PROTOCOLS

1. **Dna Resuspension**
2. **Competent Cells**
   - *E.Coli*
   - *Pichia Pastoris*
3. **Transformation**
   - *E.Coli*
   - *Pichia Pastoris*
4. **Cryopreservation**
   - *E.Coli*
   - *Pichia Pastoris*
5. **Screening**
6. **Miniprep**
7. **Culture Media**
   - *E.Coli*
   - *Pichia Pastoris*
8. **Antibiotic Stocks**

Long Protocols

Quick Protocols

Quick Protocols

Resuspending Dry DNA

Reagents

1) TE buffer (10mM Tris: 0.1 mM EDTA; pH 8.0)

2) Dry-Freeze oligonucleotides

3)High – grade water (nuclease-free)

Components

1) Centrifuge

2) Centrifuge sterile conical tubes

3) Pipettes

Protocol

1) Centrifugation of the tubes containing the oligonucleotides

2) Resuspend the oligos in TE buffer in a sterile tube.

3) Alternatively, use nuclease-free water, this means high grade water

4) To make the resuspension in the desired concentration, Resuspension calculations can be made using yield information contained on IDT product specification sheets and on the oligo tube or use a web tool: http://www.idtdna.com/analyzer/Applications/resuspensioncalc/

5) Storage of the resuspended DNA is at 4 C, not exceed more than 7 months.

Notas Importantes:

It's important to centrifuge the tubes containing the oligonucleotides because during the shipping of the tubes, some oligonucleotides could be loose in the tube.

DEPC water should be avoided during resuspension due to the overly acidic nature of the compound, this could harm the oligonucleotides.

For hard to suspend oligos, heat the oligonucleotide at 55 C for 1 – 5 minutes, then vortex thoroughly.

Reference http://www.idtdna.com/pages/decoded/decoded-articles/core-concepts/decoded/2011/03/16/dna-oligonucleotide-resuspension-and-storage

Preparation of competent *E. coli* cells

Reagents

1) Luria Broth (LB) medium

2) *E. coli* strain

3) Sterile 60 mM cold CaCl2 solution (60 mM CaCl2, 15% glycerol, 10 mM piperazine-N, N´- bis (2- hydroxypropanesulfonic acid)

Components

1) Shaker and water bath shaker

2) Spectrophotometer

3) Centrifuge

4) Erlenmeyer flask

5) 5- ml glass or plastic pipettes precooled in refrigerator (sterile)

Protocol:

1) Inoculate 5 ml of LB media with *E. coli* and grow overnight at 37°C in a shaker.

2) Inoculate 1 ml of overnight culture into a sterile Erlenmeyer flask containing 100 ml of LB broth.

3) Shake culture in a 37°C water bath until cell density reaches mid-log growth phase (about 5 x 10^7cells/ml). This should take 2 to 4 hours. The growth rate of the culture is determined by removing a 1-ml aliquot at various times and reading the optical density at 550 nm wavelength using a spectrophotometer. The relationship between optical density and cell number will vary depending on the bacterial strain.

4) Chill the culture on ice for 10 minutes.

5) Spin the cell suspension at 4,000 g in a centrifuge for 5 minutes at 4°C.

6) Discard the supernatant.

7) Resuspend the cells in half the volume (50 ml) of the original culture with ice-cold sterile 60 mM $CaCl_2$.

8) Place the cell suspension in an ice bath for 30 minutes.

9) Centrifuge the suspension at 4,000 g for 5 minutes at 4°C. Discard the supernatant

10) Gently resuspend the cells in 5 ml of sterile ice-cold $CaCl_2$ using precooled pipettes. Cells will remain competent for up to 24 hours at 4°C. Transformation efficiency increases four- to six-fold during this time. For long-term storage, dispense 250-ml aliquots into prechilled, sterile microfuge tubes and store at -70°C until needed. Depending on the strain used, some *E. coli* cells will remain competent to take up DNA for as long as 6 months.

Notes

The transformation efficiency is about 1-5x10^6 /ul DNA when using the competent cells prepared with this method.

Important: all steps after harvesting the cell should be done on ice (or at 4 °C)

The frozen competent cells are stable for 6 months, but once a tube is taken from the freezer and thawed, any unused portion should be discarded.

References

Transformation of Escherichia coli Made Competent by Calcium Chloride Protocol by Anh-Hue T. Tu

http://www.microbelibrary.org/component/resource/laboratory-test/3152-transformation-of-escherichia-coli-made-competent-by-calcium-chloride-protocol

Preparation of competent *Pichia* cells

Reagents

1) YPD (Yeast Extract Peptone Dextrose) medium

2) Sorbitol solution containing ethylene glicol and DMSO for the preparation of competent cells 75 mL*

3) Pichia pastoris strain

* Invitrogen Kit Components

Components

1) 30°C rotary shaking incubator

2) 50 ml, Centrifuge sterile conical tubes (floor or table-top)

3) 1.5 ml sterile screw-cap micro centrifuge tubes

4) -80 C freezer

5) Styrofoam box or paper towels

Protocol:

1) Streak a YPD plate with your Pichia pastoris strain such that isolated, single colonies will grow.

2) Incubate the plate at 28-30°C for 2 days.

3) Equilibrate Solution 2 to room temperature.

4) Inoculate 10 ml of YPD with a single colony of your Pichia strain. Grow overnight at 28-30°C in a shaking incubator (250-300 rpm)

5) Dilute cells from the overnight culture to an OD600 of 0.1-0.2 in 10 ml of YPD. Grow the cells at 28-30°C in a shaking incubator until the OD600 reached 0.6-1.0. This will take approximately 4 to 6 hours.

6) Pellet the cells by centrifugation at 500 x g for 5 minutes at room temperature. Discard the supernatant.

7) Resuspend the cell pellet in 10 ml of the Solution 2. No incubation time is required.

8) Pellet the cells by centrifugation at 500 x g for 5 minutes at room temperature. Discard the supernatant.

9) Resuspend the cell pellet in 1 ml of the Solution 2. The cells are now competent.

10) Aliquot 50 to 200 µl of competent cells into labeled 1.5 ml sterile screw-cap micro centrifuge tubes.

11) The cells may be kept at room temperature and used directly for transformation or frozen for future use. To freeze cells, place tubes in a Styrofoam box or wrap in several layers of paper towels and place in an -80°C freezer. It is important that the cells freeze down slowly. **Do not snap-freeze the cells in liquid nitrogen.**

Notes

Cells can be thawed and refrozen several times without significant loss in transformation efficiency.

higher transformation efficiencies are often obtained with frozen versus freshly prepared cells.

References

http://tools.invitrogen.com/content/sfs/manuals/easycomppp_man.pdf

E. coli Transformation

Reactants, solutions and media:

1)SOC medium

2) LB medium

3) Transforming plasmid

4) Competent cells

Equipment and Materials

1) Micropipette

2) Water bath

3) Media Plates

4) Microcentrifuge tubes

5) Shaking incubator

6) Centrifuge

Protocol

1) Desfrost the CaCl2 treated 50µl aliquots of competent cells previously stored at -80°C at ice for 30 min.
2) Pipette five µl of plasmid that contain a total of 50pg DNA over competent cells.
3) Mix these cells gently by tapping 4-5 times.
4) Incubate on ice for 30 min
5) Follow by a heat shock treatment at 42°C for 30 seconds.
6) After treatment, incubate the cells at ice for 2 min and by add 250 µl of SOC media to each vial.
7) Incubate the vials at 37°C for 1 hr at 225 rpm in a shaking incubator.
8) Dilute the cultures in LB medium and plate 50-100µl of each culture in triplicates on ampicillin containing media plates.
9) Incubate overnight at 37°

Notes:

Times on ice and water bath are critical, so be careful.

Reference

Singh, M. et al "Plasmid DNA Transformation in Escherichia Coli: Effect of Heat Shock Biotechnology and Biochemistry ISSN 0973-2691 Volume 6 Number 4 (2010) pp. 561–568

Yeast Transformation

Reactants, solutions and media:

1)YPAD/SC

2) Transformation Mix

-PEG 3500

-LiAc

-SS-carrier DNA

-Plasmid DNA

Equipment and Materials

1)Rotatory Shaker

2)Microcentrifuge

3)Vortex

4)Ice

5)Water bath

6)Microp

Protocol

1) Biol a sample of carrier DNA for 5 mins and chill in an ice/water bath.

2) Pipette cell suspensión to a 1.5ml microcentrifuge tube, centrifuge for 30sec and discard supernatant.

3) Add water until 1ml of volume and vortex vigorously to resuspend cells.

