Uppsala iGEM 2019

Protein Expression Protocols

Uppsala iGEM 2019 **1**

Protein Expression Protocols **1**

1. Agarose gel 3
2. Buffers and solutions 3
    2.1. 0.02% Biotin 3
    2.2. 1 M CaCl2 3
    2.3. 0.5 M EDTA pH 8.0 3
    2.4. 50% Glycerol 4
    2.5. 1 M H2KPO4 pH 6 4
    2.6. 1 M KCl 4
    2.7. 5% methanol solution 4
    2.8. 1 M MgSO4 4
    2.9. 0.2 M NaOH 1% SDS 4
    2.10. PBS buffer 5
    2.11. 50 mM phosphate buffer with 1 M ammonium sulphate, pH 7.5 5
    2.12. 1 M potassium phosphate buffer, pH 6.0 5
    2.13. SDS sample buffer **5**
        2.13.1. 1.25X Sample Loading Buffer (SLB) with 0.8% Triton 5
        2.13.2. 5X Sample Loading Buffer (SLB) 5
    2.14. SDS 10x Running buffer 6
    2.15. Sodium acetate, 3M 6
    2.16. 3M Sodium Acetate solution pH 5.3 6
    2.17. 1M Sorbitol 6
    2.18. Sorbitol freezing buffer [1] 6
    2.19. TE buffer 7
    2.20. Tris-HCl, 0.5M pH 6.8 7
    2.21. Tris-HCl, 1M pH 8.0 7
    2.22. YNB [1] 7
3. Colony PCR of Pichia Pastoris [1] 8
4. Electroporation yeast (including making competent cells) [1] 9
5. Ethanol precipitation of DNA 9
6. Expression of yeast [1] 10
7. Gel extraction [2] 11
8. Glycerol stock [3] 11

9. Harvest expression culture 11
10. Horseradish Peroxidase extraction from horseradish 11
11. Linearization of plasmid 12
12. Medias [1] 12
12.1. Buffered Glycerol-complex Medium (BMGY) 12
12.2. Buffered Methanol-complex Medium (BMMY) 12
12.3 Minimal Methanol (MM) and Minimal Dextrose (MD) plates 12
12.4. Yeast Extract Peptone Dextrose Medium (YPD) 13
12.5. Yeast Extract Peptone Dextrose Medium with Sorbitol (YPDS) 13
13. Midiprep [4] 13
14. Miniprep E.coli [5] 14
15. MutS and Mut+ testing [1] 14
16. Native SDS-Page gel preparation 14
17. PCR for analysis [1] 15
18. PCR Purification [6] 16
19. Phenol/chloroform extraction of DNA 16
20. Restriction digestion [3] 16
21. SDS-gel preparation 16
22. SDS-sampling and handling of samples 17

Sources 18

1. Agarose gel

Materials:

- 50 mL 1x TBE buffer
- 0.5 g agarose
- 5 µL SYBR safe

Procedure:

1. Add 1x TBE buffer and agarose to a e-flask.
2. Heat the solution in the microwave until the agarose is completely dissolved
3. Let cool until cool enough to touch.
4. Add SYBR safe.
5. Pour into the tray.
6. Load samples
    a. 4 µL 6x purple loading dye #B7025S was added to each of the 20 µL digested samples and to 20 µL samples of 200 ng undigested plasmid in water
7. Gel put in electrophoresis machine and ladder and samples loaded. Run at 120 Volt, 100 milliampere for about 1 h.

2. Buffers and solutions

2.1. 0.02% Biotin

Materials for 100 mL:

- 20 mg biotin

Procedure:

1. Dissolve 20 mg biotin in 100 ml of water and filter sterilize using 0.45 µm filter. Store at 4°C.

2.2. 1 M $CaCl_2$

Materials for 200 mL:

- 22.2 g $CaCl_2$

2.3. 0.5 M EDTA pH 8.0

Materials for 100 mL:

- 14.61 g of EDTA

Procedure:

1. Dissolved EDTA in 60 mL of water, adding NaOH pellets to raise the pH to ~8.0, diluting to a final volume of 100 mL
2. Final pH adjustment with 10% HCl and 1 M NaOH to pH 8.0.
3. The EDTA was made to dissolve by the high pH and by heating the measuring cylinder with hot water streaming along the sides. The solution was then autoclaved.

