Calcofluor White Stain Protocol

Introduction

Calcofluor white stain is a nonspecific dye that binds to chitin and cellulose in cell walls of specimens such as fungi, yeast, and parasites. It fluoresces in the presence of UV light.

Materials

- Testing sample
- 10% potassium hydroxide (Sigma Aldrich)
- Calcofluor White stain (Sigma Aldrich)
  - Fluorescent Brightener 28
  - Ethyl 4-(2-chloroacetamido)benzoate
  - Evans Blue dye
- Glass microscope slide
- Coverslip
- UV light microscope

Procedure

1. Put a drop of the sample to be tested onto a clean glass slide
2. Add one drop of Calcofluor White Stain and one drop of 10% potassium hydroxide
3. Place a coverslip over the sample and let stand for 1 minute
4. Examine the slide under UV light at x100 to x400 magnification. Any chitin present should fluoresce blue.

E. coli Transformation Protocol

Purpose

To transform plasmids into cells.

Discussion

When transforming a plasmid into cells, it is important to understand what happens in a transformation. Before transformation, purified DNA is typically attached, through one of many mechanisms, to a plasmid backbone that contains a gene expressing resistance to a specific antibiotic. The gene is only expressed, however, when the plasmid binds to the DNA inserted. Any bacteria that express antibiotic resistance must therefore also express the inserted DNA. In order to make this assumption, it is important to make certain conclusions by constructing 'positive' and 'negative' control tests.

- **Negative** control: used to prove that the antibiotic employed works. A negative control is typically created by plating **wild-type bacteria** on a **plate with the antibiotic**. If the antibiotic works, nothing will grow on the plate. If the antibiotic doesn't work there will be growth on the plate, meaning that it is impossible to conclude that growth on experimental plates is due to a successful transformation
- **Positive control**: used to prove that the competent bacteria and plates used are viable. A positive control is typically created by plating **wild-type bacteria** on a **plate without the antibiotic**. Bacterial growth indicates that the cells and plates used are viable whereas lack of growth indicates that either the cells or plates were not viable.

Materials

- Purified DNA 'parts' (5uL of each)
- Plates with antibiotic resistance (1 for negative control, 1 for each part to be transformed)
    - Common antibiotics include: ampicillin (amp), chloramphenicol (chlor), kanamycin (kan), and spectinomycin (spec)
- Plate with no antibiotic resistance (1 for positive control)
- Eppendorf tubes (1 total for both controls, 1 for each part)
- Competent cells (50uL total for both controls, 50uL for each part)
- SOC media (200uL total for both controls, 200uL for each part)
- Ice
- Glass beads
- Pipette tips
    - Filtered if possible
- Deionized water

(Optional) If Using DNA From Kit Plates

- Kit plate
- PCR tubes (1 per part taken from kit plate)

Equipment

- 42℃ Water Bath [BIND 134 balance room or B025]
- 37℃ Incubator with shaker [B025]

Protocol

1. Prepare lab space
    a. Wipe counter with ethanol
    b. Light flame
2. Get ice from autoclave room in 134
3. Warm water bath to 42℃
    a. If using water bath in 134, adjust knob to desired temperature and press to display current water bath temperature
    b. If using water bath in B025, adjust knob to ~4.9; if too hot when arriving at the heat-shock step, replace hot water with cold water
4. Place plates in 37℃ incubator
    a. 1 broth/agar plate for positive control
    b. 1 antibiotic/broth/agar plate for the negative control and 1 for each part
5. (If using kit plate) Resuspend freeze-dried DNA
    a. Discover and mark part location on kit plate with sharpie
    b. Use pipette tip to puncture kit plate and move around to clear all foil from opening
    c. Add 10µL of DI Water to Kit Plate well xand pipette up and down until orange liquid is present in pipette tip
    d. Let stand for 5 minutes
    e. Label PCR tube for each part
    f. Pipette 10µL of DNA suspension into corresponding PCR tube
6. Retrieve competent cells
    a. Bring the ice bucket to the -80℃ freezer in 134
    b. Immediately put needed competent cells on ice when removing from freezer and record the manufacturer name (NEB/Invitrogen)
        i. Most competent cells are in the white-lidded container in the back of the freezer
7. Add competent cells to Eppendorf Tubes
    a. Label Eppendorf tubes
        i. Each will eventually contain both controls, or a part
    b. Mark the lid of the competent cell container to be used with a black dot
    c. Add 50µL of competent cells to each of the Eppendorf tubes
        i. Keep competent cells on ice as much as possible