4) Pipette 100µl samples into 1.5ml microfuge tubes, one for each transformation, centrifuge for 30 sec and remove supernatant.

5) Make enough Transformation Mix using 2/3 of PEG 3500 50% w/v, 1/10 of LiAc 1.0M 5/36 of SS-carrier DNA, 17/180 of plasmid DNA and keep it in ice.

6) Add 360µl of Transformation Mix to each tube and resuspend cells by vortex.

7) Incubate in a 42°C water bath for 40 min.

8) Microcentrifuge for 30 sec and remove Transformation mix with a micropipette.

9) Pipette 1ml of sterile water into each tube and vortex.

10) Plate onto SC médium, incúbate at 30°C for 3-4 days.

Important Notes:

The optimum water bath time can vary for different yeast strains. Please test this if you need high efficiency from your transformations.

Reference*:* Gietz, R.D. and R.A. Woods. (2002) TRANSFORMATION OF YEAST BY THE Liac/SS CARRIER DNA/PEG METHOD. Methods in Enzymology 350: 87-96.

Cryopreservation of *E.coli*

Reactans, Solutions and Meduims

1) 100% sterile glycerol *

2) LB broth

3) 75% Ethanol

Equipment and Materials

1)Inoculation loop and bunsen burner

2) Laminar flow cabinet

3) 15 ml Tubes

4) Incubator with con shaker (37°C – 250rpm)

5) Cryovials (or eppendorf tubes)

6) Autoclave

7) -80°C Freezer

8) Pippetes and tips

9) Permanent marker for labeling

Protocol

~14- 18 hours before

1) In the laminar flow cabinet, with an inoculation loop, take a colony of the bacteria and set it free into the 15 ml tube with LB broth. (Is not necessary to fill all the tube with the medium)
2) Shake the tubes at 250rpm at 37°C for a period between 14 and 18 hours.

Cryopreservation

1) Label a cryovial with the name of the E. coli strain, the plasmid name (if applicable), the date and your name. (Eppendorf tubes can also be used)

2) Pipette 0.4ml of an overnight culture of the desired E.coli strain in the labelled cryovial (~14-16h growth when starting from a colony). Cells can be frozen as soon as the culture medium (usually LB) becomes turbid.

3) Add an equal volume (0.4ml) of 100% sterile glycerol. (Glycerol should be pipetted using a cut tip)

4) Vortex vigorously (Check that the solution is homogeneous).

5) Transfer to the -80ºC freezer.

Relevant Notes:

Unlike eukaryotes, bacteria are resistant to changes in temperature and there is thus no need to freeze the sample slowly . Likewise, the **glicerol concentration can vary from 30- 50%. ***

REFERENCE

Lanctôt Lab, 05.2010<. http://lanctotlab.org/en/protocoles/prot_molbio_ecolifreezing.pdf>

Cryopreservation of yeast cells

Reactants, Solutions and Mediums

1) Yeast culture

2) YPD medium (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% glucose)

3) ice-cold sterilized water

4) ice-cold cryoprotectant solution: sorbitol(0.6 and 2.0 M) or glycerol (10±20% v/v)

Equipment y Materials

1) Centrifuge

2) Pippetes

3) Microcentrifuge tubes

4) -80°C freezer

Protocol

1) Grow cells in YPD medium to a density of approximately $1x10^7$ cells/ml at 30°C.

2) Place the culture on ice for 15 min just before harvesting. Collect the cells by centrifugation at 1600rg for 5 min, and wash the resulting pellet three times with ice-cold sterilized water.

3) Resupend this pellet in ice-cold cryoprotectant solution to give approximately $5x10^8$cells/ml.

4) When the cells are frozen in glycerol, place the cell suspension on ice for 30 min to allow glycerol to permeate into the cells.

5) Dispense aliquots of 0.1 ml of the cell suspension to 1.5 ml microcentrifuge tubes and then froze and store by placing them directly into a -80°C freezer.

Important Notes:

To maintain a high eficiency, electro-competent cells should be frozen in a non-permeating cryoprotectant, such as sorbitol, instead of in the commonly used permeating cryoprotectants, such as glycerol and DMSO.

REFERENCES: Suga M, Isobe M., and Hatakeyama t. Cryopreservation of competent intact yeast cells for eficient electroporation. Department of Materials and Biosystem Engineering, Toyama University, 3190 Gofuku Toyama-City, Toyama 930-8555, Japan. < http://pef.aibn.uq.edu.au/support/material/download/Cryopreservation_of_competent_intact_yeast_cells_for_efficient_electroporation_Suga-2000.pdf>

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (Laemmly Buffer System, Discontinuous Gel)

Reagents

1.Acrylamide/Bis (30% T, 2.67% C)

2. 10% (w/v) SDS

3. 1.5 M Tris-HCl, pH 8.8

4. 0.5 M Tris-HCl, pH 6.8

5. deionized water

6. glycerol

7. 0.5%(w/v) bromophenol blue

8. SDS

9. ammonium persulfate

10. TEMED

Materials and Equipment

1. Gel Casting

i. Place a comb completely into the assembled gel cassette. Mark the glass plate 1 cm below the comb teeth. This is the level to which the resolving gel is poured. Remove the comb.

ii. Prepare the resolving gel monomer solution by combining all reagents except APS and TEMED. (Refer to Section 4 for gel formulations.) Degas the solution under vacuum for at least 15 minutes. Do not use a sink water aspirator.

iii. Add APS and TEMED to the degassed monomer solution and pour to the mark using a glass or disposable plastic pipette. Pour the solution smoothly to prevent it from mixing with air.

iv. Immediately overlay the monomer solution with water or t-amyl alcohol.

Note: If water is used, add it slowly and evenly to prevent mixing. **Do not overlay**

v. Allow the gel to polymerize for 45 minutes to 1 hour. Rinse the gel surface completely with distilled water. Do not leave the alcohol overlay on the gel for more than 1 hour because it will dehydrate the top of the gel.

Note: At this point the resolving gel can be stored at room temperature overnight. Add 5ml of a 1:4 dilution of 1.5MTris-HCl, pH 8.8 buffer (for Laemmli System) to the resolving gel to keep it hydrated. If using another buffer system, add 5 ml 1x resolving gel buffer to the resolving gel surface for storage.

vi. Prepare the stacking gel monomer solution. Combine all reagents except APS and TEMED. Degas under vacuum for at least 15 minutes.

vii. Before casting the stacking gel, insert a piece of filter paper to dry the area in between the glass plates above the resolving gel. Take care not to touch the surface of the gel.

viii. Add APS and TEMED to the degassed stacking gel monomer solution and pour the solution between the glass plates. Continue to pour until the top of the short plate is reached.

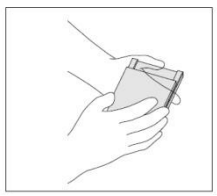
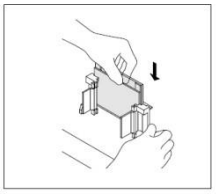
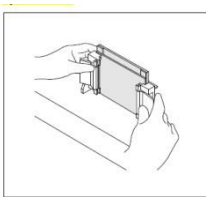
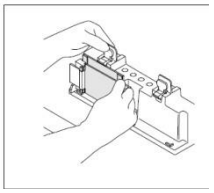
ix. Insert the desired comb between the spacers starting at the top of the Spacer Plate, making sure that the tabs at the ends of each comb are guided between the spacers. It is easiest to insert the combs starting at an angle and insert well 1 first, then 2, 3, and so on until the combs is completely inserted. Seat the comb in the gel cassette by aligning the comb ridge with the top of the Short Plate.

x. Allow the stacking gel to polymerize for 30–45 minutes.

xi. Gently remove the comb and rinse the wells thoroughly with distilled water or running buffer.

xii. Rinse the Casting Frame(s) and Stand with distilled, deionized water after use.

Assembly of equipment to make the gel

For 10 ml monomer solution:

Resolving Gel: 50 µl 10% APS and
5 µl TEMED

Stacking Gel: 50 µl 10% APS and
10 µl TEMED

Swirl gently to initiate polymerization.

Note: Prepare any desired volume of monomer solution by using multiples of the 10 ml recipe. The volumes of APS and TEMED must be adjusted accordingly.
Warning: The catalyst concentration is very important! Webbing and incomplete well formation can result from inaccurate catalyst concentration.

2. Gel electrophoresis assembly

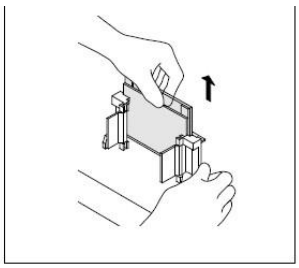
1. Remove the gel cassette from the casting stand.

