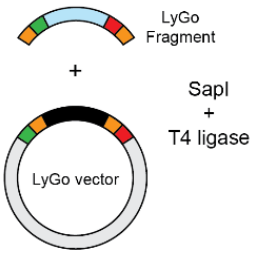
Oct 27, 2020

LyGo cloning



In 1 collection

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Protocol status: Working
We use this protocol routinely and it works for us

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Abstract

Lytic polysaccharide monooxygenses (LPMOs) are enzymes that play a critical role in breaking the chemical bonds of the most abundant polymers found in recalcitrant biomass, such as cellulose and chitin. LyGo cloning (Lytic Polysaccharide Monooxygenase Golden Gate cloning) is a versatile heterologous expression platform for LPMOs, which is compatible with cloning both PCR products and synthetic gene fragments with a simple 15-minute assembly step. The method allows for parallel construction of multiple expression vectors, enabling exploration of several expression strategies. The open-source LyGo collection consists of vectors for some of the most relevant model organisms used for protein production in both academic and industrial settings.

This protocol describes how to clone LyGo fragments into LyGo vectors.

Guidelines

The expression vectors used in this protocol have to be compatible with LyGo cloning. Making vectors compatible is done by inserting the LyGo cassette between the N-terminal histidine and the stop codon of the CDS. The LyGo fragments consist of SapI recognition sites flanking the LPMO of interest. Furthermore, we include a few basepairs of junk DNA flanking the SapI sites. The N-terminal extension (including the SapI site) is: "5'-ATCGTCTCTGCTCTTCG-3'" and the C-terminal extension is "5'-CGAAGAGCGAAGCAGAAC-3'". The addition of these sequences potentially facilitates recognition and allows for standardized primers to be used for amplification of all LyGo fragments.

Materials

MATERIALS

- T4 Ligase
- FastDigest Buffer (10X) **Thermo Fisher Catalog #**B64
- T4 DNA Ligase Buffer (10X) **Thermo Fisher Catalog #**B69
- FastDigest LguI **Thermo Fisher Catalog #**FD1934
- LyGo vector
- LyGo fragment

Safety warnings

This protocol describes the construction of GMO classified organisms. Make sure that the local GMO and safety legislations are respected.

Before start

Construct or acquire a LyGo vector, and order or amplify a LyGo fragment with the required N-terminal and C-terminal extensions (see Guidelines). Have competent *E. coli* cells on-hand.

Mix reactions

1 Prepare a reaction mix in the following proportions:

	Component	Volume (µL)
	LyGo vector	1
	LyGo fragment	3
	FastDigest SapI	0.5
	10x T4 ligase	1
	10x FastDigest buffer	1
	10x T4 buffer	1
	MQ	2.5
	Total	10

Note

The exact volume of LyGo vector and LyGo fragment depends on their kb sizes and DNA concentrations. The optimal molar ratio of vector:fragment is between 1:3 and 1:6, so dilutions/additions should be made accordingly. The NEB Ligation Calculator (https://nebiocalculator.neb.com/#!/ligation) is a useful tool to perform these calculations.

Reaction

2 Incubate the reactions for at least 00:15:00 at Room temperature .

3 The reactions are now ready to be transformed directly into competent *E. coli* cells, using established protocols e.g.

Protocol

NAME

E. coli Heat Shock Transformation

CREATED BY

Alex Rajewski

PREVIEW

Note

In our experience, it is helpful to transform all 10 µL of the reactions, although this might depend on the ligation and transformation efficiencies.

Note

Subsequent construct verification should also be performed (e.g. PCR, digestion and/or sequencing), before transforming the expression vectors into the expression host.

3.1 Thaw competent cells on ice. This takes about 10 minutes. Also thaw the plasmid to be transformed.

3.2 Warm SOC media to room temperature.

250 µL SOC media per reaction

Note

LB media can also be used. Anecdotally, we haven't seen a difference between LB and SOC.

3.3 Heat a dry block to 42ºC, a shaker-incubator to 37ºC, and a cabinet-style incubator to 37ºC.

3.4 Label a 1.5mL eppendorf tube for each transformation reaction with the construct name and place it on ice. Label an agar plate with the construct, date, your initials, and plate media type (with antibiotic, if applicable).

Expected result

Plate: pYPQ131A 13 March 2018 AR LB+Tet

3.5 Add plasmid to the labeled eppendorf tube on ice.

2 µL plasmid

3.6 Add thawed competent cells to the tube with the plasmid and gently swirl with the pipet tip to mix.

45 µL competent cells

Note

DO NOT vortex. Competent cells have very specific and fragile cell membranes that can be damaged by vortexing.

3.7 Incubate the mixture on ice for 30 minutes.

00:30:00 Ice

Note

This time is fungible. Longer is better, but as short as 5 minutes will also work.

3.8 Place the mixture in the dry block at 42ºC for 30 seconds

00:00:30 Dry Block

3.9 Incubate the mixture on ice for 2 minutes.

00:02:00 Ice

3.10 Add SOC to the mixture and shake in an incubator

01:00:00 Shake at 37º & 250 rpm

250 µL SOC per reaction

3.11 (Optional) Spin the bacterial broth for 30 seconds at 6000 rpm, removed 180µL of supernatant, and gently resuspend the pellet in the remaining media to concentrate.

00:00:30 spin at 6000 rpm

3.12 Immerse a plate spreader in 70% ethanol to sterilize and then burn off the excess alcohol.

3.13 Pipet at least 100µL of the broth onto the labeled agar plate and spread it evenly across the surface of the agar with the sterile spreader. Restilerize the spreader.

Note

A larger volume can be used, or you can make two plates with different volumes (e.g. 100 and 50µL) to prevent getting colonies that are too close together.

3.14 Place the prepared plate (lid-down) into a 37ºC incubator overnight (16 hours)

Note

The plates can also be incubated at room temperature for several days (the weekend).