

## Protocol

### Agarose Gel Electrophoresis:

1. Measure TAE buffer.
2. Weigh the agarose powder with a volume to mass ratio of 1% to 2% and add agarose powder to the buffer.
3. The mixture is heated several times in a microwave oven until the solution becomes clear.
4. Cool the solution to about 40-50 ° C and add the nucleic acid dye.
5. Place the appropriate comb on the gel tray and pour the solution into the tray gel and allow the gel to cool until it becomes hard.
7. Carefully pull out the comb, do not damage the glue hole, the gel into the electrophoresis room.
8. Add enough TAE buffer to make it no gel.
9. Remove the appropriate amount of the loading buffer and the DNA (Gene Finder) mixed DNA sample into the pores of the gel; take the appropriate amount of marker into the gel pore as a control;
10. Run the gel at 120V, 115 milliamperes for 20 minutes.

### Gel Purification

1. Excise gel slice containing the DNA fragment using a clean scalpel or razor blade. Cut as close to the DNA as possible to minimize the gel volume. Place the gel slice into a pre-weighed 1.5 ml tube and weigh. Record the weight of the gel slice.

Note: If the purified fragment will be used for cloning reactions, avoid damaging the DNA through UV light exposure. Minimize UV exposure to a few seconds or keep the gel slice on a glass or plastic plate during UV illumination.

2. Add 1:1 volume of Binding Buffer to the gel slice (volume: weight)

Note: For gels with an agarose content greater than 2%, add 2:1 volumes of Binding Buffer to the gel slice.

3. Incubate the gel mixture at 50-60° C for 10 min or until the gel slice is completely dissolved. Mix the tube by inversion every few minutes to facilitate the melting process. Ensure that the gel is completely dissolved. Vortex the gel mixture briefly before loading on the column.

Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10ul of 3M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.

4. Optional: Use this step only when DNA fragment is  $\leq 500\text{bp}$  or  $\geq 10\text{kb}$  long.

If the DNA fragment is  $\leq 500\text{bp}$ , add 1gel volume of 100% isopropanol to the solubilized gel solution. Mix thoroughly.

If the DNA fragment is  $\geq 10\text{kb}$ , add 1gel volume of 100% water to the solubilized gel solution. Mix thoroughly.

5. Transfer up to 800ul of the solubilized gel solution(from step 3 to 4) to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.

Note: If the total volume exceeds 800ul, the solution can be added to the column in stages. After each application, centrifuge the column for 30 -60s and discard the flow-through after each spin. Repeat until the entire volume has been applied to the column membrane. Do not exceed 1g of

total agarose gel per column.

Close the bag with GeneJET Purification Columns tightly after each use.

6. Optional: Use this additional binding step only if the purified DNA will be used for sequencing. Add 100ul of Binding Buffer to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.

7. Add 700ul of Wash Buffer to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.

8. Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove residual wash buffer.

Note: This step is essential to avoid residual ethanol in the purified DNA solution. The presence of ethanol in the DNA sample may inhibit downstream enzymatic reactions.

9. Transfer the GeneJET purification column into a clean 1.5ml microcentrifuge tube (not included). Add 50ul of Elution Buffer to the center of the purification column membrane. Centrifuge for 1 min.

Note: For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50ul does not significantly reduce the DNA yield. However, elution volumes less than 10ul are not recommended.

If DNA fragment is  $\geq 10$ kb, prewarm Elution Buffer to  $65^{\circ}\text{C}$  before applying to column. If the elution volume is 10ul and DNA amount is  $\leq 5$ ug, increase column for 1 min at room temperature before centrifugation.

10. Discard the GeneJET purification column and store the purified DNA at  $-20^{\circ}\text{C}$ .

## PCR

### Overlap Extension PCR:

System: Mixed Template: 2ul; Fastpfu: 1ul;  $5\times$ Fastpfu Buffer:10ul; Forward primer: 2ul;  
Reverse primer: 2ul; dNTP:4ul; Sterile water: 29ul; Total: 50ul

Procedure:  $95^{\circ}\text{C}$ 3min; cycle: $95^{\circ}\text{C}$ 30s;  $(T_m-5)^{\circ}\text{C}$ 30s;  $72^{\circ}\text{C}$ 1K/min;  $72^{\circ}\text{C}$ 7min;  
 $16^{\circ}\text{C}$   $\infty$ ;

### Site-specific Mutagenesis PCR:

System: Template: 2ul; Fastpfu: 1ul;  $5\times$ Fastpfu Buffer:10ul; Forward primer: 2ul;  
Reverse primer: 2ul; dNTP:4ul; Sterile water: 29ul; Total: 50ul

