Protocol for BioBrick tutorial 2017

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Introduction

The goal of this exercise to get familiar with the 3A assembly method. We will do this by creating a new BioBrick, which will make transformants express a purple-blue chromoprotein that is visible with the naked eye. To obtain this new BioBrick we will assemble parts from 3 different BioBricks:

1. A constitutive promoter (BBa_K1330002)
2. A reporter BioBrick contain a strong RBS and amilCP CDS (BBa_K592025)
3. The pSB1C3 plasmid backbone (destination plasmid) from a RFP coding device (BBa_J04450).

We will use 3A assembly with a little twist, since all the parts that are assembled are in the same backbone (pSB1C3). The twist is the an extra enzyme which is added in the digestion step to reduce the chance of religation of the used parts.

The exercise consist of 3 parts today:

1. Digestion
2. Ligation
3. Transformation

And tomorrow we will check if we successfully created the new amilCP expressing BioBrick.

1. Digestion

Purpose:
The purpose of the digestion is to digest three plasmids: constitutive promoter plasmid, amilCP plasmid and the destination plasmid. Each digestion is done with a different set of enzymes. The promoter and the amilCP plasmid is digested with three restriction enzymes to reduce the chance of religation, while the destination plasmid is only digested with two enzymes.

Materials

- Restriction enzymes - located at the common table in the lab
- 2.1 buffer (NEBuffer 2.1) - in your icebox
- DNA - all in your icebox
    - Constitutive promoter plasmid: **Tube D9** (BBa_K1330002)
        - Concentration: 54 ng/ul
    - amilCP plasmid **Tube D12** (BBa_K592025)
        - Concentration: 35 ng/ul
    - Destination plasmid: **Tube A1** (BBa_J04450)
        - Concentration: 28 ng/ul
- 4 eppendorf tubes
- H2O

Procedure

Calculations

- Each digestion reaction should contain **100 ng** plasmid DNA. What volume of the 3 plasmids should you use? Write your answer in the tables below.
    - *Stock concentrations of plasmid can be found in materials*
- Each reaction should have a **total volume** of **10 ul**, calculate the amount of water needed for each reaction. Write your answer in the tables below.

Digestion 1	
Promoter plasmid (D9)	
EcoRI-HF	0.2 ul
SpeI	0.2 ul
PstI	0.2
10x NEBuffer 2.1	1 ul
H2O	

Digestion 2	
amilCP plasmid (D12)	
EcoRI-HF	0.2 ul
XbaI	0.2 ul
PstI	0.2 ul
10x NEBuffer 2.1	1 ul
H2O	

Digestion 3	
Destination plasmid (A1)	
EcoRI-HF	0.2 ul
PstI	0.2
10x NEBuffer 2.1	1 ul
H2O	

1. Mark 3 eppendorf tubes with appropriate names
2. Make the 3 digestion reactions as stated in the tables above on ice the 3 eppendorf tubes.
    a. NB add enzymes last!
    b. Enzymes can be found on the common table.
    c. IMPORTANT when pipetting enzymes!
        i. it is very important to keep the enzyme tubes cold at all time
        ii. Only stick very tip of the pipet tip into liquid when taking enzyme. Since extra liquid will stick to the outside of the pipet tip, resulting in a over use of enzyme.
3. Incubate the tube at 37 °C for 60 min
    a. *This is done in one of the 37 °C incubators under the common table*
4. Heat inactivate the restriction enzymes by heating the tubes to 80 °C for 20 min
    a. Use common heating block located at the common table.
5. Put the tubes on ice until they are used in the next exercise.

2. Ligation

Purpose

Now that we have digested the 3 different plasmids we want to recombine them to make the desired BioBrick (which expresses the blue color protein - amilCP). To make this biobrick we will need to mix all 3 digestion reaction in a ligation reaction that then ligates the desired fragment into our new BioBrick. Unfortunately, our new BioBrick is not the only plasmid that will be assembled in this reaction, we will have a total of five different outcomes:

- The new amilCP expressing BioBrick
- Reassembly of one of the 3 digested BioBrick
- Assembly of the two small fragments from the digest between: EcoRI-Xbal and SpeI-PstI

Later we will discuss how to differentiate between the different outcomes.