2.4. 50% Glycerol

Material for 30 mL:

- 17.65 mL of 85% glycerol

Procedure:

1. Dissolve 85% glycerol in water to a final volume of 30 mL.
2. Autoclave the glycerol solution.

2.5. 1 M H_2KPO_4 pH 6

Materials for 800 mL:

- 108.87 g H_2KPO_4

Procedure:

1. H_2KPO_4 was dissolved in about 0.5 L ddH_2O and the pH was adjusted to pH 6 using 5M NaOH. The solution was diluted to a final concentration of 800 mL and then autoclaved.

2.6. 1 M KCl

Materials for 200 mL:

- 14.9 g KCl

2.7. 5% methanol solution

Material for 200 mL:

- 10 mL 100% methanol

Procedure:

1. The methanol was diluted to a final volume of 200 mL.

2.8. 1 M $MgSO_4$

Materials for 200 mL:

- 24.7 g $MgSO_4$

2.9. 0.2 M NaOH 1% SDS

Materials for 100 mL:

- 20 mL 1 M NaOH
- 5 mL 20% SDS
- 75 mL distilled water

Procedure:

1. Solutions mixed to the final volume.

2.10. PBS buffer

Materials for 500 mL:

- 4 g NaCl
- 0.1 g KCl
- 1.36 g Na_2HPO_4 (hydrated)
- 0.12 g KH_2PO_4
- HCl for pH adjustment

Procedure:

1. Mix all chemicals and adjust pH to 7.4

2.11. 50 mM phosphate buffer with 1 M ammonium sulphate, pH 7.5

Material for 50 mL:

- 0.505 g Sodium (Hydrogen) Phosphate 7-hydrate
- 0.739 g sodium phosphate monobasic
- 6.757 g Ammonium sulphate

Procedure:

1. Deionized water and chemicals mixed to final volume of 50 mL. Sterile filtered with 0.45 µm filter.

2.12. 1 M potassium phosphate buffer, pH 6.0

Materials for 1 L:

- 132 ml of 1 M K_2HPO_4
- 868 ml of 1 M KH_2PO_4

Procedure:

1. Combine 132 ml of 1 M K2HPO4, 868 ml of 1 M KH2PO4 and confirm that the pH = 6.0 ± 0.1 (if the pH needs to be adjusted, use phosphoric acid or KOH).
2. Sterilize by autoclaving and store at room temperature.

2.13. SDS sample buffer

2.13.1. 1.25X Sample Loading Buffer (SLB) with 0.8% Triton

Materials for 2 mL:

- 500 µl 5X SLB
- 16 µl Triton X-100, 100%
- 1 484 µl ddH_2O

Procedure:

1. Components mixed to the final volume.

2.13.2. 5X Sample Loading Buffer (SLB)

Materials:

- 0.25 M Tris-HCl pH 6.8
- 0.5 M DTT
- 10 % SDS
- 50 % Glycerol
- 0.5 % bromophenol blue

2.14. SDS 10x Running buffer

Materials för 1 L:

- 30 g Tris base
- 144 g glycine
- 10 g SDS

Procedure:

1. 200 mL deionized water was added to a 1 L measuring cylinder.
2. The chemicals were added to the cylinder and 750 mL water was added to the solution.
3. The pH was adjusted using concentrated HCl to a final pH of 8.3. Water was added to the final volume of 1 L.

2.15. Sodium acetate, 3M

Materials for 10 mL:

- 2.46 g of sodium acetate.

