Synthetic Biology Lab Protocols

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Preparing LB and LB agar

1. The following is required for preparing LB (liquid broth)

Yeast Extract	5g
Tryptone	10g
NaCl	10g
ddH_2O	To 1L

The following is required for preparing LB agar

Yeast Extract	5g
Tryptone	10g
NaCl	10g
Agar	15g
ddH_2O	To 1L

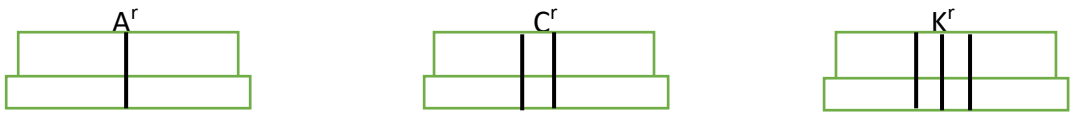
2. Antibiotics are added into LB right before the bacterial culture. For LB agar, antibiotics are added during preparation.
   Dilute the antibiotic 1000-fold.

Ampicillin(A^r)	1ml
Chloramphenicol(C^r)	1ml
Kanamycin(K^r)	1ml

3. Put magnetic stir bars inside the mixed solution from step 2. Heat and evenly stir the solution on a hot plate stirrer.

※Observe closely to make sure there aren't any suspended powders in the bottom of the solution.

4. After the powders are completely dissolved, retrieve the stir bars with the magnetic retriever. Seal with aluminum foil, apply autoclave tape, and autoclave for 1.5 hrs. For LB, label the date of preparation and store in 4℃ freezer. For LB agar, prepare 2 strips of empty plates while autoclaving.
5. Label date and resistance on the plastic bag containing the strip of plates. Label resistance on plate as below.



Pour the LB agar till the plate is half-full. Place the plates back into the plastic bag and store in 4℃ freezer after the LB agar solidify.

Culturing Single Colony

1. Make sure there are sample tubes, toothpicks, and alcohol lamp inside the sterile hood.
2. Sterilize liquid broth (LB), antibiotics, culture plate, pipets, and your hands with alcohol. Place the equipment mentioned in the sterile hood. Light up the alcohol lamp to disinfect toothpick after the alcohol on your hands fully evaporates.
3. Choose the single colony desired for culture from the plate and mark clearly.
4. Label group number, date, sample name, and resistance on the sample tube.
   ※Make sure the labelling is clear and can be traced back to the marks on the culture plate.
5. Add 3ml of LB into the sample tube.
6. Add 3μl of antibiotic into the sample tube, gently pipet to mix.
7. Use the disinfected toothpick to pick out the marked single colony in the plate.
8. Tilt the sample tube, stick the toothpick inside the liquid and stir.
9. Incubate the sample tube, tilted, at the appropriate temperature (depends on the bacterial strand and the purpose of the experiment, usually 37℃) for 12~16hrs with shaking. Observe its growth.

※Wipe down the sterile hood counter with alcohol-drenched tissue paper from in to out after use. Close the hood, turn off the suction and visible light, and turn on the UV light.

Plasmid Extraction (Miniprep)

1. Prepare two eppendorfs and one spin column for each sample on alcohol-sprayed tissue paper.
2. Label sample details (including name, date, resistance and group number) on the eppendorfs and spin columns.
3. Add 1.5 ml of cells into the eppendorf. Centrifuge for 1 minute at 14,000 rpm, room temperature. Decant the supernatant. Repeat the process until all cells in the sample tube are processed.
4. Add 200μl of buffer S1. Vortex till pellet disappears.

   ※ **buffer S1 is stored in the 4℃ freezer, put it back immediately after use.**

5. Add 200μl of buffer S2, mix by inversion. (About ten times. Do not mix violently) The solution should be transparent at this point. Place in room temperature for 2 minutes.
6. Add 300μl of buffer S3, mix by inversion. (About ten times. Do not mix violently) A white precipitate should form. Centrifuge for 10 minutes at 14,000 rpm, room temperature.
7. Transfer the supernatant to spin column. (Avoid the white precipitate.) Centrifuge for 30 seconds at 14,000 rpm, room temperature. Discard the flow-through.
8. Add 400μl of buffer W1. Centrifuge for 1 minute at 14,000 rpm, room temperature. Discard the flow-through.
9. Add 600μl of buffer W2. Centrifuge for 1 minute at 14,000 rpm, room temperature.
10. Centrifuge for and additional 1 minute at 14,000 rpm, room temperature to completely remove any residual buffer W2. Leave the lid open for a short while to allow any residual ethanol to evaporate.
11. Transfer the spin column to the readily labelled Eppendorf. Elute with 40 μl of ddH_2O (add the ddH_2O to the center of the column). Stand the spin column in room temperature for 2 minutes. Centrifuge for 2 minutes at 14,000 rpm, room temperature.

