Cloning using restriction enzymes

Protocoles

Fragment amplification via PCR.....p2

Insert digestion.....p4

Plasmid digestion.....p6

Ligation.....p7

Bl21(DE3) E. coli heat shock transformation.....p8

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Fragment amplification via PCR

Introduction

This is the protocol for fragment amplification with NEB® Hot Start Taq 2X Master Mix to get your insert ready for molecular cloning using restriction enzymes..

Material

- Reverse and Forward primers
- Template DNA (100-200ng/µL)
- NEB® Hot Start Taq 2X master mix
- Nuclease-free water
- Bucket of ice
- Promega Wizard® SV Gel and PCR Clean-Up System kit

Procedure

1- Add to a sterile thin-walled PCR tube on ice:

component	50 µL reaction
20 µM Forward Primer	1.5µL
20 µM Reverse Primer	1.5µL
Template DNA	2µL
NEB Hot Start Taq 2X master mix	25µL
Nuclease-free H20	20µL

Gently mix the reaction and perform a quick spin to collect all liquid to the bottom of the tube.

2- Transfer PCR tubes to a PCR machine and run the following thermocycling protocol:

Step	Temperature (°C)	time
Initial denaturation	95°C	30 secondes
35 cycles	95 °C 55°C 68 °C	30 secondes 30 secondes 50 secondes
Final extension	68 °C	5 minutes

Hold	10°C	infinite

3- Perform a PCR clean-up using the Promega Wizard® SV Gel and PCR Clean-Up System.
Add 50µL of Membrane Binding Solution to your PCR amplification product and follow the 5.A. DNA Purification by Centrifugation part of the protocol given in the kit.

4- Nanodrop your sample to make sure of the purity of your sample and to know the concentration. Your insert can now be digested.

Insert digestion

Introduction

This is the protocol for fragment digestion to get ready for molecular cloning with restriction enzymes.

Material

- Ice bucket
- DNA insert
- NEB® rCutSmart Buffer
- NEB® 3.1 Buffer
- NEB® XhoI
- NEB® XhoI
- NEB® XhoI
- NEB® EcorI
- Nuclease-free water
- TAE 1X
- Low Melting Agarose powder
- SYBR™ Safe DNA Gel Stain
- Promega Wizard® SV Gel and PCR Clean-Up System

Procedure

1- Add to a sterile 1.5mL tube on ice:

Component	72 µL reaction (GFP)	60 µL reaction (variable part of the toehold switch)
Insert	60 µL	50 µL
Buffer	rCutSmart = 7.2 µL	3.1 buffer = 6 µL
Restriction enzymes	XhoI = 0.6 µL EcoRI = 1.2 µL	BglII = 1 µL BamHI = 1.5 µL
Nuclease-free H20	3 µL	1.5 µL

2- Incubate 12 hours at 37°C.

3- Purify your digested insert via gel migration. Prepare a 1.25% low melting gel, run your sample for 45 minutes et 25V.

4- Under UV, cut the insert band. Weight your gel slice using a precision scale.

5- Perform a PCR clean-up using the Promega Wizard® SV Gel and PCR Clean-Up System.
Add x µL of Membrane Binding Solution per x mg of gel to your gel slices. Heat your sample at 50°C for at least 10 minutes and follow the 5.A. DNA Purification by Centrifugation part of the protocol given in the Promega Wizard® SV Gel and PCR Clean-Up System kit.

Plasmid digestion

Introduction

This is the protocol for plasmid pET14d(+) digestion using restriction enzymes for molecular cloning.

Material

- Ice bucket
- DNA insert (~100-200 ng/µL)
- NEB® rCutSmart Buffer
- NEB® 3.1 Buffer
- NEB® XhoI
- NEB® XhoI
- NEB® XhoI
- NEB® EcorI
- Nuclease-free water
- TAE 1X
- Low Melting Agarose powder
- SYBR™ Safe DNA Gel Stain
- Promega Wizard® SV Gel and PCR Clean-Up System

Procedure

1- Add to a sterile 1.5mL tube on ice:

component	60 µL reaction (cloning with GFP)	60 µL reaction (cloning with variable part of the toehold switch)
DNA plasmid	50 µL	50 µL
Buffer	rCutSmart = 6 µL	3.1 Buffer = 6 µL
Restriction enzymes	EcorI = 1.5 µL XhoI = 1 µL	BglII = 1 µL BamHI = 1.5 µL
Nuclease-free H2O	1.5 µL	1.5 µL

2- Incubate 12 hours at 37°C.

4- Under UV, cut the upper band (open plasmid). Weight your gel slice using a precision scale.

5- Perform a PCR clean-up using the Promega Wizard® SV Gel and PCR Clean-Up System.

Add x µL of Membrane Binding Solution per x mg of gel to your gel slices. Heat your sample at 50°C for at least 10 minutes and follow the 5.A. DNA Purification by

Centrifugation part of the protocol given in the Promega Wizard® SV Gel and PCR Clean-Up System kit.

Ligation

Introduction

This is the protocol for the ligation of the digested plasmid with the digested insert using NEB® T4 DNA ligase.

Material

- NEB® T4 DNA ligase
- NEB® 1X T4 DNA Ligase Reaction Buffer
- Digested plasmid (20ng/µL)
- Digested insert (40ng/µL)

Procedure

1- Add to a sterile 1.5mL tube:

Component	20 µL reaction
Digested plasmid	4 µL
Digested insert	5 µL
NEB® 1X T4 DNA Ligase Reaction Buffer	10 µL
NEB® T4 DNA ligase	1 µL

2- Leave at room temperature for 10 minutes

3- Store at -20°C

Bl21(DE3) E. coli heat shock transformation

Introduction

This is the protocol for the heat shock transformation of Bl21(DE3) E. coli with your plasmid assembled using the golden gate method previously detailed.

Material

- Bucket of ice
- Bl21(DE3) E. coli (120 µL)
- Plasmid DNA (~100 ng/µL)
- Terrific Broth (TB) media
- Round-bottom tube
- Petri dishes (LB + antibiotic of your plasmid resistance cassette)

Procedure

1- Store all your material on ice under the hood. Bacteria should thaw slowly (~10 minutes).

At the same time, prepare aliquots of TB and incubate them at 37°C. Turn on your water bath at 42°C.

2- Add 2 µL of the plasmid at the bottom of the P20 pipette

3- Add 120 µL of the bacteria, gently tap the tube to mix.

4- Leave to incubate on ice for 30 minutes. Gently tap the tube every 10 minutes.

5- Heat shock 30 seconds in a water bath at 42°C

6- Leave 2 minutes on ice

7- Add 150 µL of the TB media

8- Incubate on the shaker 220 RPM, 37°C for 90 minutes

9- Plate 120 µL on your petri dish

10- Incubate at 37°C overnight