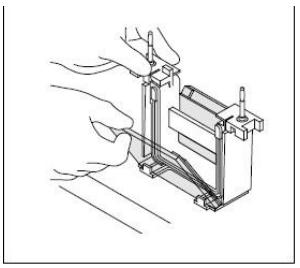
2. Place a gel cassette sandwich into the slots at the bottom of each side of the electrode assembly.
* In this step it is important to place the gel with the lower plates facing each other.

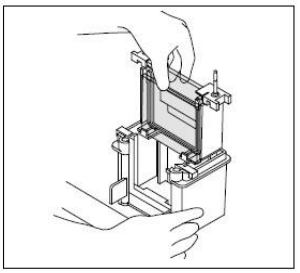
3. Lift the gel cassette sandwich into place against the green gaskets and slide into the clamping frame.

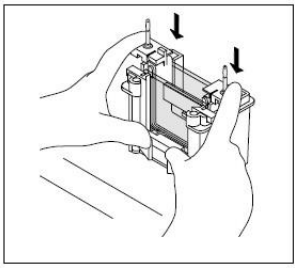
4. Press down on the electrode assembly while closing the two cam levers of the clamping frame to form the inner chamber and to insure a proper seal of the short plate against the notch on the U-shaped gasket. The short plate must align with notch in gasket.

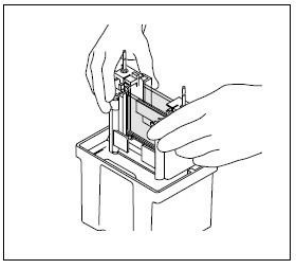
Assembly to run the gel











Note: Gently pressing the top of the electrode assembly while closing the clamping frame cams forces the top of the short plate on each gel cassette sandwich to seat against the rubber gasket properly and prevents leaking.

5. Lower the inner chamber assembly into the mini tank. Fill the inner chamber with ~125 ml of running buffer until the level reaches halfway between the tops of the taller and shorter glass plates of the gel cassettes.

Note: Do not overfill the Inner Chamber Assembly. Excess buffer will cause the siphoning of buffer into the lower chamber which can result in buffer loss and interruption of electrophoresis.

6. Add ~200 ml of running buffer to the mini tank (lower buffer chamber).

3. Sample Loading

1. Load the samples into the wells with a Hamilton syringe or a pipette using gel loading tips.

2. If using a sample loading guide, place it between the two gels in the electrode assembly.

3. Use the sample loading guide to locate the sample wells. Insert the Hamilton syringe or pipette tip into the slots of the guide and fill the corresponding wells.

Note: Load samples slowly to allow them to settle evenly on the bottom of the well. Be careful not to puncture the bottom of the well with the syringe needle or pipette tip.

4. Mini tank assembly

1. Place the lid on the mini tank. Make sure to align the color coded banana plugs and jacks.

*Apply power to the Mini tank cell and begin electrophoresis; 200 volts constant is recommended for SDS-PAGE and most native gel applications. Run time is approximately 35 minutes at 200 volts for SDS-PAGE.

Referencias :

http://www.bio-rad.com/prd/es/MX/LSR/PDP/5cf78e19-7ed5-4373-a988-3e62456a488e/Mini-PROTEANreg_Tetra_Cell

Miniprep DNA Purification System

Reactives

1) Cell Resuspension Solution
2) Cell Lysis Solution
3) Alkaline Protease Solution
4) Neutralization Solution
5) Wash Solution
6) Nuclease-Free Water

Material & Equipment

1)Microcentrifuge
2)Collection Tube
3)Spin Column
4)Sterile 1.5mL microcentrifuge tube
5)Micropipettes

Protocolo

Production of Cleared Lysate

1. Pellet 1–10ml of overnight culture for 5 minutes.
2. Thoroughly resuspend pellet with 250µl of Cell Resuspension Solution.
3. Add 250µl of Cell Lysis Solution to each sample; invert 4 times to mix.
4. Add 10µl of Alkaline Protease Solution; invert 4 times to mix. Incubate 5 minutes at room temperature.
5. Add 350µl of Neutralization Solution; invert 4 times to mix.
6. Centrifuge at top speed for 10 minutes at room temperature.

Binding of Plasmid DNA

7. Insert Spin Column into Collection Tube.
8. Decant cleared lysate into Spin Column.
9. Centrifuge at top speed for 1 minute at room temperature. Discard flowthrough, and reinsert Column into Collection Tube.

Washing

10. Add 750µl of Wash Solution (ethanol added). Centrifuge at top speed for 1 minute. Discard flowthrough and reinsert column into Collection Tube.

11. Repeat Step 10 with 250µl of Wash Solution.

12. Centrifuge at top speed for 2 minutes at room temperature.

Elution

13. Transfer Spin Column to a sterile 1.5ml microcentrifuge tube, being careful not to transfer any of the Column Wash Solution with the Spin Column. If the Spin Column has Column Wash Solution associated with it, centrifuge again for 1 minute at top speed, then transfer the Spin Column to a new, sterile 1.5ml microcentrifuge tube.

14. Add 100µl of Nuclease-Free Water to the Spin Column. Centrifuge at top speed for 1 minute at room temperature.

Notes: Avoid repeated freezing and thawing of DNA by storing the purified DNA at 4°C for immediate use or by preparing aliquots of DNA for long-term storage at -20°C

15. Discard column, and store DNA at –20°C or below.

Wizard® Plus SV Minipreps DNA Purification System. 2009. **Promega**. Quick Protocol.

E.coli Media Recipes: LB (Luria Bertani)

Reactives

1)Tryptone

2)Yeast Extract

3)NaCl

4)NaOH

5)Deionized water

Equipment

1)1L beaker

2)Autoclave

Protocol:

1. For 1 liter, dissolve the following in 950mL deionized water:
    a. 10g tryptone
    b. 5g yeast extract
    c. 10g NaCL
2. Adjust the pH of the solution with NaOH and bring the volume up to 1 liter.
3. Autoclave the medium for 20min at 15 lbs./sq.in. Let it cool to ~55°C and add desired antibiotics at this point.
4. Store medium at room temperature or at 4°C

Note: *For LB agar plates:*

1. Make LB Medium and add 15g/liter agar before autoclaving.
2. Autoclave the medium for 20min at 15 lbs./sq.in
3. Let it cool to ~55°C and add desired antibiotics. Pour into 10cm Petri plates. Let the plates harden, invert, and store at 4°C

Reference: *http://tools.invitrogen.com/content/sfs/manuals/pich_man.pdf p. 59*

E.coli Media Recipes: SOB Medium

Reactives

1)Bacto Tryptone

2)Bacto Yeast Extract

3)NaCl

4)KCl

5)$MgCL_2$

6) $MgSO_4$

7) Deionized water

Equipment

1)1L beaker

2)Autoclave

Protocol

1. Dissolve the following in 900mL deionized water:
    a. 20g Bacto Tryptone
    b. 5g Bacto Yeast Extract
    c. 2ml of 5M NaCl
    d. 2.5ml of 1M KCl
    e. 10ml of 1M MgCl2
    f. 10ml of 1M MgSO4
2. Adjust to 1L with deionized water

Sterilize by autoclaving and store at room temperature or 4°C

Note: For growing *E.coli* DH5 alpha in order to make competent cells, then you should use SOB or 2YT media and transform them using SOC media

E.coli Media Recipes: 2YT Medium

Reactives

1)Bacto tryptone

2)Bacto Yeast Extract

3)NaCl

4)NaOH

5)Deionized Water

Equipment

1)1L beaker

2)Autoclave

Protocol

1. Dissolve the following in 900mL deionized water:
   a. 16g Bacto Tryptone.
   b. 10g Bacto Yeast Extract.
   c. 5g NaCl.
2. Adjust pH to 7.0 with 5N NaOH.
3. Adjust to 1L with deionized water
4. Sterilize by autoclaving. Store at room temperature or 4°C

E.coli Media Recipes: SOC
(Super Optimal Broth with Catabolic Represor) Medium

Reactives

1)Bacto tryptone

2)Bacto Yeast Extract

3)NaCl

4)KCl

5) $MgCl_2$

6) $MgSO_4$

7) Glucose

5)Deionized Water

Equipment

1)1L beaker

2)Autoclave

Protocol

1. Dissolve the following to 900ml of deionized water
    a. 20g Bacto Tryptone
    b. 5g Bacto Yeast Extract
    c. 2ml of 5M NaCl.
    d. 2.5ml of 1M KCl.
    e. 10ml of 1M MgCl2
    f. 10ml of 1M MgSO4
    g. 20ml of 1M glucose
2. Adjust to 1L with deionized water
3. Sterilize by autoclaving. Store at room temperature or 4°C

Pichia Pastoris Media Recipes: Yeast Extract Peptone Dextrose Medium (YPD)

Reactivos

1) Ampicillin 4 grams

2) Ultra Pure Water 80 milliliters

Equipo y Materiales

1) Small weight boat

2) 250 milliliter bottle

3) falcon tube

4) filter (200nm)

5) 20ml syringe

6) Small tubes for aliquot.