    ※accelerate the elution with 55℃ ddH_2O

    ※cut off the lid of the eppendorf or arrange the eppendorf according to the spinning direction before combining the eppendorf with the spin column to prevent the lid from breaking inside the centrifuge

12. Store plasmid DNA at **-20℃.**

Digestion

1. Label eppendorfs and prepare proper pipets.
2. Premix the following in the labelled eppendorf.

Plasmid to be digested	5μl
Cut smart	2μl
10X BSA	2μl
ddH_2O	10μl

3. Add the following into the labelled eppendorf on ice. (Use ice bucket from -20℃ freezer.)

   Upstream part **(Enzyme:EcoRI-HF and SpeI)**

EcoRI-HF	0.5μl
SpeI	0.5μl

   Downstream part **(Enzyme:XbaI and PstI)**

XbaI	0.5μl
PstI	0.5μl

   Vector part **(Enzyme:EcoRI-HF and PstI)**

EcoRI-HF	0.5μl
PstI	0.5μl

   ※Do not take the enzymes out of the ice bucket when adding. Immediately re-fasten the cap after use to avoid vapor that would affect the enzyme's freezing point.
4. Incubate for 4~6 hrs at the appropriate temperature (depends on the bacteria strain and purpose of the experiment, usually 37℃)

Ligation

1. Prepare proper pipets and label eppendorfs.
2. Premix the following solution in the labelled eppendorfs.

Vector part	1μl
Insert part	16μl
10X T4 DNA ligase buffer	2μl(contain ATP)

3. Add 0.5μl of T4 DNA ligase into the eppendorf in step 2 on ice. (Use ice bucket from -20℃ freezer.)

T4 DNA ligase	1μl

※Do not take the ligase out of the ice bucket when adding. Immediately re-fasten the cap after use to avoid vapor that would affect the enzyme's freezing point.

4. Incubate for 10 hrs at the appropriate temperature (depends on the bacteria strain and purpose of the experiment, usually 37℃)

PCR (Polymerase Chain Reaction) for colonies

1. Label PCR tubes and circle the colonies desired for amplification.
2. Add the following into the PCR tubes

Fast-Run Taq Master Mix	5μl
ddH_2O	4μl
VR	0.5μl
VF_2	0.5μl

3. Sterilize the PCR tubes and plates before placing them inside the sterile hood. Light up the alcohol lamp to disinfect toothpick. Use the disinfected toothpick to pick out the circled colony. Stick the toothpick into the PCR tube and slightly stir.
4. Setting the PCR thermal cycle
   i. Denature : 95℃, 10 mins
   ii. Annealing (15~30 cycles) : 95℃, 30secs→ 55℃, 30secs (Tm minus 5℃)→ 72℃, 1 min/kb + 30sec (for DNA<1kb, 1min30sec)
   iii. Elongation : 72℃, 10 mins
   iv. 4℃, ∞
5. Run gel electrophoresis after the PCR is completed. Observe and record by image acquisition.

Transformation

1. Preparation:
   -place the plates face-down to dry
   -set the water bath to 42℃
   -prepare an ice bucket
   -prepare a pre-cooled and labelled eppendorf
   -take competent cell from -80℃ freezer until it's half-thawed
   ※ **Make sure to fully close the -80℃ freezer immediately after use.**
2. Add 35μl of competent cell into the labelled eppendorf and place on ice. Label and store the remaining competent cells back into the 80℃ freezer.
   ※Place the remaining competent cells back into the 80℃ freezer as soon as possible to prevent cell death.
3. Take 2μl of plamid (mini) or 10μl of ligation product from -20℃ freezer. Gently mix by pipetting.
   ※Do this step on ice.
4. Place the mixed cells on ice for 5 mins. Heat-shock in 42℃ water bath for 30 secs
5. Label the plates to be spread with group numbers, dates, sample name and resistance. Light up the alcohol lamp to disinfect the spreader and wait for the spreader to cool. Spread the cells evenly on the labelled plates.
6. Incubate for 12~16 hrs at the appropriate temperature (depends on the bacteria strain and purpose of the experiment, usually 37℃). Observe growth.
   ※Wipe down the sterile hood counter with alcohol-drenched tissue paper from in to out after use. Close the hood, turn off the suction and visible light, and turn on the UV light.
   ※If the transformant DNA is larger than 6000 mb or has a low copy number, change step 4 into add 200μlof LB, incubate mixed cells for 12 mins with shaking, then place on ice for 5 mins.