Procedure:

1. Sodium acetate mixed with deionized water to a final volume.

2.16. 3M Sodium Acetate solution pH 5.3

Materials for 50 mL:

- 12.30 g of sodium acetate
- HCl and NaOH for pH adjustment

Procedure:

1. Dissolved sodium acetate to 30 mL of water, adding 10% HCl to lower pH to 5.3.
2. Adding water to final volume of 50 mL, adjusting last pH with 10% HCl and 1 M NaOH.

2.17. 1M Sorbitol

Materials for 100 mL:

- 18.21 g of sorbitol

Procedure:

1. Dissolve sorbitol into water to get 100 mL of final solution.
2. Sterile filter with 0.45 µm filter.

2.18. Sorbitol freezing buffer [1]

Materials for 100 mL:

- 72.83 g sorbitol
    - Sorbitol (143 mL solution was prepared separately)
- 0.111 g $CaCl_2$
- 0.238 g HePeS
- NaOH and HCl for pH adjustment

Procedure:

1. A solution of $CaCl_2$ and HePeS was prepared and autoclaved.
2. Flask contains 50 mL of solution (for final volume 100 mL) with pH 7.5. Sorbitol not added. Stored on shelf over lab-bench.
3. The sorbitol was prepared separately and sterile filtered. Concentration 2.80 M.

2.19. TE buffer

Materials for 200 mL:

- 2 mL 1M Tris-HCl (pH 8.0)
- 0.4 mL EDTA

Procedure:

1. Mixing 2 mL 1M Tris-HCl (pH 8.0) and 0.4 mL EDTA and adding water to the final volume of 200 mL.
2. The solution was then autoclaved.

2.20. Tris-HCl, 0.5M pH 6.8

Materials for 250 mL:

- 15.14 g tris base.

Procedure:

1. Tris base was dissolved in ~half the final volume of water, pH adjusted with 12% HCl and concentrated NaOH, diluted to almost the total volume and the pH adjusted once more.

2.21. Tris-HCl, 1M pH 8.0

Materials for 250 mL:

- 45.43 g tris base.

Procedure:

1. The needed amount of Tris base was dissolved in ~half the final volume of water, pH adjusted with 12% HCl and concentrated NaOH, diluted to almost the total volume and the pH adjusted once more.

2.22. YNB [1]

Materials for 1 L:

- 134 g yeast nitrogen base with ammonium sulphate, without amino acids

Procedure:

1. 134 g of yeast nitrogen base with ammonium sulfate, without amino acids mixed with water to final volume 1000 mL. Sterile filtered with 0.45 µm filter. Stored in cold room.

3. Colony PCR of *Pichia Pastoris* [1]

Material:

- Extracting DNA:
  - 0.2 M Lithium acetate 1% SDS solution.
  - Ethanol 96-100 % and 70 %
- PCR master mix for 7 reactions:
  - 35 µL dNTPs
  - 1.75 µL forward primer
  - 1.75 µL reverse primer
  - 35 µL reaction buffer
  - 1.75 µL Taq polymerase
  - 267.75 µL nuclease free water
- For each reaction:
  - 49 µL master mix
  - 1 µL of template or water

Procedure:

1. Extracting DNA:
   a. Pick one yeast colony from the plate or spin down 100-200 µL of liquid yeast culture (OD_{600}=0.4). Suspend cells in 100 µL of 200mM LiOAc, 1 % SDS solution.
   b. Incubate for 5 minutes at 70°C.
   c. Add 300 µL of 96-100 % ethanol, vortex.
   d. Spin down DNA and cell debris at 15 000 g for 3 minutes.
   e. Wash pellet with 70 % ethanol
   f. Dissolve pellet in 100 µL of H_2O or TE and spin down cell debris for 15 seconds at 15 000 g.
   g. Use 1 µL of supernatant for PCR
2. PCR reaction
   a. Make a mastermix with the chemicals stated above.
   b. Add DNA/water to the tubes. Transfer 49 µL master mix to the 6 different tubes.
   c. PCR made according to instructions for pPICZaB in P. Pastoris:
      - 95 °C 5 min.
      - 95 °C 30 s
      - 55 °C 1 min
      - 72 °C 1 min 30 s
      - Goto step 2 X30
      - 72 °C 7 min
      - 4 °C ∞

