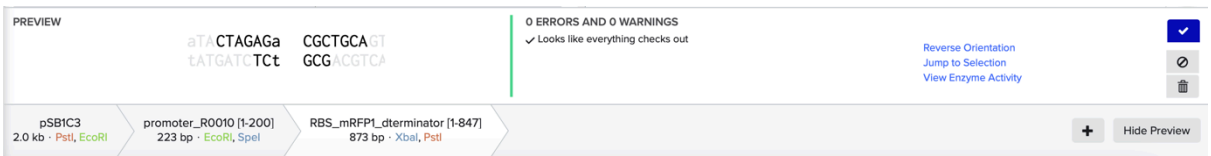

🔧Go to your digested backbone sequence click on "*ASSEMBLY*" > "*Assembly wizard*" and start "*Digest and Ligate*" assembly.


🔧As before, begin by setting the fragments you want to ligate, making sure to choose the correct fragment resulting from the digestion.

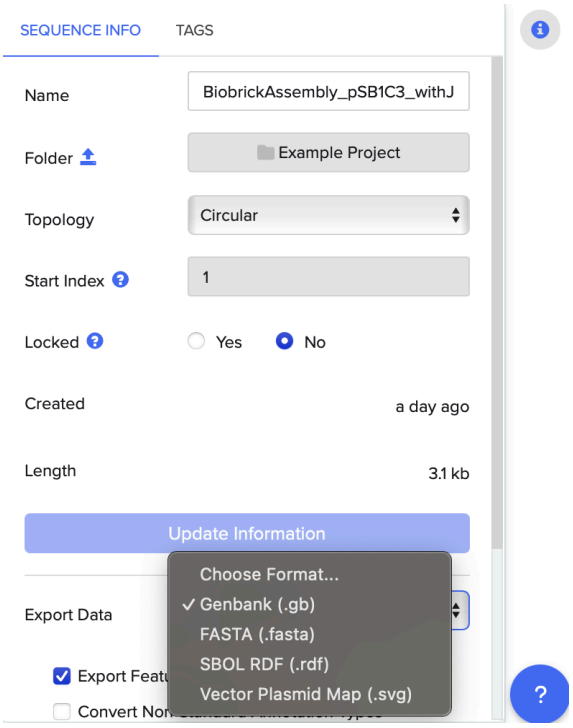
1) Use pSB1C3 as backbone.
2) For insert, select the promoter, again ensuring you've chosen the appropriate fragment generated by the restriction digest.
3) Add another insert tab by clicking on the "+" sign. The second insert will be the subparts sequence.

Something like this:



Lastly, click on "*Done*" and your BioBrick Assembly is completed. 🥳


🔎To export the construct, click on the right tool bar > "*Information*" and choose the preferable format.




With primers and plasmid design in place, you're all set to move forward with cloning and expression—let the experiments begin! 🥳