# 2020 Fudan Protocols

# Catalog



## Cell Cryopreservation

Cryopreservation is a technique that store cells at a very low temperatures (-80℃) to reduce cell metabolic damage and enable long-term storage.

- Add cryoprotectant: Add 80% glycerin 500 μL and bacterial fluid 1 000 μL into Cryopreservation vials. Mix upside down gently.
- Seal the vials: Stick sellotape around the label.
- Store the vials: Store prepared vials in refrigerator at -80℃.

## Cell Recovery

Bacteria preserved at -80℃ need to be recovery to restores cell growth.

- Prepare plate: Take out the plate with relevant resistance from 4℃ and wait for the temperature to the room temperature. Mark at the bottom of the plate.
- Recover the cell: The -80℃ frozen strain was removed and placed on the ice quickly. Take a ring of the upper layer of melt bacteria liquid by the sterilized inoculation ring. Coated the plate and draw a line.
- Culture the recovered cell: Incubate at 37℃ overnight.

## PCR

PCR is performed for amplify DNA fragments in our project.

- Set up PCR system (25 μL):

| Reagent | Volume |
|---|---|
| $ddH_2O$ | To 25 μL |
| 2 x Phanta Max Buffer | 12.5 μL |
| dNTP | 0.5 μL |
| Phanta | 0.5 μL |
| Template | Plasmid: 0.5 μL<br>PCR product: 1 μL |
| Forward Primer | 1 μL |
| Reverse Primer | 1 μL |

- Place the PCR tubes in a PCR amplifier.
- Set up reaction program:

| Procedure | Temperature | Time | Cycle |
|---|---|---|---|
| Initialization | 95℃ | 30s | 1 |
| Denaturation | 95℃ | 15s | 40 |
| Annealing | 72℃ | 15s | |
| Extension | 72℃ | 30s/kb | |
| Final elongation | 72℃ | 5min | 1 |

- Incubate: Incubate at 16℃ until the PCR product is picked up.

## Colony PCR

Colony PCR is performed to determine whether we insert DNA into plasmid successfully.

- Set up colony PCR system (10 μL):

| Reagent | Volume |
|---|---|
| $ddH_2O$ | To 10 μL |
| 10 x Taq Buffer | 1 μL |
| dNTP | 0.2 μL |
| Taq DNA Polymerase | 0.2 μL |
| Colony Template | 1 μL |
| Forward Primer | 0.4 μL |
| Reverse Primer | 0.4 μL |

- Place the PCR tubes in a PCR amplifier.
- Set up reaction program:

| Procedure | Temperature | Time | Cycle |
|---|---|---|---|
| Initialization | 94℃ | 5min | 1 |
| Denaturation | 94℃ | 30s | 25 |

| | | | |
|---|---|---|---|
| Annealing | 72℃ | 30s | |
| Extension | 72℃ | 1min/kb | |
| Final elongation | 72℃ | 7min | 1 |

- Incubate: Incubate at 8℃ until the PCR product is picked up.

## Overlap Extension PCR (OE PCR)

OE PCR is used to fuse fragments together.

- First round PCR: Use primer a/b to amplify fragment AB by PCR, and c/d to amplify fragment CD. Set up PCR system:

| Reagent | Volume |
|---|---|
| $ddH_2O$ | To 25 μL |
| 2 x Phanta Max Buffer | 12.5 μL |
| dNTP | 0.5 μL |
| Phanta | 0.5 μL |
| Template | Plasmid: 0.5 μL<br>PCR product: 1 μL |
| Forward Primer (a or c) | 1 μL |
| Reverse Primer (b or d) | 1 μL |

Set up reaction program:

| Procedure | Temperature | Time | Cycle |
|---|---|---|---|
| Initialization | 95℃ | 30s | 1 |
| Denaturation | 95℃ | 15s | 40 |
| Annealing | 72℃ | 15s | |
| Extension | 72℃ | 30s/kb | |
| Final elongation | 72℃ | 5min | 1 |