4. Electroporation yeast (including making competent cells) [1]

Making competent cells:

1. Grow 5 ml of your Pichia pastoris strain in YPD in a 50 ml conical at 28°C overnight.
2. Inoculate 500 ml of fresh medium in a 2 liter flask with 0.1–0.5 ml of the overnight culture. Grow overnight again to an OD_{600} = 1.3–1.5.
3. Centrifuge the cells at 1,500 × g for 5 minutes at 4°C. Resuspend the pellet with 500 ml of ice-cold, sterile water.
4. Centrifuge the cells as in Step 3, then resuspend the pellet with 250 ml of ice cold, sterile water.
5. Centrifuge the cells as in Step 3, then resuspend the pellet in 20 ml of ice-cold 1 M sorbitol.
6. Centrifuge the cells as in Step 3, then resuspend the pellet in 1 ml of ice-cold 1 M sorbitol for a final volume of approximately 1.5 ml. Keep the cells on ice and use that day. Do not store cells.

Transformation protocol:

1. Mix 80 µl of the cells from Step 6 (previous page) with 5–10 µg of linearized DNA (in 5–10 µl sterile water) and transfer them to an ice-cold 0.2 cm electroporation cuvette.
   Note: For circular DNA, use 50–100 µg.
2. Incubate the cuvette with the cells on ice for 5 minutes.
3. Pulse the cells using the manufacturer's instructions for Saccharomyces cerevisiae.
4. Immediately add 1 ml of ice-cold 1 M sorbitol to the cuvette. Transfer the cuvette contents to a sterile 15-ml tube and incubate at 30°C without shaking for 1 to 2 hours.
5. Spread 10, 25, 50, 100, and 200 µl each on separate, labeled YPDS plates containing 100 µg/ml Zeocin™. Plating at low cell densities favors efficient Zeocin™ selection.
6. Incubate plates from 3–10 days at 28°C until colonies form.
7. Pick 10–20 colonies and purify (streak for single colonies) on fresh YPD or YPDS plates containing 100 µg/ml Zeocin™.

The instrument settings for the electroporation machine in step 2 was V = 1.5 kV; C=25 µF; R=200 Ω; t=4-10 ms.

5. Ethanol precipitation of DNA

Materials:

- 3 M sodium acetate pH 5.3
- 100% ethanol
- 70% ethanol

Procedure:

1. 1/10 volume of 3 M sodium acetate pH 5.3 added
2. 2.5 volumes of 100% ethanol added
3. Centrifuged to pellet DNA - 10min, 17 000 g
4. 300 µL of 70% ethanol added to wash, air dried for 15 min
5. Resuspended in 10 µL sterile ddH_20
6. Left -20 ˚C, 3 hours

6. Expression of yeast [1]

Easyselect Man protocol.
Mut^+ intracellular or Secreted:

1. Using a single colony, inoculate 25 ml of BMGY in a 250 ml baffled flask. Grow at 28°C in a shaking incubator (200 rpm) until culture reaches an OD_{600} = 2–6. The cells will be in log-phase growth.
2. Harvest the cells by centrifuging at 1,500–3,000 × g for 5 minutes at room temperature. Decant supernatant and resuspend cell pellet to an OD_{600} of 1.0 in BMMY medium to induce expression (approximately 100–200 ml).
3. Place culture in a 1 liter baffled flask.
4. Add 100% methanol to a final concentration of 0.5% methanol every 24 hours to maintain induction.
5. At each of the times indicated below, transfer 1 ml of the expression culture to a 1.5 ml microcentrifuge tube. Use these samples to analyze expression levels and determine the optimal time post-induction to harvest. Centrifuge at maximum speed in a tabletop microcentrifuge for 2–3 minutes at room temperature.
   Time points (hours): 0, 6, 12, 24 (1 day), 36, 48 (2 days), 60, 72 (3 days), 84, and 96 (4 days).
6. For secreted expression, transfer the supernatant to a separate tube. Store the supernatant and the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N_2 or a dry ice/alcohol bath.
   For intracellular expression, decant the supernatant and store just the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N2 or a dry ice/alcohol bath.
7. Analyze the supernatants and cell pellets for protein expression by Coomassie-stained SDS-PAGE.