To prepare 1 liter:

1. Dissolve 10g yeast extract and 20g of peptone
   in 900mL of water. (Add 20g of agar if making YPD plates).
2. Autoclave for 20 minutes.
3. Add 100mL of 10X D*
4. Store the liquid medium at room temperature. Store the YPD plates at 4°C

Notes: Add 20g of agar if making YPD plates. It should last for several months

http://tools.invitrogen.com/content/sfs/manuals/pich_man.pdf p. 59-66

Pichia Pastoris Media Recipes: Minimal Glycerol Medium + Histidine (MGY & MGYH)

Reactives

1) 10X YNB*

2)500X B *

3)Water

4)10X GY*

Equipo y Materiales

1)Flasks

2)Autoclave

Protocol:

To prepare 1 liter:

1. Combine aseptically 800mL autoclaved water with 100mL of 10X YNB*, 2mL of 500X B, and 100mL of 10X GY
2. Store at 4°C.

Notes: For growth of his4 strains in this medium, a version can be made that contains histidine by adding 10mL of 100X H* stock solution.

It should last for several months

http://tools.invitrogen.com/content/sfs/manuals/pich_man.pdf p. 59-66

Preparation of Antibiotics Stocks : Ampicillin

Reactives

1) Yeast Extract

2)Peptone

3)Water

4)10X D*

Equipo y Materiales

1)Flasks

2)Autoclave

Protocol:

Stock concentration 50mg/ml in Water.

1) Ampicillin is kept at 4ºC fridge.

2) Weight 4 grams of Ampicillin in a small weight boat.

3) Add 80 milliliters of Ultra Pure Water in a 250 milliliter bottle.

4) Add the 4 grams of Ampicillin to the 250 milliliter bottle.

5) Mix the solution.

6) Sterilize the solution into a falcon tube by using a filter (200nm) and a 20ml syringe.

7) Aliquot into small tubes.

Notas Importantes:

Warning: Ampicillin solution is unstable, prepare just before use. Add ampicillin to sterilized media that has been cooled to approximately 55ºC. Avoid more than 10 freezing-thawing cycle.

Working Concentration (dilution)

Escherichia coli:

The working concentration is: 100 µg /ml. Make a dilution of (1/500)

Pichia pastoris:

The working concentration is: 50 to 100 µg /ml. Make a dilution of (1/1000) to (1/500).

Preparation of Antibiotics Stocks : Streptomycin

Reactivos

1) Streptomycin 2 grams

2) Ultra Pure Water 80 milliliters

Equipo y Materiales

1) Small weight boat

2) 250 milliliter bottle

3) falcon tube

4) filter (200nm)

5) 20ml syringe

6) small tubes for aliquot.

Stock concentration 50mg/ml in Water.

Protocolo:

1. Streptomycin is kept at 4ºC fridge.

2. Weight 2 grams of Streptomycin in a small weight boat.

3. Add 40 of Ultra Pure Water in a 250 milliliter bottle

4. Add the 2 grams of Streptomycin to the 250 milliliter bottle.

5. Mix the solution.

6. Sterilize the solution into a falcon tube by using a filter (200nm) and a 60ml syringe.

7. Aliquot into small tubes.

8. Store the mixture at -20ºC.

Notas Importantes:

Warning: Add Streptomycin to sterilized media that has been cooled to approximately 55ºC. Avoid more than 10 freezing-thawing cycle.

Working Concentration (dilution)

Escherichia coli:

The working concentration is: 50 µg /ml to 100 µg /ml. make a dilution of (1/1000) to (1/500)

Preparation of Antibiotics Stocks : Tetracycline

Reactivos

1) Tetracycline ,HCL 400mg

2) 70% Ethanol ,80 milliliters

Equipo y Materiales

1) Small weight boat

2) 250 milliliter bottle

3) falcon tube

4) filter (200nm)

5) 20ml syringe

6)Small tubes for aliquot.

Protocolo:

Stock concentration 5mg/ml in 70% ethanol.

1. Tetracycline HCL is kept at 4ºC fridge.
2. Weight 400mg of Tetracycline HCL in a small weight boat.
3. Dilute 95% Ethanol to 70% using Ultra Pure Water. (add 200 milliliter of Water and 60 milliliter of ethanol gives 80 milliliter of 71% ethanol.)
3. Add 80 milliliters of 70% Ethanol to a 250 milliliter bottle.
4. Add the 400mg of Tetracycline HCL to the 250 milliliter bottle.
5. Mix the solution.
6. Sterilize the solution into a falcon tube by using a filter (200nm) and a 20ml syringe.
7. Aliquot into small tubes.
8. Store the mixture at -20ºC.

Notas Importantes:

Warning: Add Tetracycline to sterilized media that has been cooled to approximately 55ºC. Avoid more than 10 freezing-thawing cycle.

Working Concentration (dilution)

Escherichia coli:

The working concentration is: 50 µg /ml to 100 µg /ml. make a dilution of (1/1000) to (1/500)

Referencia: Foster, T. 1983. Plasmid determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. 47:361-409. Garrett, R., S. Douthwaite, A. Liljas, A. Matheson, P. Moore, and H. Noller. 2000. The Ribosome: Structure, Function, Antibiotics, and Cellular Interactions. ASM Press, Washington, D.C. Russell, A., and I. Chopra. 1990. Understanding Antibacterial Action and Resistance. Ellis Horwood, NY.Zahner, H., and W. Maas. 1972. Biology of Antibiotics. Springer-Verlag, NY.

Long Protocols

LP: Preparation of competent *E. coli* cells

Introduction

The protocol is for preparing competent E. Coli , for optimal results the protocol should be followed very carefully. It is essential that all the glass ware is toughly rinsed and autoclaved filled with water.

Since E. coli is not naturally transformable, the ability to take up DNA or competency must be induced by chemical methods using divalent and multivalent cations (calcium, magnesium, manganese, rubidium, or hexamine cobalt). Alteration in the permeability of the membranes allows DNA to cross the cell envelope of E. coli which is composed of an outer membrane, an inner membrane, and a cell wall.

Components

1) Shaker and water bath shaker

2) Spectrophotometer

3) Centrifuge

4) Erlenmeyer flask

5) 5- ml glass or plastic pipettes precooled in refrigerator (sterile)

Reagents

1) Luria Broth (LB) medium

2) E. coli strain

3) Sterile 60 mM cold CaCl2 solution (60 mM CaCl2, 15% glycerol, 10 mM piperazine-N, N´- bis (2-hydroxypropanesulfonic acid)

LB medium

10g/L Bacto®-tryptone

5g/L Bacto®-yeast extract

5g/L NaCl

Adjust the pH to 7.5 with NaOH. Autoclave to sterilize. For LB plates, include 15g agar prior to autoclaving.

Protocol

Before starting:

1) Inoculate 5 ml of LB media with E. coli and grow overnight at 37oC in a shaker.

2) Inoculate 1 ml of overnight culture into a sterile Erlenmeyer flask containing 100 ml of LB broth.

3) Shake culture in a 37oC water bath until cell density reaches mid-log growth phase (about 5 x 107cells/ml). This should take 2 to 4 hours. The growth rate of the culture is determined by removing a 1-ml aliquot at various times and reading the optical density at 550 nm wavelength using a spectrophotometer. The relationship between optical density and cell number will vary depending on the bacterial strain.

Note: It is vital not to overgrow the culture; this may lead into less efficiency of the protocol.

Preparation of competent cells

Note: From this point forward, every step needs to be done with the cells at low temperature near 4 C to avoid lysis of the cells.

4) Chill the culture on ice for 10 minutes.

5) Spin the cell suspension at 4,000 g in a centrifuge for 5 minutes at 4oC.

6) Discard the supernatant.

7) Resuspend the cells in half the volume (50 ml) of the original culture with ice-cold sterile 60 mM CaCl2.

8) Place the cell suspension in an ice bath for 30 minutes.

