2019 Fudan-TSI Protocols

Molecular Cloning

- **Fragment PCR (25 μl)**
  a) For a 25 μl fragment PCR reaction, add 12.5 μl Vazyme® 2 x Phanta Buffer, 0.5 μl template (if the template is plasmids) or 1 μl template (if the template is previous PCR products), 1 μl of both forward and reverse primers, 0.5 μl of dNTPs (10mM), 0.5 μl Vazyme® Phanta Super Fidelity DNA Polymerase and ddH_2O up to 25 μl into a PCR tube to set up the reaction.
  b) Place the PCR tube(s) in a PCR amplifier.
  c) Set up the reaction program:
    i. Incubate at 95℃ for 30 s.
    ii. Incubate at 95℃ for 15 s.
    iii. Incubate at the annealing temperature for 15 s.
    iv. Incubate at 72℃ for the elongation time (2000bp/min).
    v. Return to step ii for another 39 cycles.
    vi. Incubate at 72℃ for 5 min.
    vii. Incubate at 16℃ until the PCR product is picked up.
- **Vector PCR (25 μl)**
  a) For a 25 μl vector PCR reaction, add 12.5 μl 2xPhanta Buffer, 0.5 μl plasmid template, 1 μl of both forward and reverse primers, 0.5 μl of dNTPs (10mM), 0.5 μl Vazyme® Phanta Super Fidelity DNA Polymerase and ddH_2O up to 25 μl into a PCR tube to set up the reaction.
  b) Set up the reaction program:
    i. Incubate at 95℃ for 30 s.
    ii. Incubate at 95℃ for 15 s.
    iii. Incubate at the annealing temperature for 15 s.
    iv. Incubate at 72℃ for the elongation time (1000bp/min).
    v. Return to step ii for another 29 cycles.
    vi. Incubate at 72℃ for 10 min.
    vii. Incubate at 16℃ until the PCR product is picked up.
- **Colony PCR (10 μl)**
  a) For a 10 μl colony PCR reaction, add 1 μl 10xTaq Buffer, 0.4 μl of both forward and reverse primers, 0.2 μl of dNTP solution (10mM), 0.2 μl Vazyme® Taq DNA Polymerase, 1 colony template and ddH_2O up to 10 μl into a PCR tube to set up the reaction.
  b) Set up the reaction program:
    i. Incubate at 94℃ for 5 min.
    ii. Incubate at 94℃ for 30 s.
    iii. Incubate at the annealing temperature for 30 s.
    iv. Incubate at 72℃ for the elongation time (1000bp/min).

v. Return to step ii for another 24 cycles.
vi. Incubate at 72℃ for 7 min.
vii. Incubate at 8℃ until the PCR product is picked up.

- **Agarose Gel Electrophoresis**

a) Add 0.7g agarose, 70ml TAE buffer into a glass bottle and heat in a micro-oven for 2 min to prepare a liquid agarose gel.
b) Cool the liquid agarose gel to lower than 60℃ and decant the liquid agarose gel into a agarose gel tank.
c) Add 8 μl EB into the liquid agarose gel and place the electrophoresis comb to form 6 or 10 sample loading chambers after the solidification of the agarose gel.
d) Place the solid agarose gel in an electrophoresis device.
e) Add 10xDNA Loading Buffer in the DNA sample, mix them up gently and carefully pipette the sample into the sample loading chambers.
f) Cover the lid of the electrophoresis device, set the electrophoresis time and start electrophoresis.

- **DNA Gel Extraction**

a) After DNA agarose gel electrophoresis, place the gel under UV light and find the DNA band of the desired nucleotide length.
b) Cut the gel containing desired DNA and put it into an Eppendorf tube.
c) Purify the DNA gel with Vazyme® DNA Gel Extraction Kit:
i. Add 300 μl buffer GDP to the Eppendorf tube and incubate at 55℃ until the gel completely dissolved in the solution (shake the tube for each about 3 min to help with the dissolving process).
ii. Spin briefly to bring the sample to the bottom of the tube, insert a FastPure DNA Mini Columns-G into a 2 ml Collection Tube, carefully transfer the solution maximally of 700 μl once a time to a filtration column, centrifuge at 12,000 x g for 30 ~ 60 sec.
iii. Discard the filtrate and reuse the Collection Tube, add 600 μl of Buffer GW (with ethanol added) to the filtration column, centrifuge at 12,000 x g for 60 sec.
i. Repeat step iii.
ii. Discard the filtrate and reuse the Collection Tube, centrifuge the empty column at 12,000 x g for 2 min.
iii. Insert the column into a clean 1.5 ml Eppendorf tube and incubate at 55℃ for 5 min. to completely dry the column. Also incubate ddH_2O at 55℃ for better extraction efficiency.
iv. Add 7 μl ~ 30 μl of ddH2O to the center of the column membrane, incubate at room temperature for 2 min, and then centrifuge at 12,000 x g for 1min. Discard the filtration column, store DNA at -20℃.
v. Use Nanodrop 2000 to measure the DNA concentration of the extracted product.