Apart for methanol, 5 µL Vitamin B1 and Trace Metal mix to a final concentration of 0.05% was added to cultures containing HRP in step 4.
In step 5 and 6 the the samples were treated for SDS-PAGE according to Protocol 21: SDS-sampling and handling of samples.

MutS Intracellular or Secreted:

1. Using a single colony, inoculate 100 ml of BMGY in a 1 liter baffled flask. Grow at 28°C in a shaking incubator (200 rpm) until the culture reaches an OD_{600} = 2–6.
2. Harvest the cells by centrifuging at 1,500–3,000 × g for 5 minutes at room temperature. To induce expression, decant the supernatant, and resuspend cell pellet in BMMY medium using 1/5 to 1/10 of the original culture volume (approximately 10–20 ml).
3. Place in a 100 ml baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator to continue to grow.
4. Add 100% methanol to a final concentration of 0.5% every 24 hours to maintain induction.
5. At each of the times indicated below transfer 1 ml of the expression culture to a 1.5 ml microcentrifuge tube. These samples will be used to analyze expression levels and determine the optimal time post-induction to harvest.
   Centrifuge at maximum speed in a tabletop microcentrifuge for 2–3 minutes at room temperature.

Time points (hours): 0, 24 (1 day), 48 (2 days), 72 (3 days), 96 (4 days), 120 (5 days), and 144 (6 days).

6. For secreted expression, transfer the supernatant to a separate tube. Store the supernatant and the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N_2 or a dry ice/alcohol bath.
   For intracellular expression, decant the supernatant, and store just the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N2 or a dry ice/alcohol bath.
7. Analyze the cell pellets for protein expression by Coomassie-stained SDS-PAGE

Apart for methanol, 5 µL Vitamin B1 and Trace Metal mix to a final concentration of 0.05% was added to cultures containing HRP in step 4.
Samples treated for SDS-PAGE according to Protocol 21 in step 5 and 6.

7. Gel extraction [2]

Gel extraction made with *GeneJET Gel Extraction Kit* from Thermo Scientific according to manufacturer's instructions.

8. Glycerol stock [3]

Materials for 1 mL:

- 400 µL of 50% glycerol

Procedure:

1. Added 400 µL of 50% glycerol to 600 µL culture and mixed by pipetting.

9. Harvest expression culture

Procedure:

1. Cultures divided into falcon tubes and then harvested by centrifugation at 2500 g for 5 min in room temperature.
2. 2 mL of supernatant snap frozen and stored in -80°C.
3. Supernatant and pellet divided and the rest stored in -20°C.

10. Horseradish Peroxidase extraction from horseradish

Procedure:

1. Cut horseradish into small pieces, remove outer layer
2. Mix in blender with appropriate amount of PBS (Approximately 50 mL was used for one root)
3. Always keep on ice to prevent protein degradation
4. Sonicate 15 min on ice
5. Centrifuge 4000g 5 min 4 °C
6. Ammonium sulfate was added to supernatant for precipitated (30 % saturation)
7. 1 h incubation stirring at 4 °C
8. Pellet at 16100 g 30 min 4 °C
9. Supernatant ammonium sulfate precipitated (65 %)
10. 1 h incubation stirring at 4 °C