9) Centrifuge the suspension at 4,000 g for 5 minutes at 4oC. Discard the supernatant

10) Gently resuspend the cells in 5 ml of sterile ice-cold CaCl2 using precooled pipettes. Cells will remain competent for up to 24 hours at 4oC. Transformation efficiency increases four- to six-fold during this time. For long-term storage, dispense 250-ml aliquots into prechilled, sterile microfuge tubes and store at -70oC until needed. Depending on the strain used, some E. coli cells will remain competent to take up DNA for as long as 6 months.

Note: The negative charges of the incoming DNA, however, are repelled by the negatively charged portions of the macromolecules on the bacterium's outer surface. The addition of CaCl2 serves to neutralize the unfavorable interactions between the DNA and the polyanions of the outer layer.

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 2002. Short protocols in molecular biology, 5th ed. John Wiley & Sons, New York, NY.

T, Anh Hue. "Transformation of Escherichia coli Made Competent by Calcium Chloride Protocol." n. page. Web. 7 Mar. 2012. <http://www.microbelibrary.org/component/resource/laboratory-test/3152-transformation-of-escherichia-coli-made-competent-by-calcium-chloride-protocol>.

LP: Preparation of P. pastoris competent cells

Introduction

Yeast cells can be made competent for transformation by treatment with ethylene glycol and dimethyl sulfoxide (DMSO) and then frozen in small aliquots and stored at - 70oC. While the highest transformation efficiencies are obtained with freshly grown cultures, the moderately efficient transformation of frozen competent cells saves time.

Reagents

1) YPD (Yeast Extract Peptone Dextrose) medium

2) Sorbitol solution containing ethylene glicol and DMSO (Dimethyl Sulfoxide) for the preparation of competent cells 75 mL*

3) Pichia pastoris strain

* Invitrogen Kit Components

YPD Medium recipe

Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract

2% peptone

2% dextrose (D-glucose)

1. Dissolve the following in 900 ml of water:
    10 g yeast extract
    20 g of peptone
2. Autoclave for 20 minutes on liquid cycle.
3. Add 100 ml of 10X D.

Store the medium at room temperature. The shelf life is several months.

Protocol

Before Beginning

Streak a YPD plate with your Pichia pastoris strain such that isolated, single colonies will grow. Incubate the plate at 28-30°C for 2 days.

Equilibrate Solution 2 to room temperature.

Preparation of competent cells

1) Inoculate 10 ml of YPD with a single colony of your Pichia strain. Grow overnight at 28-30°C in a shaking incubator (250-300 rpm)

2) Dilute cells from the overnight culture to an OD600 of 0.1-0.2 in 10 ml of YPD. Grow the cells at 28-30°C in a shaking incubator until the OD600 reached 0.6-1.0. This will take approximately 4 to 6 hours.

3) Pellet the cells by centrifugation at 500 x g for 5 minutes at room temperature. Discard the supernatant.

4) Resuspend the cell pellet in 10 ml of the Solution 2. No incubation time is required.

5) Pellet the cells by centrifugation at 500 x g for 5 minutes at room temperature. Discard the supernatant.

6) Resuspend the cell pellet in 1 ml of the Solution 2. The cells are now competent.

Note: Cells can now be frozen bellow - 70 C for storage, slow freezing improves efficiency.

Transformation efficiencies are often obtained with freshly versus frozen prepared cells. You may choose to use some of the cells immediately following preparation and freeze the remaining cells in small aliquots.

References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1990) Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, New York.

"tools invitrogen." . N.p., n.d. Web. <http://tools.invitrogen.com/content/sfs/manuals/easycomppp_man.pdf>.

LP: Transformation of E. coli

Introduction

Although the transformation mechanism is not known, previous studies indicate that these channels allow for the transport of DNA molecules across the cell membrane (Bayer, 1968)

The negative charges of the incoming DNA, however, are repelled by the negatively charged portions of the macromolecules on the bacterium's outer surface. The addition of CaCl2 serves to neutralize the unfavorable interactions between the DNA and the polyanions of the outer layer. The DNA and competent cells are further incubated on ice for thirty minutes to stabilize the lipid membrane and allow for increased interactions between calcium ions and the negative components of the cell. The reaction mixture is then exposed to a brief period of heat-shock at 42oC. The change in temperature alters the fluidity of the semi-crystalline membrane state achieved at 0oC thus allowing the DNA molecule to enter the cell through the zone of adhesion

Bayer, M. E. 1968. Adsorption of bacteriophages to adhesions between the wall and membrane of Escherichia coli. J. Virol. 2:346–356.

Equipment and Materials

1) Micropipette
2) Water bath
3) Media Plates
4) Microcentrifuge tubes
5) Shaking incubator
6) Centrifuge

Reactants, solutions and media:

1)SOC medium
2) LB medium
3) Transforming plasmid
4) Competent cells

SOC medium

2.0g Bacto®-tryptone
0.5g Bacto® -yeast extract

1ml 1M NaCl

0.25ml 1M KCl

1ml Mg2+stock

(1M MgCl2 • 6H2O,

1M MgSO4 • 7H2O)

filter-sterilized

1ml 2M glucose,

filter-sterilized

Bring to 100ml with distilled water. Add Bacto®- tryptone, Bacto®- yeast extract, NaCl and KCl to 97ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg2+ stock and 2M glucose stock, each to a final concentration of 20mM. Filter the complete medium through a 0.2µm filter unit. The pH should be 7.0. (Promega)

LB medium with ampicillin

10g/L Bacto®-tryptone

5g/L Bacto®-yeast extract

5g/L NaCl

Adjust the pH to 7.5 with NaOH. Autoclave to sterilize. Allow the autoclaved medium to cool to 55°C, and add ampicillin (final concentration 100µg/ml). For LB plates, include 15g agar prior to autoclaving.

Protocol:

1. Desfrost the CaCl2 treated 50µl aliquots of competent cells previously stored at -80°C at ice for 30 min.
2. Pipette five µl of plasmid that contain a total of 50pg DNA over competent cells.
3. Mix these cells gently by tapping 4-5 times.
4. Incubate on ice for 30 min
5. Follow by a heat shock treatment at 42°C for 30 seconds.
6. After treatment, incubate the cells at ice for 2 min and by add 250 µl of SOC media to each vial.
7. Incubate the vials at 37°C for 1 hr at 225 rpm in a shaking incubator.
8. Dilute the cultures in LB medium and plate 50-100µl of each culture in triplicates on ampicillin containing media plates.

9. Incubate overnight at 37°

Tips

All liquid and glassware that come into contact with the E. coli cells must be prechilled. For maximum transformation efficiency, the culture must be collected in early logarithmic phase and cell density should be low .

Depending on the strain of E. coli used, transformation efficiency can be improved by addition of other chemicals such as rubidium chloride, magnesium, manganese, hexamine cobalt, dimethyl sulfoxide, and dithiothreitol to the calcium chloride buffer

Careful handling is important since cells are fragile due to treatment with calcium chloride.

DNA or plasmid size also has an effect on transformation efficiency. The number of transformants decreases with increasing DNA size

Reference: Singh, M. et al "Plasmid DNA Transformation in Escherichia Coli: Effect of Heat Shock Temperature, Duration, and Cold Incubation of CaCl2 Treated Cells" International Journal of Biotechnology and Biochemistry ISSN 0973-2691 Volume 6 Number 4 (2010) pp. 561–568

LP: Transformation of *Pichia*

Introduction

The protocol can be used to generate sufficient transformants in a single reaction to screen multiple yeast genome equivalents for plasmids that complement a specific mutation, it can also be used to transform DNA fragments and oligonucleotides for yeast genome manipulation.

Required Reagents and Equipment

1) YPAD/SC

2) Transformation mix

- PEG 3500
- LiAc
- SS-carrier DNA
- Plasmid DNA

Number of transformations			
Reagents	1	5 (6X)	10 (11X)
PEG 3500 50 % w/v	240 ul	1440 ul	2640 ul
LiAc 1.0 M	36 ul	216 ul	396 ul
Boiled SS – carrier	50 ul	300 ul	550 ul
Plasmid DNA plus water	34 ul	204 ul	374 ul
Total	360 ul	2160 ul	3960 ul

3) Rotary Shaker

4) Microcentrifuge

5) Vortex

6) Ice

7) Water bath

8) Micropipette

Before starting

Inoculate the yeast strain into 5 ml of liquid medium (2x YPAD or SC selection medium) and incubate overnight on a rotary shaker at 200 rpm and 30 C. Place a bottle of double strength YPAD broth (2x YPAD) and a 250 ml culture flask in the incubator as well.