- **ClonExpress Ligation**

a) Dilute 1μl of both DNA fragment and vector in 2 (or 3 if there are 2 different fragments) different PCR tubes to the mole ratio at 1:1 with calculated amount of ddH_2O.
b) Add 1μl of both diluted DNA fragment and vector into another PCR tube and short spin to ensure the reagents are mixed at the bottom of the tube.

c) Add 1μl of mixed fragment and vector and 1μl of Vazyme® 2 x ClonExpress Mix. Short spin to ensure the reagents are mixed at the bottom of the tube.
d) Incubate the reaction mixture at 50℃ for 30 min.
e) Place the reaction product on ice immediately when the reaction is over and be ready for the plasmid transformation.

- **Restriction Enzyme Double Digest**
  a) Add DNA fragment and vector at a mole ratio of 2:1~4:1 (total 1μg), 5 μl NEB CutSmart buffer, 1 μl of both restriction enzymes and ddH_2O up to 50 μl to set up the reaction.
  b) Incubate at 37℃ overnight.
- **T4 Ligase Ligation**
  a) Add 5 μl NEB 2 x Quick Ligation Buffer, 4.5 μl restriction enzyme double digest product and 0.5 μl NEB T4 DNA Ligase to set up a 10 μl reaction.
  b) Incubate at room temperature for 6 ~ 8 hours.
- **Plasmid Transformation**
  a) Add 20 μl competent *E.coli* cells into the ligation product if the ligation is done by Vazyme® ClonExpress kit, or 50 μl competent *E.coli* cells into the ligation product if the ligation is done by NEB T4 DNA Ligase.
  b) Incubate on ice for 30 min.
  c) Heat shock at 42℃ for 30 sec. if using Vazyme® Fast-T1 Competent Cell; for 45 sec. if using Vazyme® DH5α Competent Cell or 90 sec. if using Vazyme® BL21(DE3) Competent Cell.
  d) Cool the mixture on ice for 2 min. without shaking or vibrating.
  e) Add 200 μl liquid SOC medium into the mixture and shaking culture for 1h for the recovery of the cells and expression of antibiotic genes.
  f) Evenly spread the liquid culture on a solid culture medium and incubate at 37℃ overnight for colonies forming on the plate.
- ***E.coli* Liquid Culture and Induction**
  a) Add 3~10 ml liquid culture medium such as LB and 2 x YT with or without antibiotics into a test tube.
  b) Pick one colony from the solid medium plate with a pipette tip and throw the pipette tip together with the colony into the liquid culture medium.
  c) (Optional) When the OD_{600} reaches 0.6~0.8, add 1 mM IPTG, 4mM arabinose or 100ng/ml aTc into the liquid culture for protein expression induction.
  d) Shaking culture overnight.
- **Plasmid Miniprep**
  a) Use Vazyme® FastPure Plasmid Mini Kit for plasmid miniprep:
    i. Harvest 1~ 5 ml overnight cultured (12 ~ 16 hours) bacterial cells into a centrifuge tube, centrifuge at 10,000 x g for 1 min, discard the supernatant and invert the tube on the absorbent paper to dry.
    ii. Add 250 μl of Buffer P1 (add RNase A before use), mix thoroughly by vortex or pipetting up and down.
    iii. Add 250 μl of Buffer P2, mix thoroughly by softly inverting the tube 8 ~ 10 times to assure complete lysis.
    iv. Add 350 μl of Buffer P3, mix gently and thoroughly by inverting the tube 8 ~ 10

times to neutralize Buffer P2 until a flocculent white precipitate forms and centrifuge at 13,000 x g for 10 min.

v. Insert a FastPure DNA Mini Column into a 2 ml Collection Tube, carefully transfer (by aspirating, avoid disturbing the precipitate) the supernatant from step 4 to the Filtration Column, centrifuge at 13,000 x g for 30 ~ 60 sec, discard the filtrate and reuse the Collection Tube.

vi. Add 600 µl of Buffer PW2 (with ethanol added in) to the Filtration Column, centrifuge at 13,000 x g for 30 ~ 60 sec, discard the filtrate and reuse the Collection Tube.

vii. Repeat step 7.

viii. Centrifuge the empty Filtration Column for l min at 13,000 x g to remove residual washing buffer PW2 .

ix. Insert the Column into a clean 1.5 ml microcentrifuge tube, add 30 ~ 100 µl of Elution Buffer to the center of the Column membrane, incubate at room temperature for 2 min, centrifuge at 13,000 x g for 1min.

x. Discard the Filtration Columns, store DNA at -20℃.