Materials:

- Promoter plasmid digestion reaction from earlier exercise
- amilCP plasmid digestion reaction from earlier exercise
- Destination plasmid digestion reaction from earlier exercise
- 10X T4 DNA Ligase Buffer (**Tube: ligase Buffer**) - in your icebox
- T4 DNA Ligase - can be found on the common table
- H2O

Procedure

1. Make one ligation reaction in an eppendorf tube containing the following:
    - *Again add enzyme (T4 DNA ligase) last and be careful when pipetting enzyme as when pipetting the restriction enzymes earlier.*

Promoter plasmid **digestion reaction**	2 ul
amilCP plasmid **digestion reaction**	2 ul
Destination plasmid **digestion reaction**	2 ul
10X T4 DNA Ligase Buffer (**Tube: ligase buffer**)	2 ul
H2O	11 ul
T4 DNA Ligase	1 ul
Total	20 ul

2. Incubate for 60 min at 37 °C

3. Heat inactivate the ligase by heating the reaction to 80 °C for 20 min
    - *Use the heating block on the common table*
4. Store the reaction on ice until they are used for transformation (Next step)

3. Transformation

Purpose

Finally, we are ready to transform. As discussed earlier the ligation reaction potentially contains 5 different ligated plasmids, to recap:

- The new amilCP expressing BioBrick
- Reassembly of one of the 3 digested BioBrick
- Assembly of the two small fragments from the digest between EcoRI-Xbal and SpeI-PstI into the destination plasmid

When transformed these will all result in a chloramphenicol (Cam) resistant strain, thus when growing the transformants on LB + Cam plates it will result in a mixture of transformants from all 5 plasmids. We are only looking for the new amilCP expressing BioBrick. Fortunately, these transformants will be blue colored, since they are expressing amilCP. Transformation of the 4 other plasmids will result in non-colored colonies (white) for transformants containing: reassembly of the promoter and the amilCP plasmids and assembly of the two small fragments between EcoRI. Xbal and SpeI-PstI into the destination plasmid. Reassembly of the destination plasmid will result in transformation of the A1 plasmid, which is the BioBrick BBa_J04450. It contain a Cam resistance gene and a strong RFP expression cassette, thus will result in red colonies.

In this exercise you will carry out 3 transformations:

1. Ligation mix
2. Positive control
3. Negative control

The ligation mix was made in the last exercise. For positive control we will use **non-digested** A1 plasmid and for negative control H2O will be used.

Materials

- Competent cells
    - NB keep them cold at all time until else is stated in the protocol.
    - Tube contains 100 ul
- Ligation reaction from the last exercise
- A1 plasmid (**NOT digested**)
- 3 eppendorf tubes
- Plates
    - 3 pcs. 6y Cam
    - 1 pcs. LB
- Liquid LB medie

Procedure

1. Take the LB media out of the ice and put on the bench to let it heat up to room temperature while you are doing the transformation.
2. Name 3 eppendorf tube one for each transformation and let them cool on ice
3. Gently divide 25 uL competent cells into the 3 eppendorf tubes keep them on ice
4. Add DNA to the competent cells
    a. 2 ul ligation mix
    b. 1 ul A1 plasmid for positive control
    c. 1 ul H2O for negative control
5. Incubate the 3 transformations on ice for 10 - 30 min
6. Heat shock at 45 °C for 1 min (yes, 45 °C)
    a. *Use the heating block common table*
7. Incubate 5 min on ice
8. Add 200 ul room temperature LB to the transformation tubes
9. Incubate at 37 °C for 1 - 2 hour
10. Name the plates with appropriate names and let them dry in 37 °C incubator
11. Spread 100 uL of each transformation on 6y Cam plates
12. Spread additional 100 ul of negative control transformation on a LB plate
13. Put the plates in a plastic bag and incubate plates at 37 °C overnight

Clean up

1. Put the content back into the redbox
2. Check that everything is there
3. Dispose all used tubes in GMO trash bins
4. Empty the ice bucket in the sink
5. Put trash in appropriate trash bins
6. Wipe your workstation with ethanol