1) Determine the titer of the yeast culture by pipetting 10 ml of cells into 1 ml of water in a spectrophotometer cuvette and measuring the OD at 600 nm. For many yeast strains a suspension containing 1 x 10^6 cells/ml will give an OD 600 of 0.1 Alternatively, titer the culture using a hemocytometer see note:

Notes (by using hemocytometer)

- Dilute the cultures 10^-1 or more in water
- Carefully place 10 ul of the cell suspensions between the cover slip and the base of hemocytometer. Let the cells settle onto the hemocytometer grid for a few minutes. The grid area is typically 1 square millimeter, divided into 25 equal – sized squares, and the volumen measured is 10 ^-4 ml
- Count the number of cells in 5 diagonal squares
- Calculate the cell titer as follows: cells counted x 5 x dilution factor x 1/volume measured by the 25 squares of the hemocytometer. 239 cells x 5 x 10 (dilution factor) x 1/10 ^-4 ml = 1.2 x 10^8 cells /ml.
-

2) Transfer 50 ml of the pre-warmed 2x YPAD to the pre-warmed culture flask and add enough cells to get 5 x 10^6 cells/ml

3) Incubate the flask on a rotary or reciprocating shaker at 30 C and 200 rpm.

Notes

- It is important to allow the cells to complete at least two divisions.
- This will take 3 to 5 hours
- This culture wills five sufficient cells for 10 transformations.
- Transformation efficiency (transformants/ug plasmids / 10^8 cells) remains constant for 3 to 4 cell divisions.

4) When the cell titer is at least 2 x 10^7 cells/ml, which should take about 4 hours, harvest the cells by centrifugation at 3000 g for 5 min, wash the cells in 25 ml of sterile water and resuspend in 1 ml of sterile water.

5) Boil a 1 ml simple of carrier DNA for 5 min and chill in an ice/water bath while harvesting the cells. **Note:** it is not necessary or desirable to boil the carrier DNA every time. Keep a small aliquot in your own box and boil after 3 – 4 freeze thaws. But keep on ice when out. (this is to avoid desnaturalization)

6) Transfer the cell suspension to a 1.5 ml microcentrifuge tube, centrifuge for 30 sec and discard the supernant.

7) Add water to a final volume of 1.0 ml and vortex mix vigorously to resuspend the cells.
Note: If the cells titer of the culture is greater than 2 x 10^7 cells/ml then increase the volume to maintain the titer of this suspension at 2 x 10^9 cells/ml. If the titer of the culture is less tan 2 x 10^7 cells/ml then decrease volume.

8. Pipette 100 µl samples (ca. 108 cells) into 1.5 ml microfuge tubes, one for each transformation, centrifuge at top speed for 30 sec and remove the supernatant.

9). Add 360 µl of Transformation Mix to each transformation tube and resuspend the cells by vortex mixing vigorously.

10). Incubate the tubes in a 42°C water bath for 40 min.
Note: The optimum time can vary for different yeast strains. Please test this if you need high efficiency from your transformations, if there is not enough efficiency, at this step, vortexing during the 40 min would improve efficiency, as well as increasing time to 1 or 1.5 hours.

11). Microcentrifuge at top speed for 30 sec and remove the Transformation Mix with a micropipettor.

12). Pipette 1.0 ml of sterile water into each tube; stir the pellet by with a micropipette tip and vortex..

Note: We like to be as gentle as possible at this step if high efficiency is important. Since excessive washing washes away transformant and needs to be avoided when looking for high efficiency

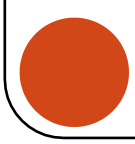
14. Plate appropriate dilutions of the cell suspension onto SC selection medium. For transformation with an integrating plasmid (YIp), linear construct or oligonucleotide, plate 200 µl onto each of 5 plates; for a YEp, YRp or YCp library plasmid dilute 10 µl of the suspension into 1.0 ml of water and plate 10 and 100 µl samples onto two plates each. The 10 µl samples should be pipetted directly into 100 µl puddles of sterile water on the SC selection medium.

Note:When spreading yeast inoculum onto the plate gently distribute the fluid completely with a sterile glass rod with a minimum of strokes. Allow the fluid to be taken up by the plate prior to incubation.

15. Incubate the plates at 30°C for 3 to 4 days and count the number of transformants.
The transformation efficiency (transformants/1 µg plasmid/108 cells) can be determined by calculating the number of transformants in 1.0 ml of resuspended cells per 1.0 microgram plasmid per 108 cells. For example, if the transformation of 1.0 x 108 cells with 100 nanogram plasmid resulted in 500 colonies on a plate of SC dropout medium spread with 1 µl of suspension (usually dispensed into a 100µl puddle of sterile water on the plate).
Transformation Efficiency = 500 x 1000 (plating factor) x 10 (plasmid factor) x 1 (cells/transformation x 108).
Transformation Efficiency = 5 x 106 transformants/1.0 µg plasmid/108 cells.

Transformation efficiency declines as plasmid concentration is increased (Gietz et al. 1995) but the actual yield of transformants per transformation increases. For example, 100 nanogram of plasmid in a transformation might give a Transformation Efficiency of 5 x 106 and a yield of 5 x 105 transformants whereas with 1 µg of plasmid the Transformation Efficiency might be 2 x 106 and the yield 2 x 106 per transformation. In order to obtain 5 x 106 transformants it is simpler to set up two or three transformations with 1 µg of plasmid DNA, or a single 3 fold scaled up transformation, than to carry out 10 reactions with 100 ng of plasmid in each.

Reference: Gietz, R.D. and R.A. Woods. (2002) TRANSFORMATION OF YEAST BY THE Liac/SS CARRIER DNA/PEG METHOD. Methods in Enzymology 350: 87-96.



LP: Cryopreservation of *E.coli*

Description.

It is of vital importance to be able to keep and store the transformed cultures so they can be ready to use when they are needed. Cryopreservation is the best way to, as the name announces, preserve bacterial cultures so they can be stored for their posterior use without dying or losing their properties. This method offers a quick, easy and cheap way to maintain cultures in a compact space and store them for years without sacrificing the functions or the viability of the cells.

Components and Storage Conditions

- **100% Sterile Glycerol**: No special requirements
- **LB broth**: Sterile. No special requirements

Equipment, Supplies and Preparation of Solutions

- **75% Ethanol**

  Create a dilution with distilled water until you get the desired ethanol concentration solution.
- **100% Sterile Glycerol**

  Concentration can vary from 30 to 50% of glycerol. To adjust this, just calculate taking as reference the amount of overnight culture in the cryovial (0.4ml). To get a 50% concentration you must add 0.4ml of glycerol; to get a 30% concentration you must add .171 ml of glycerol. You can calculate the other concentrations by a simple rule of three.

- NOTE: The laminar flow cabinet must be cleaned and prepared according to the supplier's indications.

<u>Cryopreservation of *E.coli* Protocol</u>

<u>A. Preparation of the overnight culture (~14- 18 hours before)</u>

1) In the laminar flow cabinet, with an inoculation loop, take a colony of the bacteria and set it free into the 15 ml tube with 5-10 ml of LB broth.

2) Shake the tubes at 250rpm at 37°C for a period between 14 and 18 hours (overnight).

- NOTE: The amount of LB medium may vary (5 to 1o ml) depending on how much bacteria is needed. The time of incubation may be affected too. You will know the cells had grown because of the change of the color in the medium (it becomes turbid).

-

B.Cryopreservation

1) Label a cryovial with the name of the E. coli strain, the plasmid name (if applicable), the date and your name.

- NOTE: Eppendorf tubes can also be used

2) Pipette 0.4ml of an overnight culture of the desired E.coli strain in the labelled cryovial (~14-16h growth when starting from a colony). Cells can be frozen as soon as the culture medium (usually LB) becomes turbid.

3) For a 50% concentration, add an equal volume (0.4ml) of 100% sterile glycerol.

- NOTE: Glycerol should be pipetted using a cut tip and the concentration may vary. To calculate a different concentration of glycerol, please check "Equipment, Supplies and Preparation of Solutions".

4) Vortex vigorously (Check that the solution is homogeneous).

5) Transfer to the -80ºC freezer.

Supplemental Information

- When preparing the Overnight culture, always pick a single colony for each tube.

- Unlike eukaryotes, bacteria are resistant to changes in temperature and there is thus no need to freeze the sample slowly. Likewise, the **glicerol concentration can vary from 30- 50%. ***

Troubleshooting

In case that your cells don´t survive the cryopreservation with concentrations lower than 50% of glycerol, you can increase this concentration to improve the protection for your cells.