- **Cell Colony Count**

a) Take the agar media plate with previously grown bacteria colony on it.

b) Use a white pipette tip, pick up a small colony of bacteria and inoculate in 6 ml 2 x YT broth in a 10 ml falcon tube.

c) Shake culture at 37℃ at 250 rpm for approximately 16 hours.

d) Prepare a solid agar plate with appropriate antibiotics and pre-heat at 37℃ before use. This will promote the growth of bacteria colonies and reduce the risk of plate contamination with a different bacterial species as well.

e) Dilute the bacteria liquid culture to an appropriate concentration, pre-experiment may be required for identification of the appropriate concentration so that there will be colonies forming on the plate.

f) Add 100 ~ 200 µl diluted bacterial culture to the plate and spread across the plate.

g) After overnight culture in 37℃, count the number of colonies forming on the plate.

Protein Electrophoresis

- **SDS-PAGE**

a) Prepare the protein sample

i. Add 1 ml bacterial cells into a centrifuge tube, centrifuge at 12,000 x g for 1 min, discard the supernatant.

ii. Add 40 µl of 1 x SDS Loading, mix thoroughly by vortex or pipetting up and down until there is no visible precipitation.

iii. Incubate at 95℃ for 10 min.

iv. Centrifuge at 12,000 x g for 5 min.

b) Prepare the polyacrylamide gel.

i. Choose the proper concentration and thickness of gel.

ii. For 5 ml 10% Running Gel, add 2.32 ml ddH_2O, 1.33 ml 30% Acrylamide/Bis, 1.25 ml 1.5M Tris-HCl pH8.8, 50 µl 10% SDS, 50 µl 10% APS, 2.5 µl TEMED.

Mix them thoroughly by inverting the tube 8 ~ 10 times.

iii. Fill the 4/5 of gel cassette with the Running Gel softly and fill up the cassette with ddH_2O. Keep it still for 30 min until the water layer can be observed.

iv. For 2.5 ml 4% Stacking Gel, add 1.17 ml ddH_2O, 333.3 µl 30% Acrylamide/Bis, 945 µl 0.5M Tris-HCl pH6.8, 25 µl 10% SDS, 25 µl 10% APS, 2.5 µl TEMED. Mix them thoroughly by inverting the tube 8 ~ 10 times.

v. Pour out the ddH_2O completely and fill up the cassette with Stacking Gel. Insert the comb and take care not to catch bubbles under the teeth. Keep it still for 40 min.

c) Load the gel.

i. Take off the cassette and assemble the gel running stand.

ii. Fill the stand with 1 x SDS running buffer and remove the combs from the gel.

iii. Mix up 8 µl Marker with 7 µl 1 x SDS Loading.

iv. Load 15 µl marker mixture and 15 µl supernatant of samples into the wells

d) Run the gel.

i. Cover the lid of the electrophoresis device, and start electrophoresis at 80 V for 20 min. Then start electrophoresis again at 120 V for 60 min until the dye front is nearly at the bottom of the gel.

e) Stain the gel with 0.1% Coomassie Blue dye.

i. Submerge the whole piece of the disassembled gel with 0.1% Coomassie Blue dye and heat in the microwave oven for 2 min.

ii. Pour out the 0.1% Coomassie Blue dye and add half box of water. Heat in the microwave oven for 15 min. Do it twice.

f) Scan the gel.

Fluorescence Measurement and Quantification

- **General Green and Red Fluorescence Measurement**

a) Fluorescein Standard Curve Measurement:

i. For Green fluorescence measurement:

1. Use the fluorescein from 2019 iGEM Measurement Kit and short spin to concentrate the fluorescein at the bottom of the tube.
2. Add 1 ml PBS for dissolving the fluorescein to prepare 10 x fluorescein solution.
3. Add 100 µl 10 x fluorescein solution and 900 µl PBS into a new Eppendorf tube to prepare 1 x fluorescein solution.

ii. For Red fluorescence measurement:

1. Take 1M rhodamine solution as 10 x fluorescein salt solution.
2. Add 100 µl 10 x fluorescein solution and 900 µl PBS into a new Eppendorf tube to prepare 1 x fluorescein solution.

iii. Take a 96-well plate and add 100 µl 1 x PBS in A2 ~ A12、B2 ~ B12、C2 ~ C12、D2 ~ D12 wells.

