Protocols

Preparation of TE Buffer
Materials
・Tris-HCl: 2ml
・EDTA: 0.5ml
・dH_2O: 197.5ml
Protocol
1. Mix the above materials.
2. Place the mixture in a medium bottle and cover with aluminum foil
3. Autoclave. Following autoclave, store it at room temperature.

Preparation of LB medium
Materials
・Tryptone: 10g
・Yeast Extract: 5g
・NaCl: 5g
・dH_2O: 1000ml
Protocol
1. Put the above materials into a beaker, cover with aluminum foil, and mix with a stirrer.
2. Autoclave. Following autoclave, store it in a medium bottle at 4 ℃.

Preparation of Ampicillin
Materials
・Ampicillin sodium salt: 0.5g
・Sterile water: 5ml
Protocol
1. Mix the above materials and vortex.
2. Filter the mixture through a syringe filter.
3. Dispense the amount to be used into a tube and store at -20 ℃. (If you use 50% ethanol instead of sterile water, you do not need to dispense.)

Preparation of LB Agar Plates
Materials
・Tryptone: 4g
・Yeast Extract: 2g
・NaCl: 2g
・Agar (not agarose): 6g
・Antibiotic (ampicillin): 400$\mu\ell$
・dH_2O: 400ml
Protocol
1. Put Tryptone, Yeast Extract, NaCl, and dH_2O into a beaker, cover with aluminum foil, and mix with a stirrer.
2. Add agar and autoclave.
3. Mix with a stirrer.
4. When the solution cools to about 60 ℃, add ampicillin and mix.
5. Transfer the mixture to a Petri dish on a clean bench. When the agar has set, stack the Petri dishes, cover them with plastic wrap, and store them at 4 ℃.

Resuspending gBlocks® Gene Fragments (IDT)

Materials
・DNA
・TE Buffer

Protocol
1. Prior to opening, centrifuge the tube at a minimum of 3000×g to ensure that the material is at the bottom of the tube.
2. Add TE to reach a final concentration of 10ng/$\mu\ell$.
3. Vortex briefly.
4. Incubate at 50 ℃ for 20 minutes.
5. Briefly vortex and centrifuge.

Gibson Assembly (NEB)
Materials
・DNA
・Gibson Assembly® Master Mix (E2611)
・Sterile water
Protocol
1. Determine the amount of each material according to the table below.

	2-3 Fragment Assembly	4-6 Fragment Assembly	Positive Control
Total Amount of Fragments	0.02-0.5 pmols (X $\mu\ell$)	0.2-1 pmols	10$\mu\ell$
Gibson Assembly Master Mix (2×)	10$\mu\ell$	10$\mu\ell$	10$\mu\ell$
dH_2O	10-X$\mu\ell$	10-X$\mu\ell$	0
Total Volume	20$\mu\ell$	20$\mu\ell$	20$\mu\ell$

2. Add sterile water, DNA, and Gibson Assembly Master Mix to the microtube in that order.
3. Incubate samples in a thermocycler at 50 ℃ for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled. Following incubation, store samples at -20 ℃.

DNA purification
Materials
・DNA
・Wizard SV® Gel and PCR Clean-up System (Promega)
Protocol
1. Add an equal volume of Membrane Binding Solution to DNA.
2. Insert SV Minicolumn into Collection Tube.
3. Transfer DNA to the Minicolumn assembly. Incubate at room temperature for 1 minute.
4. Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5. Add 700$\mu\ell$ Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
6. Repeat Step 5 with 500$\mu\ell$ Membrane Wash Solution. Centrifuge at 16,000 × g for 5 minutes.
7. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.
8. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
9. Add 50$\mu\ell$ of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × g for 1 minute.
10. Discard Minicolumn and store DNA at 4℃ or –20℃.

Dilution of TAE Buffer (10×)

Materials
・TAE Buffer(10×): 100ml
・MiliQ: 900ml
Protocol
1. Mix the above materials and store them in a medium bottle at room temperature.

Preparation of agarose gel
Materials
・TAE Buffer: 20ml
・Agarose: 0.2μℓ
・Midori Green: 1μℓ
Protocol
1. Put TAE Buffer and Agarose in a beaker and cover with plastic wrap.
2. Melt the Agarose in a microwave oven.
3. When the solution is cool enough not to be hot to the touch, add the Midori Green.
4. Pour the gel into the casting stand and set the comb.
5. When the gel hardens, cover with plastic wrap and store at 4 ℃.

Agarose Gel Electrophoresis
Materials
・DNA: 1μℓ of each
・DNA Ladder: 5μℓ
・Loading Dye (6×): 1μℓ×(n+1)
・Sterile water: 4μℓ×n
・TAE Buffer
(n: Number of samples)
Protocol
1. Add sterile water4μℓ, Loading Dye (6×)1μℓ, and DNA1μℓto the PCR tube.
2. Add DNA Ladder5μℓ and Loading Dye (6×)1μℓto the PCR tube.
3. Place the agarose gel into the gel box.
4. Fill the gel box with TAE Buffer until the gel is covered.
5. Place the samples.
6. Run the gel at 100V for 15-25 minutes.
7. Check the bands by UV irradiation.