References

Lanctôt Lab, 05.2010<. http://lanctotlab.org/en/protocoles/prot_molbio_ecolifreezing.pdf>

LP: Cryopreservation of *P.pastoris*

Description

It is of vital importance to be able to keep and store the transformed cultures so they can be ready to use when they are needed. Cryopreservation is the best way to, as the name announces, preserve cell cultures so they can be stored for their posterior use without dying or losing their properties. This method offers a quick, easy and cheap way to maintain cultures in a compact space and store them for years without sacrificing the functions or the viability of the cells.

Components and Storage Conditions

- **Ice cold sterilized water**: -20°C freezer.
- **Ice cold cryoprotectant solution**: cryoprotectant and distilled water. -20°C freezer.
- **YPD Meduim**: 1% Bacto Yeast Extract, 2% Bacto Peptone, 2% glucose. Sterile. No special requirements. For growing cells: Keep at 30°C.
- **Cryovials with cells**: Store in a -80°C Freezer

Equipment, Supplies and Preparation of Solutions

Cryoprotectant Solution

-Sorbitol: 0.2 M solution = Add 0.2 moles of sorbitol for each liter of solution.

-Glycerol: 20% v/v = Add 2 ml of glycerol and 8 ml of distilled water for 10 ml solution.

- NOTE: The laminar flow cabinet must be cleaned and prepared according to the supplier's indications.
- NOTE: You can change the concentration of the solution by manipulating the amount of cryoprotectant and distilled water added by simply following a rule of three.

Cryopreservation of *P.Pastoris* Protocol

A. Preparation of the culture

3) Grow cells in YPD medium to a density of approximately $1x10^7$ cells/ml at 30°C.

- NOTE: This step may vary depending on the growth rate of the cells. It could take more than one day.

B.Cryopreservation

1) Place the culture on ice for 15 min just before harvesting. Collect the cells by centrifugation at 1600rg for 5 min, and wash the resulting pellet three times with ice-cold sterilized water.

2) Resupend this pellet in ice-cold cryoprotectant solution to give approximately $5x10^8$cells/ml.

3) When the cells are frozen in glycerol, place the cell suspension on ice for 30 min to allow glycerol to permeate into the cells

4) Dispense aliquots of 0.1 ml of the cell suspension to 1.5 ml microcentrifuge tubes and then froze and store by placing them directly into a -80°C freezer.

Supplemental Information

- To maintain a high eficiency, electro-competent cells should be frozen in a non-permeating cryoprotectant, such as sorbitol, instead of in the commonly used permeating cryoprotectants, such as glycerol and DMSO.

Troubleshooting

In case that you experience any problems with the viability or activity of the cells, try different concentrations in the cryoprotectants solutions

For Sorbitol: 0.6 M - 2.0 M

For Glycerol: 10- 20% v/v

References

Suga M, Isobe M., and Hatakeyama t. Cryopreservation of competent intact yeast cells for eficient electroporation. Department of Materials and Biosystem Engineering, Toyama University, 3190 Gofuku Toyama-City, Toyama 930-8555, Japan. < http://pef.aibn.uq.edu.au/support/material/download/Cryopreservation_of_competent_intact_yeast_cells_for_efficient_electroporation_Suga-2000.pdf>

LP: Screening- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

Reagents and Materials

1.Acrylamide/Bis (30% T, 2.67% C)

2. 10% (w/v) SDS

3. 1.5 M Tris-HCl, pH 8.8

4. 0.5 M Tris-HCl, pH 6.8

5. deionized water

6. glycerol

7. 0.5%(w/v) bromophenol blue

8. SDS

9. ammonium persulfate

10. TEMED

1. Gel Casting

i. Place a comb completely into the assembled gel cassette. Mark the glass plate 1 cm below the comb teeth. This is the level to which the resolving gel is poured. Remove the comb.

ii. Prepare the resolving gel monomer solution by combining all reagents except APS and TEMED. (Refer to Section 4 for gel formulations.) Degas the solution under vacuum for at least 15 minutes. Do not use a sink water aspirator.

iii. Add APS and TEMED to the degassed monomer solution and pour to the mark using a glass or disposable plastic pipette. Pour the solution smoothly to prevent it from mixing with air.

iv. Immediately overlay the monomer solution with water or t-amyl alcohol.

v. Allow the gel to polymerize for 45 minutes to 1 hour. Rinse the gel surface completely with distilled water. Do not leave the alcohol overlay on the gel for more than 1 hour because it will dehydrate the top of the gel.

vi. Prepare the stacking gel monomer solution. Combine all reagents except APS and TEMED. Degas under vacuum for at least 15 minutes.

vii. Before casting the stacking gel, insert a piece of filter paper to dry the area in between the glass plates above the resolving gel. Take care not to touch the surface of the gel.

viii. Add APS and TEMED to the degassed stacking gel monomer solution and pour the solution between the glass plates. Continue to pour until the top of the short plate is reached.

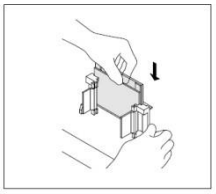
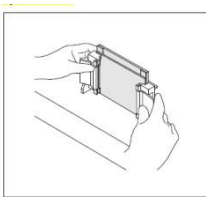
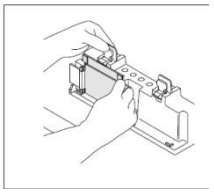
ix. Insert the desired comb between the spacers starting at the top of the Spacer Plate, making sure that the tabs at the ends of each comb are guided between the spacers. It is easiest to insert the combs starting at an angle and insert well 1 first, then 2, 3, and so on until the combs is completely inserted. Seat the comb in the gel cassette by aligning the comb ridge with the top of the Short Plate.

x. Allow the stacking gel to polymerize for 30–45 minutes.

xi. Gently remove the comb and rinse the wells thoroughly with distilled water or running buffer.

xii. Rinse the Casting Frame(s) and Stand with distilled, deionized water after use.

Assembly of equipment to make the gel

<u>For 10 ml monomer solution:</u>

Resolving Gel: 50 µl 10% APS and 5 µl TEMED

Stacking Gel: 50 µl 10% APS and 10 µl TEMED

Swirl gently to initiate polymerization.

Note: Prepare any desired volume of monomer solution by using multiples of the 10 ml recipe. The volumes of APS and TEMED must be adjusted accordingly.

Warning: The catalyst concentration is very important! Webbing and incomplete well formation can result from inaccurate catalyst concentration.

2. Gel electrophoresis assembly

1. Remove the gel cassette from the casting stand.

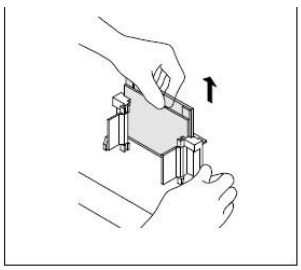
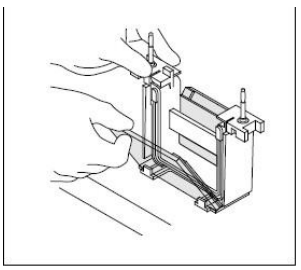
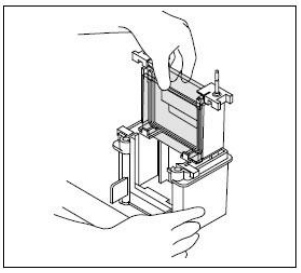
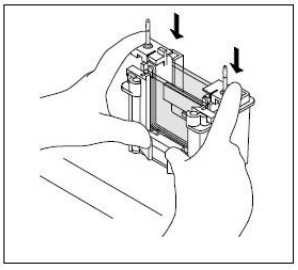
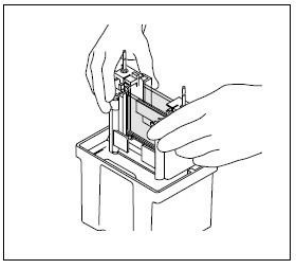
2. Place a gel cassette sandwich into the slots at the bottom of each side of the electrode assembly.

* In this step it is important to place the gel with the lower plates facing each other.

3. Lift the gel cassette sandwich into place against the green gaskets and slide into the clamping frame.

4. Press down on the electrode assembly while closing the two cam levers of the clamping frame to form the inner chamber and to insure a proper seal of the short plate against the notch on the U-shaped gasket. The short plate must align with notch in gasket.

Assembly to run the gel

5. Lower the inner chamber assembly into the mini tank. Fill the inner chamber with ~125 ml of running buffer until the level reaches halfway between the tops of the taller and shorter glass plates of the gel cassettes.

6. Add ~200 ml of running buffer to the mini tank (lower buffer chamber).

3. Sample Loading

1. Load the samples into the wells with a Hamilton syringe or a pipette using gel loading tips.

2. If using a sample loading guide, place it between the two gels in the electrode assembly.

3. Use the sample loading guide to locate the sample wells. Insert the Hamilton syringe or pipette tip into the slots of the guide and fill the corresponding wells.

