Protocol of Enzymatic Digestion (EcoR I, Xba I, Spe I, Pst I) Takara™

General Reaction Mixture:

Restriction enzyme	1 μl
10 x Buffer	5 μl
DNA	1 μl
Steriled Water	Up to 50 μl

Reaction temperature: 37℃
Note: 2 types of 10 x Buffer are used. 10 x M Buffer is used for Xba I and Spe I, 10 x H Buffer is used for EcoR I and Pst I.
Reaction Time: Generally, 5min reaction under 37oC is enough to digest target DNA completely. If it doesn't work well for special substrate DNA, the reaction time can be extended to 1h.

Protocol of Ligation T4 DNA Ligase

ThermoTM #EL0014

Linear Vector DNA	20-100 ng
Insert DNA	1:1 to 5:1 molar ratio over vector
10X T4 Ligase Buffer	2 μl
T4 DNA Ligase	1 Weiss U
Water, nuclease-free	To 20 μl
Total volume	20 μl

1. Prepare the following reaction mixture:
2. Incubate 10 min at 22℃.
3. Use up to 5 μL of the mixture for transformation of 50 μL of chemically competent cells or 1-2 μL per 50 μL of electrocompetent cells.

Gibson Assembling

1. We gain the Gibson Cloning Master Mix from Luo's lab, and it consists of three different enzymes:

1) T5 Exonuclease - creates single-strand DNA 3' overhangs by chewing back from the DNA 5' end. Complementary DNA fragments can subsequently anneal to each other.
2) Phusion DNA Polymerase - incorporates nucleotides to "fill in" the gaps in the annealed DNA fragments.
3) Taq DNA Ligase - covalently joins the annealed complementary DNA fragments, removing any nicks and creating a contiguous DNA fragment.

Gibson Cloning Master Mix	5 μl
Insert DNA	1:1 to 5:1 molar ratio over vector
Water, nuclease-free	To 10 μl

2. Preparing the following reaction mixture:
3. Incubate the mix for 30 mins at 50°C or follow manufacturer's instructions.
4. Transform the DNA into bacteria and screen for the correct plasmid product by Restriction Digest.
5. Sequence the important regions of the final plasmid

PCR

KOD Neo Plus

To prepare several parallel reactions and to minimize the possibility of pipetting errors, prepare a PCR master mix by mixing water, buffer, dNTPs, primers and template DNA. KOD Neo Plus DNA polymerase should be the last component added. Prepare sufficient master mix for the number of reactions plus one extra to allow for pipetting error.

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Place a thin-walled PCR tube on ice and add the following components for each 50 µl reaction: variable
3. Gently vortex the samples and spin down.

10X Buffer	5 µl
MgCl2	3 µl
2mM dNTP	5 µl
Primer 1	1.5µl
Primer 2	1.5µl
DNA template	< 1 µg
KOD Neo Puls(1U/µl)	1µl
Water, nuclease-free	up to 50 µl

4. If using a thermal cycler that does not use a heated lid, overlay the reaction mixture with 25 µl of mineral oil.
5. Perform PCR using the following thermal cycling conditions

	Temperature/℃	Time	Number of cycles
Initial denaturation	94	2 min	
Denaturation	98	10s	
Annealing	Tm	30s	25-45 cycles
Extension	68	30s/kb	
Final extension	68	3min	