Digestion
Materials
・Restriction enzyme
(EcoRⅠ-HF, HindⅢ)
・NEB Buffer r2.1 (10×)
・DNA
・Sterile water
Protocol
1. Determine the amount of each material according to the following table.

	Insert	Vector
①Restriction enzyme	0.4μℓ of each (fixed)	0.4μℓ of each (fixed)
②Buffer (10×)	1(~2μℓ)	1(~2μℓ)
③DNA	<1μg	<1μg
④Sterile water	Total-(①+②+③)	Total-(①+②+③)
Total Volume	10(~20)μℓ	10(~20)μℓ

2. Add sterile water, Buffer, restriction enzyme, and DNA to the microtube in that order.
3. Incubate samples in a thermocycler at 37 ℃ for 1 hour. Following incubation, store samples at -20 ℃.

Ligation

Materials

・Ligation Mix: 3μℓ
・Sterile water: 18μℓ
・DNA

Protocol

1. Prepare Mighty Mix by mixing Ligation Mix and sterile water.
2. Determine the amount of each material according to the table below.

				Negative Control
Vector	50ng (fixed)	Aμℓ	X mol	A μℓ
Insert		B μℓ	5X~10X mol	B μℓ (TE Buffer)
Mighty Mix		(A+B) μℓ		(A+B) μℓ

3. Add vector, insert, and Mighty Mix to the microtube. (For negative controls, add TE Buffer instead of Insert.)
4. Incubate samples in a thermocycler at 16 ℃ for 30 minutes. Following incubation, store samples at -20 ℃.

Transformation

Materials

・Plasmid: 6μℓ of each (Recombinant plasmid and Negative Control)
・Competent cells: 50μℓ ×n
・SOC medium (or LB medium) 1ml×n
・LB Agar Plates: 2 Plates ×n
(n: Number of samples)

Protocol

1. Add plasmid and competent cells to the microtubes.
2. Place the microtubes on ice for 30 minutes.
3. Heat shock at 42 ℃ for 45 seconds
4. Add SOC medium to the microtubes.
5. Place the microtubes at 37 ℃ for 30 minutes.
6. Spread 100μℓ onto the plate.
7. Centrifuge the rest of the mixture at 27°C, 3000 rpm for 1 minute.
8. Discard about 700μℓ of the supernatant.
9. Dissolve the precipitate by pipetting slowly and spread 200μℓ onto the plate.
10. Incubate at 37℃ for 14~18 hours. Following incubation, cover samples in plastic wrap and store them at 4 ℃.

Inoculation

Materials

・LB medium: 3ml×n
・Antibiotic (ampicillin): 3μℓ×n
・LB Agar Plates with colony
(n: Number of samples)

Protocol

1. Mix LB medium and ampicillin.
2. Dispense 3ml into each culture tube.
3. Pick the colonies from LB Agar Plates using a yellow tip and eject them into a culture

tube.
4. Incubate at 37 ℃ for 12~16 hours in a shaking incubator.

Miniprep (Promega)
Materials
・Bacterial culture 2.4ml×n
・PureYield™ Plasmid Miniprep System
(n: Number of samples)
Protocol
1. Centrifuge 1.2ml of bacterial culture for 30 seconds at maximum speed in a microcentrifuge.
2. Discard the supernatant.
3. Add an additional 1.2ml of bacterial culture to the same tube. Repeat Steps 1 and 2.
4. Add 600$\mu\ell$ of TE buffer or water to the cell pellet, and resuspend completely.
5. Add 100$\mu\ell$ of Cell Lysis Buffer (Blue), and mix by inverting the tube 6 times.
6. Add 350$\mu\ell$ of cold (4–8°C) Neutralization Solution, and mix thoroughly by inverting.
7. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.
8. Transfer the supernatant (~900$\mu\ell$) to a PureYield™ Minicolumn without disturbing the cell debris pellet.
9. Place the minicolumn into a Collection Tube, and centrifuge at maximum speed in a microcentrifuge for 15 seconds.
10. Discard the flowthrough, and place the minicolumn into the same Collection Tube.
11. Add 200$\mu\ell$ of Endotoxin Removal Wash (ERB) to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 15 seconds.
12. Add 400$\mu\ell$ of Column Wash Solution (CWC) to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 30 seconds.
13. Transfer the minicolumn to a clean 1.5ml microcentrifuge tube, then add 30$\mu\ell$ of Elution Buffer or nuclease-free water directly to the minicolumn matrix. Let stand for 1 minute at room temperature.
14. Centrifuge for 15 seconds to elute the plasmid DNA. Cap the microcentrifuge tube, and store eluted plasmid DNA at –20 ℃.

