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Protocol for Gibson Assembly (PrimeStar + In-Fusion) V.1

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

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Abstract

Procedure for cloning using Gibson Assembly using PrimeStar and In-Fusion kits

Materials

- Plasmids
- DNA template
- Restriction Enzymes (New England Biolabs)
- 10x Cutsmart Buffer (New England Biolabs)
- PrimeSTAR Max DNA Polymerase (TaKaRa, #R045A)
- Agarose
- EtBr
- NEB Monarch DNA Gel Extraction kit (NEB #T1020)
- In-Fusion Snap Assembly Master Mix (TaKaRa, #638947)
- TOP10 cells

Vector digestion

30m

1 1. Double digest Vector with New England Biolabs Restriction Enzymes: 80uL reaction 30m

	A	B
	Component	Volume (uL)
	DNA plasmid	X ul for 4 ug of plasmid
	10x Cutsmart Buffer	8 uL
	Enzyme 1	4 uL
	Enzyme 2	4 uL
	NucleaseFree H2O	64-X uL

2. Incubate for 00:30:00 at 37 °C .

Notes:

- Not all two enzymes can be used together in a double digestion. Think about it ahead and you can test your two enzymes here: https://nebcloner.neb.com/#!/redigest.
- Enzymes are different, but most can be digested in CutSmart buffer at 37°C within 30 minutes. This is a general protocol. Please check the best digestion conditions for your enzymes in this web：https://nebcloner.neb.com/#!/redigest. It will provide the temperature, buffer, and incubation time specific to the two enzymes you are using.

Insert fragment PCR with PrimeSTAR Max DNA polymerase

10m 30s

2 Set up PCR Reaction (50uL): 10m 30s

	A	B
	PrimeStar Mix (2X)	25 uL
	F primer	1 uL
	R Primer	1 uL
	DNA template	20-50 ng
	H20	up to 50uL

Run PCR Conditions:

a) 98 °C 00:00:10

b) 98 °C 00:00:10

c) 55 °C 00:00:10

d) 72 °C X seconds

e) Repeat step b-d 30 times

f) 72 °C 00:10:00

g) 4 °C hold

Note:

- You might to increase the amount of DNA template if your template is cDNA.
- X depends on the length of your PCR product, 15s per kb.
- Step C: Using 55°C has worked perfectly for me for over 10 years with PrimeSTAR DNA polymerase. However, the success also depends on how your primers are designed.

Run DNA gel

40m

3 40m

1. Add 6x loadig Dye to digestion and PCR reaction and vortex briefly to mix.
2. Make 1% 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 2 µL of EtBr to agarose and pour into DNA gel mold with 10 well comb.

d) Let gel solidify.

3. Load 100 µL of reaction into well of gel
4. Run gel for 00:40:00 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.

Purification DNA from gel with Monarch DNA Gel Extraction kit

4

1. Weigh the gel slice.
2. Add 4 volumes of Monarch Gel Dissolving Buffer to the tube with the gel slice (e.g., 400 µl buffer per 100 mg agarose). If the gel slice is >150 mg, consider reducing the amount of Gel Dissolving Buffer to 3 or 3.5 volumes to minimize the guanidine salt present in the workflow.
3. Incubate the sample between 37–55°C (typically 50°C), inverting periodically until the gel slice is completely dissolved (generally 5–10 minutes).
4. Insert the column into collection tube and load sample onto the column. Spin for 1 minute, then discard flow-through.

5. Re-insert column into collection tube. Add 200 µl DNA Wash Buffer and spin for 1 minute. Discarding flow-through is optional.
6. Repeat wash (Step 5).
7. Re-spin the column and tubes.
8. Transfer column to a clean 1.5 ml microfuge tube. Use care to ensure that the tip of the column has not come into contact with the flow-through. If in doubt, re-spin for 1 minute before placing into clean microfuge tube.
9. Add ≥ 6 µl of DNA Elution Buffer to the center of the matrix. Wait for 1 minute, and spin for 1 minute to elute DNA.

Notes:

- *Step2: If the volume of the dissolved sample exceeds 800 µl, the loading of the sample onto the column should be performed in multiple rounds to not exceed the volume constraints of the spin column.*
- *Step3: For DNA fragments > 8 kb, an additional 1.5 volumes of water should be added after the slice is dissolved to mitigate the tighter binding of larger pieces of DNA (e.g., 100 mg gel slice: 400 µl Gel Dissolving Buffer: 150 µl water). Failure to dissolve all the agarose will decrease the recovery yield due to incomplete extraction of the DNA and potential clogging of the column by particles of agarose.*
- For PCR products, if you have only one specific band, you can skip the gel running step and purify DNA directly from the PCR products.

Gibson Assembly with TAKARA In-Fusion

1h 48m 30s

5

1h 48m 30s

1. Calculate the molar ratios of Vector and PCR product used
   a) https://nebiocalculator.neb.com/#!/ligation
   b) I usually use 1:1 or 1:2 ratio, and the vector amount I use is 50-60ng

Reaction component	Cloning reaction	Negative control reaction	Positive control reaction
Purified PCR fragment	10–200 ng	–	2 µl of 2 kb control insert
Linearized vector	50–200 ng	1 µl	1 µl of pUC19 control vector
5X In-Fusion Snap Assembly Master Mix	2 µl	2 µl	2 µl
Deionized Water	to 10 µl	to 10 µl	to 10 µl

2. Mix Mater Mix, vector, insert and H20 together.
3. Incubate for 00:15:00 at 50 °C .
4. Transform Product into E. coli

a) Add 2 µL of product to 50 µL of TOP10 cells.

b) Incubate for 00:30:00 on ice.

c) Heat shock bacteria in 42 °C waterbath for 00:00:30 .

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

e) Add 500 µL of SOC media and shake in warm room for 01:00:00 .

f) Spin down the bacteria and discard the supernatant, leaving only 50-100 µL. Then, resuspend the bacteria in the remaining volume.

g) Plate bacteria onto LB-Antibiotic Plate. And incubate overnight in 37 °C warm room.

Note:

- Please read the In-Fusion Snap Assembly User Manual before your start.
- This kit allows the insertion of more than one fragment; refer to the User Manual for detailed instructions.
- Incubation time at 50°C could be extend to 30min, but longer time may not that helpful, think about other things if you didn't get clones.
- The incubation time at 50°C can be extended to 30 minutes if you have more than one insertion or the plasmids is big, but longer durations may not significantly improve results. If you do not get clones, consider troubleshooting other things of your experiment.

Protocol references

PrimeSTAR Max DNA Polymerase protocol:
https://www.takarabio.com/documents/User%20Manual/R045Q/R045Q_e.v1108Da.pdf?srsltid=AfmBOop6Kkf6MyrY101Gl9kzHGl3tWCIt48RmNlmzJrUF1vp7YRCu4kW
Monarch DNA Gel Extraction Kit Protocol
https://www.neb.com/en-us/protocols/2015/11/23/monarch-dna-gel-extraction-kit-protocol-t1020?srsltid=AfmBOopHTsWwc4YwVyiO2Plq7zqd6dZL-3cMFxWIUQeZdJGdAVZ68Ltz
In-Fusion Snap Assembly User Manual
https://www.takarabio.com/documents/User%20Manual/In/In-Fusion%20Snap%20Assembly%20User%20Manual.pdf