Procedure:  $95^{\circ}\text{C}$ 3min; 30 cycle: $95^{\circ}\text{C}$ 30s;  $(T_m-5)^{\circ}\text{C}$ 30s;  $72^{\circ}\text{C}$ 1K/min;  $72^{\circ}\text{C}$ 7min;  
 $16^{\circ}\text{C}$   $\infty$ ;

### Colony PCR

System:  $2\times$ Taq mix:10ul; Forward primer: 1ul; Reverse primer: 1ul; Sterile water: 8ul;  
Total: 20ul;

Procedure:  $95^{\circ}\text{C}$ 10min; 30 cycle: $95^{\circ}\text{C}$ 30s;  $(T_m-5)^{\circ}\text{C}$ 30s;  $72^{\circ}\text{C}$ 2K/min;  $72^{\circ}\text{C}$ 7min;  
 $16^{\circ}\text{C}$   $\infty$ ;

## Transformation

### Yeast

1.The cells were ligated with 2 to 5 ml of liquid YPD medium and incubated overnight at  $30^{\circ}\text{C}$  with a shaker.

2.The cultured cells were seeded in fresh YPD medium at a volume of 10% by volume.

3. Cultured at 30°C and 200 rpm for 4-5 hours.

Note: To make the cells complete at least twice mitosis, the conversion efficiency will remain unchanged after 3-4 mitosis

4. Add the cell culture solution to a 1.5 ml centrifuge tube, centrifuge at 4000 rpm for 5 minutes or 5000 rpm for 2 min.

5. Discard the supernatant and resuspend the cells with 1 ml of sterile water and centrifuge again.

6. The supernatant was discarded and the cells were resuspended with 1 ml of 100mM LiAc and allowed to stand for five minutes.

7. 4000 rpm for three minutes, discard the supernatant.

8. The SS-DNA was boiled for five minutes and rapidly cooled on ice.

9. Add the conversion mixture in sequence:

240ul PEG(50% w/v), 36ul 1.0M LiAc; 10ul SS-DNA (10.0mg/ml); x ul DNA (1~5ul);  
(74-x) ul Sterile water; Total:360ul;

10. Centrifuge the tube for one minute to allow the ingredients to mix well.

11. Incubated in a 30 degree incubator for 30 minutes; 20 to 30 minutes in a 42 degree water bath.

Note: The time of thermal shock and the type of bacteria related to the specific circumstances can be optimized to improve the conversion efficiency.

12. 4000 rpm for three minutes, and the supernatant was removed with a micropipette.

13. 4000 rpm for five minutes, discard the medium and wash twice with sterile water.

14. 100ul of sterile water to resuspend the cells (action to be gentle), coated plate, and then cultured 2 to 4 days.

Ecoli

1. Remove the loaded competent cells (BMTOP10, 100ul) from the -80 ° C refrigerator and insert into the ice immediately for 5 to 10 minutes.

2. Add 10ul of the ligation product and gently shake the ice for 30 min.

3. gently shake into the 42 ° C water bath heat shock 90s, quickly put back to the ice, standing for 5min.

4. Add 600ul of LB medium to the tube, mix gently and incubate at 37 ° C for 1 h.

5. Remove the coating rod from the alcohol, lit on the alcohol lamp, place it aside, take 50-300ul of the mixture, add to the LB plate containing antibiotics and apply evenly.

6. Marked and cultured in 37 ° C incubator for 18 h.

Ligation

T4 DNA:

System: Sterile water: 5ul; Digested fragments: 9ul; Carrier: 3ul;

T4 DNA ligase: 1ul; T4 DNA ligase buffer: 2ul; Total:20ul;

Procedure: Connect with an adapter for an hour.

Digestion

System: the plasmid and fragment needed to be digest : 16ul;

Restriction endonuclease: former and reverse both 1ul; 10× Buffer: 2ul; Total:20ul;

Procedure: The water bath was allowed to run for 4 h at the optimum temperature of the enzyme (usually 37 degrees). enzyme was inactivated for 20 min at 80 degrees.

## Plasmid Extraction

Before the Buffer PW is used, add the appropriate amount of ethanol (96-100%) according to the bottle label of the Buffer PW.