- Annealing of homologous regions in Second round PCR: Separate and purify fragment AB and CD by agarose gel electrophoresis and DNA gel extraction. Measure the concentration of the two fragments. Dilute them into 1:1 as template. Set up PCR system:

| Reagent | Volume |
|---|---|
| $ddH_2O$ | To 50 μL |
| 2 x Phanta Max Buffer | 25 μL |
| dNTP | 1 μL |
| Phanta | 1 μL |
| Template | 0.08 pmol + 0.08 pmol |

Set up reaction program:

| Procedure | Temperature | Time | Cycle |
|---|---|---|---|
| Initialization | 94℃ | 5min | 1 |
| Denaturation | 94℃ | 30s | 12 |
| Annealing | 60/64/68/72℃ | 30s | |
| Extension | 72℃ | 1min/kb | |
| Final elongation | 72℃ | 7min | 1 |

- Amplification in second round PCR: Add primer 2 + 2 μL a/d into the system. Set up reaction program:

| Procedure | Temperature | Time | Cycle |
|---|---|---|---|
| Initialization | 94℃ | 5min | 1 |
| Denaturation | 94℃ | 30s | 40 |
| Annealing | 60/64/68/72℃ | 30s | |
| Extension | 72℃ | 1min/kb | |
| Final elongation | 72℃ | 7min | 1 |

## Vector PCR (25 μL)

Vector PCR is used for generating a linear product.

- Set up PCR system (25 μL):

| Reagent | Volume |
|---|---|
| dd$H_2O$ | To 25 μL |
| 2 x Phanta Max Buffer | 12.5 μL |
| dNTP | 0.5 μL |
| Phanta | 0.5 μL |
| Template | 0.5 μL |
| Forward Primer | 1 μL |
| Reverse Primer | 1 μL |

- Place the PCR tubes in a PCR amplifier.
- Set up reaction program:

| Procedure | Temperature | Time | Cycle |
|---|---|---|---|
| Initialization | 95℃ | 30s | 1 |
| Denaturation | 95℃ | 15s | 30 |
| Annealing | 72℃ | 15s | |
| Extension | 72℃ | 30s/kb | |
| Final elongation | 72℃ | 5min | 1 |

- Incubate: Incubate at 16℃ until the PCR product is picked up.

## Agarose Gel Electrophoresis

Agarose gel electrophoresis is performed to separate and confirm whether our plasmids were constructed properly.

- Make gel solution: Add 0.7g agarose, 70 mL TAE buffer into a glass bottle and heat in a micro-oven for 2 min. Cool the liquid agarose gel to lower than 60℃ and decant the liquid agarose gel into an agarose gel tank. Add 8 μl EB into the liquid agarose gel and place the electrophoresis comb.
- Load the gel: Place the solid agarose gel in an electrophoresis device. Add 10xDNA Loading Buffer in the DNA sample, mix them up gently and carefully pipette the sample into the sample loading chambers.

- Electrophoresis: Cover the lid of the electrophoresis device, set the electrophoresis time and start electrophoresis.

## DNA Gel Extraction

DNA Gel Extraction is to extract desired DNA from an agarose gel after agarose gel electrophoresis.

- Cut the DNA gel: 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.
- Melt the DNA gel: Use Vazyme® DNA Gel Extraction Kit. Add 300 μl buffer GDP to the Eppendorf tube and incubate at 55℃. Spin briefly.
- Pure the DNA gel: Insert a Fast Mini Columns-G into a 2 mL Collection Tube, transfer the solution maximally of 700 μl once a time to a filtration column, centrifuge at 12,000 x g for 30 ~ 60 sec. 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. Pure the left solution in the same way.
- Filtrate DNA: Discard the filtrate and reuse the Collection Tube, centrifuge the empty column at 12,000 x g for 2 min. Insert the column into a clean 1.5 mL Eppendorf tube and incubate at 55℃ for 5 min. Add 7 μl ~ 30 μl of $ddH_2O$ (incubated at 55℃ in advance) 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. Measure the DNA concentration by Nanodrop 2000.
- Store DNA at -20℃.

## Restriction Enzyme Double Digestion

Double digestion reaction is performed to create mismatch ends for directional insertion.

- 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.
- Incubate at 37℃ overnight.