4. Mini tank assembly

1. Place the lid on the mini tank. Make sure to align the color coded banana plugs and

jacks.

*Apply power to the Mini tank cell and begin electrophoresis; 200 volts constant is recommended for SDS-PAGE and most native gel applications. Run time is approximately 35 minutes at 200 volts for SDS-PAGE.

Stock Solutions and buffer

1. Acrylamide/Bis (30% T, 2.67% C)

87.6 g acrylamide (29.2 g/100 ml)

2.4 g N'N'-bis-methylene-acrylamide (0.8 g/100 ml)

Make to 300 ml with deionized water. Filter and store at 4 °C in the dark (30 days maximum.)

2. 10% (w/v) SDS

Dissolve 10 g SDS in 90 ml water with gentle stirring and bring to 100 ml with deionized water. Alternatively 10% SDS solution (250 ml) can be used (Bio-Rad catalog number 161-0416).

3. 1.5 M Tris-HCl, pH 8.8

27.23 g Tris base (18.15 g/100 ml)

80 ml deionized water

Adjust to pH 8.8 with 6 N HCl. Bring total volume to 150 ml with deionized water and store at 4 °C. Alternatively 1.5 M Tris-HCl, pH 8.8 (1 L) premixed buffer can be used (Bio-Rad catalog number 161-0798).

4. 0.5 M Tris-HCl, pH 6.8

6 g Tris base

60 ml deionized water

Adjust to pH 6.8 with 6 N HCl. Bring total volume to 100 ml with deionized water and store at 4 °C.

5. Sample Buffer (SDS Reducing Buffer)

3.55 ml	**deionized water**
1.25 ml	0.5 M Tris-HCl, pH 6.8
2.5 ml	glycerol
2.0 ml	10% (w/v) SDS
0.2 ml	0.5%(w/v) bromophenol blue
9.5 ml	Total Volume

Store at room temperature.

Use: Add 50 µl β-Mercaptoethanol to 950 µl sample buffer prior to use. Dilute the sample at least 1:2 with sample buffer and heat at 95 °C for 4 minutes.

6. 10x Electrode (Running) Buffer, pH 8.3 (makes 1 L)

30.3 g	Tris base
144.0 g	Glycine
10.0 g	SDS

Dissolve and bring total volume up to 1,000 ml with deionized water. Do not adjust pH with acid or base. Store at 4 °C. If precipitation occurs, warm to room temperature before use.

Use: Dilute 50 ml of 10x stock with 450 ml deionized water for each electrophoresis run. Mix thoroughly before use.

7. 10% APS (fresh daily)

100 mg ammonium persulfate

Dissolved in 1 ml of deionized water.

Gel Formulations (10 ml)

1. Prepare the monomer solution by mixing all reagents except the TEMED and 10% APS.

Degas the mixture for 15 minutes. (Note: add TEMED just before loading each gel)

Percent Gel	DDI H_2O (ml)	30% Degassed Acrylamide/Bis (ml)	*Gel Buffer (ml)	10% w/v SDS (ml)
4%	6.1	1.3	2.5	0.1
5%	5.7	1.7	2.5	0.1
6%	5.4	2.0	2.5	0.1
7%	5.1	2.3	2.5	0.1
8%	4.7	2.7	2.5	0.1
9%	4.4	3.0	2.5	0.1
10%	4.1	3.3	2.5	0.1
11%	3.7	3.7	2.5	0.1
12%	3.4	4.0	2.5	0.1
13%	3.1	4.3	2.5	0.1
14%	2.7	4.7	2.5	0.1
15%	2.4	5.0	2.5	0.1
16%	2.1	5.3	2.5	0.1
17%	1.7	5.7	2.5	0.1

* Resolving Gel Buffer - 1.5 M Tris-HCl, pH 8.8
* Stacking Gel Buffer - 0.5 M Tris-HCl, pH 6.8

By convention, polyacrylamide gels are characterized by %T which is the weight percentage of the total monomer including the crosslinker. The %T gives an indication of the relative pore size of the gel. In general, pore size decreases with increasing %T. %T is calculated using the following equation.
%T = g acrylamide + g crosslinker x 100% total volume (ml)

2. Immediately prior to pouring the gel, add:

For 10 ml monomer solution:

Resolving Gel: 50 µl 10% APS and 5 µl TEMED

Stacking Gel: 50 µl 10% APS and 10 µl TEMED

Swirl gently to initiate polymerization.

Note: Prepare any desired volume of monomer solution by using multiples of the 10 ml recipe. The volumes of APS and TEMED must be adjusted accordingly.

Warning: The catalyst concentration is very important! Webbing and incomplete wellformation can result from inaccurate catalyst concentration.

Referencias :

http://www.bio-rad.com/prd/es/MX/LSR/PDP/5cf78e19-7ed5-4373-a988-3e62456a488e/Mini-PROTEANreg_Tetra_Cell

LP: Miniprep - DNA Purification

Description

A plasmid preparation is a method used to extract and purify plasmid DNA. Many methods have been developed to purify plasmid DNA from bacteria. Kits are available from varying manufacturers to purify plasmid DNA, which are named by size of bacterial culture and corresponding plasmid yield.

Minipreparation of plasmid DNA is a rapid, small-scale isolation of plasmid DNA from bacteria. It is based on an alkaline lysis method. The extracted plasmid DNA resulting from performing a miniprep is itself often called a "miniprep". These minipreps are used in the process of molecular cloning to analyze bacterial clones. A typical plasmid DNA yield of a miniprep is 20 to 30 µg depending on the cell strain.

Components and Storage Conditions

- **Ice cold solution I and III**: glucose, Tris-HCl, EDTA, Potassium acetate, glacial acetic acid. Store at 4°C.
- **Solution II:** SDS, NaOH. Store at room temperature.
- **TE Buffer**: TrisHCl, EDTA.
- **Ice cold Ethanol**: 70% ethanol. Store at -20°C.

Equipment, Supplies and Preparation of Solutions

- **Solution I**

5mM Glucose, 25 mM Tris-HCl, 10 mM EDTA, distilled water, sterile filtered and pH 8.0

- **Solution II**

1% SDS, 0.2 N NaOH, distilled water.

- **Solution III**

3 M Potassium acetate, 57.5 mL glacial acetic acid, distilled water.

- **TE Buffer**

10 mM TrisHCl, 1 mM EDTA, distilled water.

- **70% Ethanol**

Create a dilution with distilled water until you get the desired ethanol concentration solution.

- NOTE: It is important to maintain solutions I and III cold.

Plasmid DNA purification

A. Harvesting and lysis of the bacteria

1. Add 1.7 mL of overnight culture into a Eppendorf tube.
2. Centrifuge max speed for 1 min.
3. Repeat until you have centrifuged a total of 3-5 mL of the overnight culture.
4. Add 0.2 mL of ice-cold Solution I, to each Eppendorf tube then, resuspend with tip.
5. Add 300µLof RNAse
6. Vortex vigorously.
7. Incubate 5min at room temperature.
8. Add 0.4 mL of Solution II (Fresh).
9. Invert 5 times and incubate 5 min at room temperature.
10. Add 0.3 mL of ice-cold Solution III.
11. Invert 5 times and then, incubate on ice for 10 min.
    - NOTE: All this steps can be done outside the laminar flow cabinet but to maximize precautions, you can do steps 1, 3 and 4 inside de laminar flow cabinet.

B.Purification

12. Centrifuge 5 min at maximum speed.
13. Prepare new Eppendorf tubes, and pass supernatants (without touching the bottom layer).
14. Fill up the tubes with ice-cold isopropanol.
15. Invert and incubate 2 min at room temperature.
16. Centrifuge 5 min at maximum speed.
17. Pour off supernatant and dry tubes on paper.
18. Add 1 mL of ice-cold 70% EtOH to each tube,
19. Invert and centrifuge for 1 minute at maximum speed.
20. Pour off supernatant and dry tubes on paper.
21. Speed vac, 10 min, with heat.
    - NOTE: You can replace the Speed Vac with an 37°C incubator for about 15 minutes.
22. Resuspend with 20-50 uL of nuclease free water or TE buffer.

Supplemental Information

It is important to measure the OD of the sample to make sure there is no contamination. Optical Density must be measured at 260 and 280 nanometers to make the relation 260/280 and get the sample´s purity.

References

Mini-Preparación de DNA plasmídico. UANL. FCB. iGEM UANL 2011.