1. Place the spin column CP3 in a clean collection tube and add 500  $\mu$ l of Buffer BL to CP3. Centrifuge at 12,000 rpm ( $\sim$  13,400 x g) for 1 minute in a benchtop microcentrifuge. After discarding the waste in the collection tube, turn the rotary column CP3 back into the collection tube. (Use the rotating column on the day).
2. Centrifuge for 1 minute at 12,000 rpm ( $\sim$  13,400 x g) in a conventional benchtop microcentrifuge at room temperature (15-25 $^{\circ}$  C), 1-5 ml of bacterial cells were harvested in a microcentrifuge tube, and then all traces The supernatant opens the centrifuge tube by reversing until all the media has drained (for large volumes of bacterial cells, collect a tube through several centrifugation steps.)
3. Use 250  $\mu$ l of Buffer P1 (to ensure that the RNase A has been added) to resuspend the bacteria. The bacteria should be completely resuspended by up or down rotation or blowing until there is no cell mass. Note: After the suspension is suspended again, the cell mass is not visible, otherwise incomplete dissolution will reduce yield and purity.
4. Add 250  $\mu$ l of buffer solution P2 and mix gently with 6-8 inverting tubes. NOTE: Mix gently by pouring the tube. Do not whirl, as this will lead to cutting genomic DNA. If necessary, continue to turn the tube until the solution becomes viscous and slightly removed. Do not let the cracking reaction be carried out for more than 5 minutes. If the lysate is still unclear, reduce the bacterial precipitation.
5. Add 350  $\mu$ l of buffer P3 and mix gently with the tube upside down 6-8 times immediately. The solution should be cloudy. Centrifuge at 12,000 rpm for 10 minutes ( $\sim$  13 $^{\circ}$  C, 400 x g) in a benchtop centrifuge. Note: To avoid localized precipitation, the solution is thoroughly mixed immediately after adding buffer P3. If there is still white precipitate in the supernatant, please centrifuge again.
6. Transfer the supernatant from step 5 to spin column CP3 (place CP3 in the collection tube by decantation or pipetting). Centrifuge at 12,000 rpm ( $\sim$  13,400 x g) for 30-60 seconds. Dispose of the circulation and set the rotary column CP3 back to the header. Transfer the supernatant from step 5 to spin column CP3 (place CP3 in the collection tube by decantation or pipetting). Centrifuge at 12,000 rpm ( $\sim$  13,400 x g) for 30-60 seconds. Dispose of the circulation and set the rotary column CP3 back to the header.
7. (Optional, practically we have almost never used) Rotate column CP3 by washing 500  $\mu$ l of buffer PD and centrifuging at 12,000 rpm ( $\sim$  13,400  $\times$  g) for 30-60 seconds. Release the circulation and return the rotary column CP3 to the collection tube. It is recommended to use endA + strains such as JM series, HB101 and its derivatives or any wild-type strain with high levels of nuclease activity or high carbohydrate content to remove trace nuclease activity..
8. The rotary column CP3 was rinsed with 600  $\mu$ l of buffer PW (ensuring that ethanol was added (96% -100%) and centrifuged at 12,000 rpm ( $\sim$  13,400  $\times$  g) for 30-60 seconds. Release the Spin Column CP3 and return it to the collection.
9. Repeat step 8.
10. Centrifuge at 12,000 rpm ( $\sim$  13,400 x g) for another 2 minutes to remove residual wash buffer PW. Note that residual ethanol from buffer PW may inhibit subsequent enzymatic reactions. We recommend opening the CP3 lid and keeping it at room temperature for a period

of time to remove residual ethanol.

11. Place the rotary column CP3 in a clean 1.5 ml microcentrifuge tube. To elute the DNA, 50-100  $\mu$ l of buffer EB was added to the center of the rotary column CP3 for 2 minutes and centrifuged at 12,000 rpm ( $\sim 13,400 \times g$ ) for 2 minutes. Note: If the volume of the elution buffer is less than 50ul, the recovery efficiency may be affected. The pH of the elution buffer has a certain effect on the eluate; buffer EB or distilled water (pH 7.0-8.5) suggests elution of plasmid DNA. For long-term preservation of DNA, it is recommended to elute in buffer EB and store at  $-20^{\circ} C$  because the DNA stored in water undergo acid hydrolysis. Step 11 is repeated to improve the efficiency of the recovery of the plasmid.

Media component:

LB medium:

1. weight the component: 5g/L Yeast Extract. 10g/L NaCl. 10g/L Tryptone

Note:solid medium: add 20g/L agar

2. The above materials are fully dissolved by water.

3. Sterilize the medium for 20 minutes at  $121^{\circ} C$ .

YPD medium

1. weight the component: 10g/L Yeast Extract. 20g/L Tryptone

Note: If glucose is needed, 5g/L glucose

Note: solid medium: add 20g/L agar

2. The above materials are fully dissolved by water.

3. Sterilize the medium for 20 minutes at  $121^{\circ} C$ .

Note:if glucose is added, Sterilize the medium for 20 minutes at  $115^{\circ} C$ .