11. Pellet at 16100 g 30 min 4 °C
12. Pellets were resuspended in 1/10 volume 50 mM phosphate buffer with 1 M ammonium sulfate pH 7,5
13. Stored at -20 °C

11. Linearization of plasmid

Material for final volume 50 µL:

- Water added to the final volume 50 µL
- Plasmid solution (µL, 500 ng plasmid)
- 5 µL Reaction buffer (for Sac1)
- 2 µL Sac1

Procedure:

1. The materials were added together in that order as above for a final volume of 50 µL.
2. The tubes were on ice while the reaction buffer and enzymes were added.
3. The tubes were then incubated at 37 °C for 2 h.
4. Heat inactivated at 80 °C for 20 min.

12. Medias [1]

12.1. Buffered Glycerol-complex Medium (BMGY)

Materials for 1 L:

- 780 mL deionized water
- 100 mL 1M KH_2PO_4 (pH 6.0)
- 100 mL 13.4% YNB
- 2 mL 0.02% Biotin
- 20 mL 50% glycerol

Procedure:

1. Solutions added to autoclaved water.

12.2. Buffered Methanol-complex Medium (BMMY)

Materials for 1 L:

- 700 mL deionized water
- 100 mL 1M KH_2PO_4 (pH 6.0)
- 100 mL 13.4% YNB
- 2 mL 0.02% Biotin
- 100 mL 5% methanol
- For plates: 20 g agar

Procedure:

1. Solutions added to autoclaved water.
    a. For plates: Water mixed with agar before autoclaving.

12.3 Minimal Methanol (MM) and Minimal Dextrose (MD) plates

Materials for 500 mL MM plates:

- 400 mL water
- 7.5 g agar
- 50 mL 10x YNB
- 50 mL 10x MeOH

- 1 mL 500x Biotin

Materials for 500 mL MD plates:

- 400 mL water
- 7.5 g agar
- 50 mL 10x YNB (13.4%)
- 50 mL 10x Dextrose (20%)
- 1 mL 500x Biotin (0.02%)

Procedure:

1. Autoclaved 400 mL water + 7.5 g agar.
2. Added to YNB, MeOH and Biotin for MM plates and YNB, dextrose and Biotin the MD plates to the solution when cold enough.
3. Poured the plates and let them dry around a flame with the lids slightly opened for a while before closing the lids when the agar had solidified.

12.4. Yeast Extract Peptone Dextrose Medium (YPD)

Materials for 800 mL:

- 8 g yeast extract
- 16 g peptone
    - in 720 mL of water
- 80 mL 20% dextrose added later
- For plates: 16 g agar
- For selection media: 800 uL Zeocin

Procedure:

1. Yeast extract and peptone were mixed to a volume of 720 mL. The solution was then autoclaved.
2. Sterile 20% dextrose solution was then added. The media stored in cold room.

12.5. Yeast Extract Peptone Dextrose Medium with Sorbitol (YPDS)

Materials for 800 mL:

- 8 g yeast extract
- 16 g peptone
- 145.8 g
    - in 720 mL of water
- 80 mL 20% dextrose added later
- For plates: 16 g agar
- For selection media: 800 uL Zeocin

Procedure:

1. Yeast extract, peptone and sorbitol solved in ddH_2O to a final volume of 720 mL. Solution autoclaved.
2. Destrose stock-solution added.

13. Midiprep [4]

Midiprep of DNA made with *PureYield™ Plasmid Midiprep System* from Promega according to manufacturer's instructions.

14. Miniprep E.coli [5]

Miniprep of DNA made with *GenElute™ Plasmid Miniprep Kit* from Sigma Aldrich according to manufacturer's instructions.