Measurement of DNA concentration (NanoDrop)
Materials
・DNA 1.5$\mu\ell$
・Buffer 1.5$\mu\ell$
Protocol
1. Start nanodrop2000.
2. Perform calibration and blank measurement by entering one drop of 1.5$\mu\ell$ buffer.
3. Clean the surface of the NanoDrop with KimWipes.
4. place 1.5$\mu\ell$ per sample on the NanoDrop and measure the concentration.

CO injection
Materials
・D-Luciferin (15mg/ml) 1$\mu\ell$
・CO Spray Can
・Bacterial culture 40$\mu\ell$
Protocol
1. Put bacterial culture and D-Luciferin into PCR tube.
2. Connect the PiCOEXPLORER to your smartphone.
3. Put the sample, PiCOEXPLORER, and CO concentration meter in a sealed container

and seal it.
4. Inject CO into the container with a spray can. (Do this in a draft.)
5. Measure the luminescence intensity of E. coli at a certain CO concentration at regular intervals.

Our material composition

①Gibson Assembly→Digestion→Ligation

Gibson Assembly

Part 1 (CooA)	2μℓ
Part 2 (Luciferase)	5μℓ
dH_2O	3μℓ
Gibson Assembly Master Mix	10μℓ
Total Volume	20μℓ

Digestion

	Insert	Vector
EcoR Ⅰ	0.4μℓ	0.4μℓ
HindⅢ	0.4μℓ	0.4μℓ
NEB Buffer r2.1 (10×)	2μℓ	1μℓ
DNA	17μg	1μℓ
Sterile water	0.2μℓ	7.2μℓ
Total Volume	20μℓ	10μℓ

Ligation

Vector (pGEM®-3Z)	0.5μℓ
Insert	9.5μℓ
Ligation Mix	10μℓ

②DNA purification (Insert)→Gibson Assembly→DNA purification→Digestion→Ligation

Gibson Assembly

Part 1 (CooA)	5μℓ
Part 2 (Luciferase)	5μℓ
dH_2O	0μℓ
Gibson Assembly Master Mix	10μℓ
Total Volume	20μℓ

Digestion

	Insert	Vector
EcoR Ⅰ	0.4μℓ	0.4μℓ
HindⅢ	0.4μℓ	0.4μℓ
NEB Buffer r2.1 (10×)	2μℓ	1μℓ
DNA	12μg	1μℓ
Sterile water	5.2μℓ	7.2μℓ
Total Volume	20μℓ	10μℓ

Ligation

Vector (pGEM®-3Z)	0.5μℓ
Insert	9.5μℓ

Ligation Mix	10$\mu\ell$

③Digestion (Vector)→DNA purification (Insert)→Gibson Assembly
Digestion (Vector)

	Vector
EcoR Ⅰ	0.4$\mu\ell$
NEB Buffer r2.1 (10×)	1$\mu\ell$
DNA	1$\mu\ell$
Sterile water	7.6$\mu\ell$
Total Volume	10$\mu\ell$

Gibson Assembly

Part 3 (CooA)	2$\mu\ell$
Part 4 (First half of luciferase)	2.5$\mu\ell$
Part 5 (Second half of luciferase)	2.5$\mu\ell$
Part 6 (pCooM)	1.5$\mu\ell$
Vector (pGEM®-3Z)	1$\mu\ell$
dH_2O	0.5$\mu\ell$
Gibson Assembly Master Mix	10$\mu\ell$
Total Volume	20$\mu\ell$

④Digestion (Vector)→DNA purification (Insert)→1st Gibson Assembly→DNA purification→2nd Gibson Assembly
Digestion (Vector)

	Vector
EcoR Ⅰ	0.4$\mu\ell$
NEB Buffer r2.1 (10×)	1$\mu\ell$
DNA	1$\mu\ell$
Sterile water	7.6$\mu\ell$
Total Volume	10$\mu\ell$

1st Gibson Assembly

Part 4 (First half of luciferase)	2.9$\mu\ell$
Part 5 (Second half of luciferase)	2.5$\mu\ell$
Part 6 (pCooM)	1.5$\mu\ell$
dH_2O	3.1$\mu\ell$
Gibson Assembly Master Mix	10$\mu\ell$
Total Volume	20$\mu\ell$

2nd Gibson Assembly

Part 4 (Gibson Assembly Product)	2.3$\mu\ell$
Part 3 (CooA)	4.4$\mu\ell$
Vector (pGEM®-3Z)	1.5$\mu\ell$
dH_2O	1.8$\mu\ell$
Gibson Assembly Master Mix	10$\mu\ell$

Total Volume	20$\mu\ell$