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PCR Fragment Generation (for inserts)

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

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Abstract

Protocol to generate inserts by PCR for cloning

Materials

Reagents:

- Taq Polymerase (Thermofisher, 11304011)
- dNTPs
- PCR Primers
- Plasmid
- Nuclease Free H20
- Wizard SV Gel and PCR Purification Kit (Promega, A9282)
- DpnI (NEB, R0176L)
- 10x Cutsmart Buffer

PCR Product (Insert generation)

47m

1 47m

1. Set up PCR Reaction:

	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. Run PCR Conditions:

a) 95 °C , 00:02:00

b) 95 °C , 00:00:30

c) 60 °C , 00:00:30

d) 72 °C , 00:02:00

e) Repeat step B-D 35 times

f) 72 °C , 00:10:00

g) 4 °C , hold

*Annealing Temp and Extension time will differ based on PCR design

3. After reaction, take 5 µL of product and add 1 µL of gel loading buffer and run on 1% agarose gel to make sure there is a correct PCR product.

4. Add 5 µL of Cutsmart buffer and 1 µL of DpnI to the reaction and incubate at 37 °C for 00:15:00 . This is remove the plasmid template from mixture.

5. 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 . Keep eluate and store at -20 °C .