15. Mut^S and Mut^+ testing [1]

Materials:

- MD plates - minimal dextrose medium
- MM plates - minimal methanol medium

Procedure:

Mut^+ in X-33

1. Use the plates containing the Zeo^R transformants and confirm the Mut^+ phenotype as described below.
2. Using a sterile toothpick, pick one colony and streak or patch one Zeo^R transformant in a regular pattern on both an MM plate and an MD plate, making sure to patch the MM plate first.
3. Use a new toothpick for each transformant and continue until 10 transformants have been patched (1 plate).
4. To differentiate Mut^+ from Mut^S, make one patch for each of the controls (GS115/MutS Albumin and GS115/pPICZ/*lac*Z Mut^+) onto the MD and MM plates.
5. Incubate the plates at 28 °C for 2 days.
6. After 2 days or longer at 28 °C, score the plates. Mut^+ strains will grow normally on both plates, while Mut^S strains will grow normally on the MD plate but show little or no growth on the MM plate.

16. Native SDS-Page gel preparation

Separation Gel

Materials for 2 gels (10 mL):

- 4.2 mL water
- 3.3 mL Acrylamide/Bis 30%
- 2.5 mL 1.5M Tris-HCl pH 8.8
- 100 µL APS
- 10 µL TEMED

Procedure

1. Prepare the gel casts in holders. Fill the holder with water to check for leakage after 5 min. After leakage check, pour all water from gel casts and dry residual with paper towel
2. To prepare separation-gel, ix the reagents listed in the table below with the proper volumes to receive a gel with desired composition.
3. NOTE! Add TEMED and APS last, solidification of the gel will occur when these reagents are added.
4. Pipette gel mixture into gel casts up to a height approximately 1 cm below the gels comb.
5. Fill the remaining cast with isopropanol (if not available use distilled water).

6. Allow to harden for 30-60 min.
7. Pour all isopropanol (or water) from gel casts and dry residual with paper towel.

Stacking Gel
Materials for 2 gels (5 mL):

- 3.1 mL water
- 650 µL Acrylamide/Bis 30%
- 1.25 mL Tris HCl 0.5M pH 6.8
- 50 µL APS 10%
- 5 µL TEMED

Procedure:

1. To prepare stacking-gel, in a tube mix the reagents listed in table 4 below with the proper volumes to receive a gel with desired composition.
2. NOTE! Add TEMED and APS last, solidification of the gel will occur when these reagents are added.
3. Pipette gel mixture on top of separating gel to fill cast and remove all air bubbles.
4. Fill the remaining cast with isopropanol (if not available use distilled water).
5. Insert the comb whilst the gel is still in liquid form. The comb will form wells to load samples when the gel turns solid.
6. Allow to harden for 45-60 min.

17. PCR for analysis [1]

Materials:

- PCR for 7 reaktions:
    - 35 µL dNTPs
    - 1.75 µL forward primer
    - 1.75 µL reverse primer
    - 35 µL reaktion buffer
    - 1.75 µL Taq polymerase
    - 267.75 µL nuclease free water
    - 1 µL of template or water for each reaction

Procedure:

1. Make a mastermix with the chemicals stated above.
2. Add DNA/water to the tubes. Transfer 49 µL master mix to the 6 different tubes.
3. PCR made according to instructions for pPICZaB in P. Pastoris:
4. The PCR settings was:
    - 95 °C 5 min.
    - 95 °C 30 s
    - 55 °C 1 min
    - 72 °C 1 min 30 s
    - Goto step 2 X30
    - 72 °C 7 min
    - 4 °C ∞

18. PCR Purification [6]

PCR Purification done with the *GeneJET PCR Purification Kit* (catalog number K0701) from Thermo Scientific according to manufacturer's instructions.