## ClonExpress ligation reaction

ClonExpress ligation reaction is performed to insert our DNA fragments into vector.

- Set up ClonExpress ligation system:

| Reagent | Volume |
|---|---|
| $ddH_2O$ | To 5 μL |
| DNA fragment | 0.02*bp ng |
| vector | 0.01*bp ng |
| 2 x ClonExpress Mix | 2.5 μL |

- Ligation reaction: Incubate the reaction mixture in a PCR amplifier at 50℃ for 30 min then at 4℃ preparing for transformation.

## Plasmid transformation

Plasmid transformation is performed to transfer plasmids into the host bacteria. We transform our plasmids into DH5α *E. coli* to amplify them and BL21 to testify function of our plasmids.

- Thaw all reagents on ice. And add 20 μl competent *E. coli* cells into the ligation product.
- Heat shock at 42℃ for 45 sec and then cool the mixture on ice for 2 min.
- Add 900 μl liquid SOC or LB medium (without antibiotic) into the mixture and shaking culture at 37℃ for 1h.
- Evenly spread the liquid culture on a solid culture medium and incubate at 37℃ overnight for colonies forming on the plate.

## Plasmid Miniprep (Vazyme® FastPure Plasmid Mini Kit)

Plasmid Miniprep is performed to is extract plasmid DNA from bacteria.

- 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.
- Add 250 μl of Buffer P1 (add RNase A before use), mix thoroughly by vortex or pipetting up and down.
- Add 250 μl of Buffer P2, mix thoroughly by softly inverting the tube 8 - 10 times to assure complete lysis.
- 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.
- Insert a FastPure DNA Mini Column into a 2 mL Collection Tube, transfer the supernatant from step 4 to the Filtration Column, centrifuge at 13,000 x g for 30 - 60s, discard the filtrate and reuse the Collection Tube.
- 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. Centrifuge the empty Filtration Column for l min at 13,000 x g.
- 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. Discard the Filtration Columns, store DNA at -20℃.

## SDS-PAGE

SDS-PAGE is performed for the separation of polypeptides and confirm whether our circuits expressed properly.

- Prepare 10 mL 10% Running Gel solution: Add 4.1 mL dd$H_2O$, 3.3 mL 30% Acrylamide/Bis (29:1 or 37.5:1), 2.5 mL 1.5M Tris-HCl pH8.8, 100 μl 10% SDS, 50 μl 10% APS, 5 μl TEMED. Mix them thoroughly.
- Prepare 2 mL 4% Stacking Gel solution: Add 1.22 mL dd$H_2O$, 0.26 mL 30% Acrylamide/Bis (29:1 or 37.5:1), 0.5 mL 0.5M Tris-HCl pH6.8, 20 μl 10% SDS, 20 μl 10% APS, 2 μl TEMED.

Mix them thoroughly.

- Prepare the protein sample: Add 1 mL bacterial cells per time into a centrifuge tube, centrifuge at 12,000 x g for 1 min, discard the supernatant. Dilute SDS sample buffer to 1x. Add 1 x SDS Loading to 3000 cells per 1ul, mix thoroughly by vortex or pipetting up and down until there is no visible precipitation. Incubate at 99℃ for 5 min.
- Make the gel: Assemble the gel cassette and make sure it not to leak. Fill the gel cassette with the Running Gel softly and fill up the cassette with dd$H_2O$. Keep it still for 10~20 min until the water layer can be observed. Pour out the dd$H_2O$ 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.
- Load the gel: Take off the cassette and assemble the gel running stand. Fill the stand with 1 x SDS running buffer and remove the combs from the gel. Mix up Marker with 1 x SDS Loading. Load 15 μl marker mixture into the wells.
- Electrophoresis: Cover the lid of the electrophoresis device, and start electrophoresis at 200 V until the dye front is nearly at the bottom of the gel.
- Stain the gel: Submerge the whole piece of the disassembled gel with 0.1% Coomassie Blue dye for 30 min.
- Destain the gel: Pour out the 0.1% Coomassie Blue dye and wash it using dd$H_2O$. Destain with destaining solution for 30min. Change the destaining solution to destain until it clear.
- Scan the gel.