Gel Electrophoresis

Making the gel

1. Prepare the following mixture in a vial

   For small gel:

1X TAE	20ml
Agarose powder	0.2g
Sybr safe	2μl

   For large gel:

1X TAE	40ml
Agarose powder	0.4g
Sybr safe	4μl

2. Mix the reagents prepared in step 1 well and microwave until the mixture starts to boil. Turn off the microwave oven, put on cotton gloves to slightly swing the vial and observe if there is any residual powder. Repeat the process until there is none.

   ※Pay close attention to the microwave, do not let the solution boil for too long

3. Wait till the mixture cools down to a temperature that is tolerable to the hand (~50℃) before pouring it into the gel casting tray and inserting the comb. Wait 40~60 minutes for the gel to solidify, remove the comb with care, and place the gel along with the gel casting tray inside the electrophoresis tank.

Running gel electrophoresis

Recommended loading volume:

	Sample	6X loading dye	Total
DNA marker	2.5μl	0.5μl	3μl
Plasmid	2.5μl	0.5μl	3μl
Digestion	5μl(10μl, if insert < 500 bp)	1μl(2μl)	6μl(12μl)
PCR product	5μl	1μl	6μl

※Load the DNA marker in the first well on the left.

※Use the following loading volume for the electrophoresis before gel extraction

	Sample	6X loading dye	Total
Digestion	15μl	1μl	16μl

4. Close the lid to the electrophoresis tank and connect the electrodes. *(**Note:** Black is negative, red is positive. Always **Run from Black to Red**.)* Turn on the power and place aluminum foil on the tank lid to avoid exposure to light. Adjust running time according to the size of your DNA sample. Pay attention to the dye line to

SDS-Polyacrylamide Gel Electrophoresis

1. **Preparation of resolving gel:**
   For 10% resolving gel, 5 ml of gel solution was prepared: 1.3 ml of 1.5 M Tris-HCl, pH 8.8, 50 µl of 10% (w/v) SDS, 1.275 ml of 29:1 acrylamide mix, and adjusted final volume to 5 ml with sterile-water.
2. **Resolving gel polymerization:**
   Gel polymerization was began as soon as by adding of 50 µl of 10% Ammonium persulfate (APS) and 4 µl of N,N,N',N'-tetramethylethylenediamine (TEMED). The resolving gel was covered with appropriate volume of sterile-water to prevent evaporation and resulted in a clear line when polymerization was complete.
3. **Preparation of stacking gel:**
   Then, 2 ml of 5% stacking gel was prepared: 0.25 ml of 1.0 M Tris-HCl, pH 6.8, 20 µl of 10% (w/v) SDS, 0.2475 ml of 29:1 acrylamide mix, and adjusted final volume to 2 ml with sterile-water.
4. **Stacking gel polymerization:**
   Stacking gel polymerization was initiated by the addition of 20 µl of 10% APS and 2 µl of TEMED. After resolving gel completely polymerized, discard the sterile-water. Poured the stacking gel solution and inserted a comb immediately into the stacking gel to polymerize for at least 30 minutes.
5. **Preparation of the protein samples for SDS-PAGE:**
   To prepare the protein samples for SDS-PAGE, every protein concentration of sample was adjusted with Phosphate-buffered saline (PBS) to the same, then adding 5 µl 5X SDS Sample Buffer [1M Tris-HCl, pH 6.8, 50% (v/v) glycerol, 10% (w/v) SDS, 1% (w/v) bromophenol blue, 12.5% (v/v) β-ME] to final volume of 25 µl. The samples were heated at 98℃ for 15 minutes.
6. **Loading and electrophoresis:**
   Finally, the protein samples and the protein ladder (BLUelf Prestained Protein Ladder, GeneDireX) were loaded and electrophoresis with an initial voltage of 90 volts in the stacking gel and 140 volts in resolving gel. SDS-PAGE was performed with 1X SDS-running buffer [25 mM Tris-base, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3] using the Bio-Rad Mini- PROTEAN® Tetra gel system.