Auxotrophic medium

1. weight the component: 6.7g/L YNB. 0.6g/L Non - essential amino acids 20mg/L His.

20mg/L Trp. 20mg/L URa. 100mg/L Leu Note:Add His Trp URa Leu according to the auxotrophic. Note: If glucose is needed, 20g/L glucose

Note: solid medium: add 20g/L agar

2. The above materials are fully dissolved by water.

3. Sterilize the medium for 20 minutes at  $121^{\circ} C$ .

Note: If glucose is added, Sterilize the medium for 20 minutes at  $115^{\circ} C$ .

Fluorescence microscopy

1.Clean the coverslip and slide with ethanol

2.The bacteria droplets were dropped on the slide and covered with a coverslip from the end of the bacteria solution gently on the slide.

3. Turn on the fluorescent actuator, place the slide on the stage, first turn on the microscope light source, turn off the fluorescent channel, use the appropriate objective lens, by adjusting the coarse focus screw and fine focus spiral to make the image clear. The image is a bright field image.

4.Close the microscope light source, call the fluorescent channel, and observe the dark field image.

Gibson Assembly

1. First prepare the following IRB and AMM buffers.

IRB: System: 1M Tris-Cl pH7.5 1.5ml; each dNTP 100mM 30ul;  
50mM NAD 300ul; PEG 8000 750mg;  
Water to 3ml (500ul);

AMM: 5× IRB 80ul; Epicentre T5 exonuclease 1u/ul (diluted in storage buffer) 1.6ul;  
NEB Phusion DNA pol 5ul; 50mM MgCl<sub>2</sub> 80ul; 100mM DTT 40ul;  
water 53.4ul;

2. Add 6.5ul AMM to 2.5ul of equimolar mixed overlapping DNA fragments, then add 1ul NEB Taq DNA ligase (40u/ul) ;

3. Pipet mix briefly .

4. Incubate 1h at 50°C

5. Transform cells with nomore than 10ul (lawn danger if more).

#### Yeast genome extraction

Ensure that Buffer GD and Buffer PW have been prepared with appropriate volume of ethanol (96-100%) as indicated on the bottle and shake thoroughly.

1. Pellet an appropriate amount of yeast cell (no more than 5 × 10<sup>7</sup> cells) in a microcentrifuge tube, centrifuge for 1 min at 12,000 rpm (~13,400 × g). Discard supernatant.

Note: If the sample is more than 700 μl, repeat the centrifugation process to collect all yeast cells into one tube.

2. Lysis (enzyme method) to crack yeast cell wall: Add 600 μl Sorbitol Buffer and 50 U Lyticase (should be prepared by user, Cat. No. RT410). Mix thoroughly, and incubate at 30° C for 30 min. Centrifuge for 10 min at 4,000 rpm (~1500 × g). Discard supernatant.

Note: Above protocol is optimized for processing 5 × 10<sup>7</sup> yeast cells. Lysis time and Lyticase concentration should be varied according to the type of strain and amount of yeast cell processed.

3. Add 200 μl Buffer GA to resuspend the cell pellet. Mix thoroughly by vortex.

Note: If RNA-free genomic DNA is required, add 4 μl RNase A (100 mg/ml, should be prepared by user, Cat. no. RT405-12). Mix by vortex for 15 s, and incubate for 5 min at room temperature (15-25° C).

4. Add 20 μl Proteinase K. Mix thoroughly.

5. Add 220 μl Buffer GB to the sample, mix thoroughly by upsides and down, and incubate at 70° C for 10 min to yield a homogeneous solution. Briefly centrifuge the microcentrifuge tube to remove drops from the inside of the lid.

Note: White precipitates may form when Buffer GB is added. They will not interfere with the procedure and will dissolve during the heat incubation at 70° C. If precipitates do not dissolve during heat

incubation, it indicates that the cell is not completely lysed and may result in low yield of DNA and impurity in DNA.

6. Add 220  $\mu$ l ethanol (96-100%) to the sample, and mix thoroughly by upsides and down. White precipitates may form on addition of ethanol. Centrifuge briefly to remove drops from the inside of the lid.

7. Pipet the mixture from step 6 into the Spin Column CB3 (in a 2 ml collection tube) and centrifuge at 12,000 rpm ( $\sim 13,400 \times g$ ) for 30 s. Discard flow-through and place the spin column into the collection tube.

8. Add 500  $\mu$ l Buffer GD (Ensure ethanol has been added) to Spin Column CB3, and centrifuge at 12,000 rpm ( $\sim 13,400 \times g$ ) for 30 s, then discard the flow-through and place the spin column back to the collection tube.