## SDS-PAGE Gradient Gel

SDS-PAGE is performed for the separation of caap and confirm whether our circuits functioned properly.

- Start the device:  Put the container on the electromagnetic heating device. Increase the speed of the rotor gradually to 260 RPM.
- Prepare Gel solution: 4% and 20% Gel solution, can be prepared directly according to SDS-PAGE protocol. And 1.5 times of APS concentration can be added in autumn.
- Gradient formation: Add 4% glue slowly into the valve, and then add 20% glue. Mix them by the rotor. Wait about 2 hours for the glue to concrete.
- Store the gel: Remove the solidified glue one by one. Soak them in 1×SDS, and put them into a refrigerator at 4℃ under the counter for preservation.

## IPTG induction experiment

- Reconnection: Take 10 mL and 5 mL LB liquid medium in two test tubes, 10 mL one is used as control group and 5 mL one is for experiment group. Add 500× ampicillin 20 μl and 10μl, respectively. Culture 6h. Add 1 mL bacteria solution to experiment group and divided evenly into two tubes (3 mL each) for ipTG-induced and non-induced groups. Another 1 mL bacteria was used to measure initial OD.
- Measure OD: Measured OD value once per 30min and once per 15min after OD reached 0.2.
- Induce: When OD value reached about 0.6, add 500 mM IPTG reach final concentration of 1

mM. Incubate for 3 hours.

## Anhydrotetracycline induction experiment

- Dilute aTc: Dilute 2 mg/ mL aTc solution into 100 ng/ mL, 200 ng/ mL and 400 ng/ mL.
- Induce: Add 3 mL LB medium (with kan), 200 μL ON culture and 32 μL corresponding inducer (in one of the test tubes add 32 μL LB medium as control) into 3 mL bacteria solution.
- Measure: Add 300 μL of each test tube to the microplate reader in triplet, and measure the $t_0$ at the correct wavelength and OD600. Put the test tubes in the shaker at 37℃ and 250 rpm. After 4 hours measure the $t_4$, after 8 hours measure the $t_8$ and after 12h measure the $t_{12}$.

## Growth Curve Measurement

Growth Curve Measurement is used to characterize our Kill Switches.

- Plasmids construction and transformation: Insert DNA fragments of BBa_K3036004, BBa_K3606027 and BBa_K3606028 into pSB1C3. Transform the two kinds of constructed plasmids into DH5α strain as experimental groups and empty pSB1C plasmids as control group. Culture three groups in 60 mL LB medium (with 50 ng/ μL ampicillin) at 37℃ overnight.
- Cold treatment: Divide each group into two test tubes for 30℃-culture groups and 37℃-culture groups. (3 for each temperature).
- Measure growth situation: Extract 5μl bacteria solution from each test tube every 1h. Diluted each bacteria solution to 10^7 times and culture them on three LB plate (with 50 ng/ μL ampicillin) at 37℃ for 24h. Count the number of colonies in 5 cm^2 per plate after cultured for 24h at 37℃.
- Draw the growth curve.

## Methylthymol Blue Method for Blood Calcium Measurement

Methylthymol Blue Method is used to determine the concentration of calcium chromogenic reaction.

- Prepare solution 1：

| Reagent | Volume |
|---|---|
| $H_2O$ | To 45 mL |
| MTB | 0.009g |
| 8-Hydroxyquinoline | 6.096g |
| HCl | 0.55785 mL |
| Urea | 8.109g |

- Prepare solution 2：

| | |
|---|---|
| H20 | To 45 mL |
| Ethanolamine | 9 mL |
| Urea | 8.109g |

- Set up chromogenic reaction: Take 1 mL solution 1 and 1 mL solution 2 to make the application

solution. Add 34ul standard calcium ion sample into the system in proportion of 60:1and mix it.

- Measure the absorbance: Wait for about 3 to 5 minutes and measure the absorbance of the solution with a spectrophotometer.
- Change the standard calcium ion concentration from 0 to 20 mM and make multiple measurements to obtain the linearity curve.