19. Phenol/chloroform extraction of DNA

Materials:

- 25:24:1 Phenol:Chloroform:Isoamyl alcohol

Procedure:

1. Added 1 volume of 25:24:1 Phenol:Chloroform:Isoamyl alcohol.
2. Vortexed ~20 seconds
3. Centrifuged 5 min. at 16.000 g. Repeated for some samples, because of difficulties with phases separation
4. Aqueous phase transferred to the new tube
5. Continued with Ethanol precipitation

20. Restriction digestion [3]

Material for final volume 20 µL:

- 200 ng DNA from mini-prep kit from Sigma-Aldrich
- 2 µL 10x reaction buffer
- 0.4 µL EcoRI
- 0.4 µL PstI
- ddH_20 to a final volume of 20 µL

Procedure:

1. Add ddH_20 to a tube.
2. Add DNA.
3. Add 10x reaction buffer
4. Add EcoRI
5. Add PstI
6. Incubate for 37 °C for 30 min.
7. Inactivate in 80 °C for 20 min.
8. Store on ice.

21. SDS-gel preparation

Separation Gel
Materials for 2 gels (10 mL):

- 4.1 mL water
- 3.3 mL Acrylamide/Bis 30%
- 2.5 mL 1.5M Tris-HCl pH 8.8
- 100 µL SDS 10%
- 100 µL APS 10%
- 10 µL TEMED

Procedure:

1. Prepare the gel casts in holders. Fill the holder with water to check for leakage after 5 min.After leakage check, pour all water from gel casts and dry residual with paper towel
2. To prepare separation-gel, ix the reagents listed in the table below with the proper volumes to receive a gel with desired composition.
3. NOTE! Add TEMED and APS last, solidification of the gel will occur when these reagents are added.
4. Pipette gel mixture into gel casts up to a height approximately 1 cm below the gels comb.
5. Fill the remaining cast with isopropanol (if not available use distilled water).
6. Allow to harden for 30-60 min.
7. Pour all isopropanol (or water) from gel casts and dry residual with paper towel.

Stacking Gel
Materials for 2 gels (5 mL):

- 3.1 mL water
- 650 µL Acrylamide/Bis 30%
- 1.25 mL Tris HCl 0.5M pH 6.8
- 50 µL SDS 10%
- 50 µL APS 10%
- 5 µL TEMED

Procedure:

1. To prepare stacking-gel, in a tube mix the reagents listed in table 4 below with the proper volumes to receive a gel with desired composition.
2. NOTE! Add TEMED and APS last, solidification of the gel will occur when these reagents are added.
3. Pipette gel mixture on top of separating gel to fill cast and remove all air bubbles.
4. Fill the remaining cast with isopropanol (if not available use distilled water).
5. Insert the comb whilst the gel is still in liquid form. The comb will form wells to load samples when the gel turns solid.
6. Allow to harden for 45-60 min.

22. SDS-sampling and handling of samples

Materials:

- 5xSDS-sample buffer
- 1.25xSDS-sample buffer with 0.8% Triton X-100

Procedure:

1. Taken 1 mL of expression culture. Sample put on ice.
2. 100 µL transformed to a eppendorf tube
3. Cells spun down 3 min, 7 000 x g
4. 80 µL of supernatant transferred to a new tube
5. The supernatant sample mixed with 20 µL 5XSLB
6. The rest of the supernatant discarded
7. The pellet sample mixed with 20 µL of 1.25xSLB + 0.8% Triton X-100
8. Samples boiled on heating block for 10 min at 95˚C

9. Samples frozen and stored at -20˚C
10. 5 µL of cell pellet and 10 µL of supernatant sample was loaded to the gel.

Sources

[1] Invitrogen Manual EasySelect™ Pichia Expression Kit, Rev. Date 18 June 2010, For Expression of Recombinant Proteins Using pPICZ and pPICZα in Pichia pastoris, Cat. no. K1740-01, Manual part no. 25-0172

[2] Sigma-Aldrich protocol for product K0691.

[3] Liljeruhm, Josefine, et al. ‘Synthetic Biology: A Lab Manual.’ World Scientific, 2014.

[4] Promega protocol for product A2492

[5] Sigma-Aldrich protocol for product PLN70

[6] Thermofischer protocol for product K0701