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pGEM-T Cloning

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Protocol status: Working
We use this protocol and it's working

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Abstract

Protocol for cloning in pGEM-T vector

Materials

Reagents:

- pGEM-T Easy Kit (Promega, A1360)
- Competent E. coli cells (NEB, C3040H)
- LB plates with necessary antibiotic

Before start

PRINCIPLES BEHIND THE PROCEDURE MUST BE UNDERSTOOD. PLEASE CONSULT WITH EXPERIENCED LAB MEMBER THE FIRST TIME YOU USE THIS PROCEDURE. UPDATE AS A GENERAL PROCEDURE AS NECESSARY BUT DO NOT MODIFY WITH SPECIFICS TO YOUR PROJECT, INSTEAD DOWNLOAD AND PASTE A MODIFIED COPY IN YOUR NOTEBOOK. Updated by MH, April 2023

Insert Generation

1h 2m

1 1. Perform PCR to generate bands of interest in total volume of 50 µL . 1h 2m

	Volume (uL)
NF H20	37
Fwd Primer (10uM)	1
Rev Primer (10uM)	1
dNTPs	1
Plasmid (20ng)	1
Buffer	5
MgCl2	3
Taq Polymerase	1

2. Make 1% low melt-agarose gel.

a) Mix 1 g of Agar with 100 mL of TAE Buffer.

b) Microwave to boil agarose and let cool until you can touch bottle, but gel is not solid.

c) Add 1.5 µL of EtBr to agarose and pour into DNA gel mold with 10 well comb.

d) Let gel solidify.

3. Add 10 µL of loading buffer to PCR reaction then Load 60 µL of reaction into well of gel

4. Run gel for 00:45:00 at 120V.

5. Visualize band with UV light and cut out section of gel with band with new razor blade and place in 1.5 mL tube.

6. Purify Band from gel with Promega Wizard SV Gel and PCR Purification Kit (A9282)

a) https://www.promega.com/products/nucleic-acid-extraction/clean-up-and-concentration/wizard-sv-gel-and-pcr-clean-up-system/?catNum=A9281

b) Weigh DNA gel fragment and add 10 µL of Membrane Binding Solution per 10 mg of gel slice.

c) Incubate mixture at 65 °C for 00:10:00 or until gel is completely melted.

d) Add melted gel mixture to SV minicolumn in Collection Tube and incubate at room temperature for 00:01:00 .

e) Centrifuge at max speed for 00:01:00 . Discard flowthrough and reinsert column into tube.

f) Add 700 µL of Membrane Wash Solution. Centrifuge at max speed for 00:01:00 . Discard flowthrough and reinsert column into tube.

g) Add 500 µL of membrane wash solution. Centrifuge at max speed for 00:01:00. Discard flowthrough and reinsert column into tube.

h) Spin empty column for 00:01:00 at max speed to remove excess ethanol.

i) Transfer column to labelled 1.5 mL tube and add 35 µL of NF H_20. Incubate for 00:01:00 at room temperature.

j) Centrifuge at max speed for 00:01:00 .

k) Keep eluate and store at -20 °C .

pGEM Ligation Protocol

2h 24m

2

1. Set up Ligation Reaction in 0.2 mL PCR tube

2h 24m

Component	Volume (uL)
2x Rapid Ligation Buffer	5
pGEM-T Vector (50ng)	1
PCR Product	X*
H20	3-X
T4 DNA Ligase	1

a) *Volume of PCR product should be 2 insert:1 pGEM vector molar ratio

b) For example:

	A	B	C	D	E	F	G
	Component	Length of DNA (bp)	Molar ratio	ng of DNA	Volume of 50ng/ul solution		
	pGEM-T Vector	3015	1	50	1 ul		
	PCR Fragment	500	2	16.58	0.33 ul		
	H20				2.67 ul		

2. Incubate for 01:00:00 at room temperature

3. Transform Product into E. coli

a) Add 2 µL of product to 50 µL of TOP10 cells and mix up and down slowly with a pipette.

b) Incubate for 00:20:00 on ice.

c) Heat shock bacteria in 42 °C waterbath for 00:01:00 .

d) Incubate on Ice for 00:03:00 .

e) Add 100 µL of SOC media and shake in warm room for 01:00:00 .

f) Plate bacteria onto LB-Antibiotic/Xgal Plate and incubate overnight at 37 °C

i. Add 40 µL of Xgal to plate and spread with plate spreader. Let dry

ii. Then plate 100 µL of bacteria onto plate.

iii. Spread cells with plate spreader to get individual colonies

g) Pick white colonies for miniprep growth and sequencing.

Protocol references

pGEM-T Cloning:
https://www.promega.com/products/pcr/pcr-cloning/pgem-t-easy-vector-systems/?catNum=A1360