9. Add 600  $\mu$ l Buffer PW (Ensure ethanol has been added) to Spin Column CB3, and centrifuge at 12,000 rpm ( $\sim 13,400 \times g$ ) for 30 s. Discard the flow-through and place the spin column back to the collection tube.

10. Repeat step 9.

11. Centrifuge at 12,000 rpm ( $\sim 13,400 \times g$ ) for 2 min to dry the membrane completely. Open lid of CB3 and stay at room temperature for a while to dry the membrane completely.

Note: The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with down-stream reactions.

12. Place the Spin Column CB3 in a new clean 1.5 ml microcentrifuge tube, and pipet 50-200  $\mu$ l Buffer TE or distilled water directly to the center of the membrane.

Incubate at room temperature (15-25°C) for 2-5 min, and then centrifuge for 2 min at 12,000 rpm ( $\sim 13,400 \times g$ ).

13. *Optional:* For increased DNA concentration, add the solution obtained from step 12 to the center of membrane again.

Incubate at room temperature (15-25°C) for 2-5 min, and then centrifuge for 2 min at 12,000 rpm ( $\sim 13,400 \times g$ ).

Note: The volume of elution buffer should not be less than 50  $\mu$ l, or it may affect the recovery efficiency. What's more, the pH value of elution buffer have influence in eluting, we suggest use buffer TE or distilled water (pH 7.0-8.5) to elute gDNA. For long-term storage of DNA, eluting in Buffer TE and storing at -20°C is recommended, since DNA stored in water is subject to acid hydrolysis.

## 1) cell preparation

1. Cultures of 5 ml of yeast to early logarithmic growth (10<sup>6</sup>-10<sup>7</sup> cells / ml).
2. Add 10 to 10 volumes of formaldehyde to the culture medium (the final concentration of formaldehyde was 3.7% and the standard mother liquor was 37%).
3. The cells were cultured in formaldehyde for at least 1 hour.
4. Cells were collected by centrifugation at 2500 rpm and washed once with 0.1 mol / L potassium phosphate (pH 7.5).
5. The cells were suspended in 1 ml of 50 mg / ml digestive enzyme 100 T or 50 units / ml of the cytolytic enzyme [dissolved in 0.1 mol / L potassium phosphate solution containing 2 ml / ml 6-mercaptoethanol].
6. And incubated at 30 ° C for 30 minutes. The generation rate of protoplasts was examined by phase contrast microscopy. Cells should be black or translucent gray, bright cells (refractors) are not fully digested. The bacteria are digested overnight.
7. Cells were collected by centrifugation at 2500 rpm and suspended in 1 ml of PBS.

## 2) dyeing

1. 10 ml of 1 mg / ml of polylysine was placed in each of the Teflon masked slides (10-well slides), washed with distilled water and dried.
2. Place 10 ml of curable cells into each well and after a few minutes, wash with PBS three times. Check the slides with a microscope to ensure proper density without aggregation.
3. This procedure is optional (no antibody is required for this experiment but is recommended): Soak the slides in cold methanol (-20 ° C) for 6 minutes and then place in cold acetone (-20 ° C) for 30 seconds. Physiological production of flat cells, contribute to the observation of cytoskeleton. For some antibody-antigen conjugates, these steps are required to reactivate and the wells are rewired with PBS.
- 4, add 15ml of PBS + 3% BSA (bovine serum albumin) composed of blocking buffer to the glass hole. In the moisturizing box, such as padded with wet tissue in a flat plate for 30 minutes. It is important that during this period the slides can not be allowed to penetrate (BSA can reduce the non-specific antibody binding to the slide by blocking the protein binding site).
5. Remove the blocking buffer and wash twice with PBS + 3% BSA.

6. Add 10 ~ 15ml of the first antibody (dissolved in PBS + 3% BSA) to the slide hole and incubate in a humidifier for 1 hour. Purify the antibody or monoclonal antibody with affinity, if possible, with a homologous control that lacks the antigen of interest. It is also helpful to make a control without a first antibody.
7. The first antibody was aspirated and washed 3 times with PBS + 3% BSA.
8. Repeat steps 6 to 7 (incubated in the dark) with fluorescent secondary antibody.
9. The second antibody was aspirated and washed 3 times with PBS + 3% BSA.
10. Add 10 ~ 15ml of 1  $\mu$ g / ml DAPI to the slide hole, culture for about 1 minute, wash with PBS 3 times.
11. Suck out the PBS, add a small drop of each hole sealant, put the slide, to avoid the bubble in the hole. Remove the extra sealant with a paper towel, be careful not to move the coverslip, clean with a clean nail polish, save at -20 ° C.