iv. Add 200 µl 1 x fluorescein solution in A1 well, mix carefully and pipette 100 µl solution from A1 to A2, from A2 to A3 and repeat until pipette 100 µl solution from A10 to A11. Dispose 100 µl solution from A11 well.

v. Repeat the same operation for B1 ~ B12、C1 ~ C12、D1 ~ D12.
vi. Measure the fluorescence of each well by a 96-well plate reader with appropriate excitation wavelength and emitting wavelength.
vii. Use iGEM official Excel table for fluorescein standard curve generating. Find the best linear region of the standard curve using linear fitting softwares.

b) Silica Beads Standard Curve Measurement
i. Use the silica beads from 2019 iGEM Measurement Kit.
ii. Add 96 µl beads suspension solution and 904 µl ddH_2O for 1 x silica beads suspension solution.
iii. Take a 96-well plate and add 100 µl ddH_2O in A2 ~ A12、B2 ~ B12、C2 ~ C12、D2 ~ D12 wells.
iv. Add 200 µl 1 x silica beads suspension solution in A1 well, mix carefully and pipette 100 µl suspension solution from A1 to A2, from A2 to A3 and repeat until pipette 100 µl suspension solution from A10 to A11. Dispose 100 µl suspension solution from A11 well.
v. Repeat the same operation for B1 ~ B12、C1 ~ C12、D1 ~ D12.
vi. Measure the OD_{600} of each well by a 96-well plate reader.
vii. Use iGEM official Excel table for silica beads standard curve generating. Find the best linear region of the standard curve using linear fitting softwares.

c) Bacteria Sample Measurement
i. Harvest 1ml bacteria-liquid culture medium suspension solution into a centrifuge tube, centrifuge at 10,000 x g for 1 min, discard the supernatant and invert the tube on the absorbent paper to dry.
ii. Resuspend the bacteria cells with 500 ~ 1000 µl PBS to prepare the sample for fluorescence detecting.
iii. Add 100 µl bacteria cell-PBS suspension solution into at least 3 wells of a 96-well plate for at least 3 repeats of each sample.
iv. Add 100 µl of the fluorescence solution of the concentration at the both ends of the standard curve linear region for calibrating the standard curve.
v. Add 100 µl of the silica beads suspension solution of the concentration at the both ends of the standard curve linear region for calibrating the standard curve.
vi. Measure the fluorescence and OD_{600} of the 96-well plate samples by a 96-well plate reader and save the measurement data for further processing.

d) Data Processing
i. Use fitting software to fit the calibrated standard curve based on the fluorescence and OD_{600} of the standard fluorescein and silica beads samples.
ii. Calculate the fluorescein concentration and the number of silica beads according to the measured fluorescence intensity, OD_{600} and the calibrated standard curves.
iii. Divide the fluorescein concentration by the number of silica beads to get the relative fluorescence intensity of each sample for further processing.

- **Degradation Tags Measurement**

a) Fluorescein Standard Curve Measurement
i. Please refer to *General Green and Red Fluorescence Measurement: a) Fluorescein Standard Curve Measurement.*

b) Silica Beads Standard Curve Measurement

i. Please refer to *General Green and Red Fluorescence Measurement: b) Silica Beads Standard Curve Measurement.*

c) Bacteria Sample Measurement

i. Harvest 1ml bacteria-liquid culture medium suspension solution into a centrifuge tube. Do not centrifuge to protect the fluorescence protein from rapid degradation.

ii. Add 100 µl bacteria cell-liquid medium suspension solution into at least 3 wells of a 96-well plate for at least 3 repeats of each sample.

iii. Add 100 µl bacteria liquid culture medium into at least 3 wells of a 96-well plate as blanks.

iv. Add 100 µl of the fluorescence solution of the concentration at the both ends of the standard curve linear region for calibrating the standard curve.

v. Add 100 µl of the silica beads suspension solution of the concentration at the both ends of the standard curve linear region for calibrating the standard curve.

vi. Measure the fluorescence and OD_{600} of the 96-well plate samples by a 96-well plate reader and save the measurement data for further processing.

d) Data Processing

i. Use fitting software to fit the calibrated standard curve based on the fluorescence and OD600 of the standard fluorescein and silica beads samples.

ii. Subtract the fluorescent intensity and the OD_{600} of the liquid culture blank from the fluorescent intensity and the OD_{600} of the bacteria samples to get the actual measurement of the samples.

iii. Calculate the fluorescein concentration and the number of silica beads according to the measured actual fluorescence intensity, OD_{600} and the calibrated standard curves.

iv. Divide the fluorescein concentration by the number of silica beads to get the relative fluorescence intensity of each sample for further processing.

v. Draw a relative fluorescence intensity curve for samples corresponding to each degradation tags.