        ii. Return competent cells to -80℃ freezer as soon as possible
8. Add DNA parts to Eppendorf Tubes
    a. Add 10µL of each DNA part to its respective Eppendorf tube and mix by gently pipetting up and down
        i. Do not add any DNA to the control tube
        ii. Store leftover DNA in -20℃ Freezer
9. Incubate tubes on ice for 30-45 minutes
10. Heat shock the cells
    a. Get the styrofoam tube holder
    b. If water bath is not at 42℃ in B025
        i. If hotter than 42℃, replace hot water with cold water to accelerate cooling
        ii. If cooler, adjust knob and update the setting specified in step 3b
        iii. Can also use water bath in 134
    c. Quickly place Eppendorf tubes into styrofoam holder and then place the styrofoam holder into the water bath for the time correlating to the type and manufacturer of the competent bacteria:
        i. E. coli - NEB: 30 sec exactly, then immediately place back into ice
        ii. E. coli - Invitrogen: 45 sec exactly, then immediately place back into ice
    d. After a minute, ensure each tube is still in contact with ice
        i. The tubes may have melted the initial surrounding ice
11. Keep cells on ice for 10 minutes
12. Add 200µL of SOC media to each tube
13. Incubate at 37℃ and ~250 rpm (~7.5 in B025) for the time corresponding to the antibiotic being used:
    a. Ampicillin (amp): this step is unnecessary
    b. Chloramphenicol (chlor): 1.5 hour
    c. Kanamycin (kan): 1 hour
    d. Spectinomycin (spec) : 2 hours
14. Plate cells
    a. Properly label plates
        i. Positive control is wild-type bacteria on broth/agar plate
        ii. Negative control is wild-type bacteria on antibiotic/broth/agar plate
        iii. Experimental trials are ‘modified’ bacteria on antibiotic/broth/agar plate
    b. Plate 125uL from the control Eppendorf tube onto both the positive and negative control plates, and 125uL from each part’s Eppendorf tube onto the respective plates
    c. Add ~5 glass beads to the plates and shake until liquid is evenly dispersed on surface
    d. Shake beads towards one side of the plate and dispense them into the appropriate container to be autoclaved
15. Incubate at 37℃ for 12-18 hours

Expected Data

- Cell growth observed on the Positive Control (Ensures that cells are growing properly)
- No cell growth observed on the Negative Control (Ensures that the antibiotic is functioning)

Paragraph Form (not updated)

The purpose of this protocol is to transform parts from the registry into competent cells. Prepare the lab space by wiping the counter with ethanol and lighting a flame. Next, add 10µL of DI water to the kit plates and pipette up and down. Transferred this to a labeled PCR tube and repeat for each part. Warm the water bath to 42℃. Transfer competent cells to ice from the -80℃ freezer. Next, add 25µL of competent cells to each Eppendorf tube. The top of the cell containers should be marked to show use, and each Eppendorf tube should be labeled with the part name of control. Return the cells to the -80℃ freezer as quickly as possible. Next, add 4 µL of DNA were to the experimental Eppendorf tubes. Mix the DNA well and store leftover DNA was in PCR tubes in the -20℃ freezer. Incubate tubes on ice for 45 minutes. Next, heat shock cells at 42℃ for 60 seconds to open the cell walls for DNA insertion. Place Eppendorf tubes into Styrofoam holders and place into the water bath. Time very precisely and immediately place tubes back into ice after 60 seconds. After about a minute, move tubes around in ice because the nearby ice has probably melted. Put cells back on ice for 5 minutes. Add 200µL of SOC media to each tube. Incubate cells for 2 hours at 37℃ at 250 rpm. Next, plate 200µL of the cells on antibiotic plates for the experimental and negative control. Plate 200µL of cells on LB plates labeled positive control. Scatter the cell suspension in drops on the plate. Pour about five glass beads onto the plate. Swirl the plate to move the glass beads around and evenly coat the media with the liquid suspension of cells. Ensure that all plates are properly labeled. Incubate plates overnight from 12-18 hours at 37℃. There should be cell growth on the positive control and no cell growth on the negative control.

Ligation Protocol WITH T4 DNA Ligase (M0202)

Introduction

Please see the NEB website for supporting information on this protocol.

Materials

- 10X T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Vector DNA (4kb)
- Insert DNA (1kb)
- Nuclease-free water

Procedure

Set up the T4 DNA Ligase Reaction

Note: T4 DNA Ligase should be added last. The table shows a ligation using a molar ratio of 1:3 vector to insert for the indicated DNA sizes. *Use NEB calculator to calculate molar ratios.*

1. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.

   Tip: Alicuote the 10x buffer less concentrated so when thawing, the DTT gets soluble more easily.

2. Set up the following reaction in a microcentrifuge tube on ice:

Table1

	A	B
1	**Component**	**Volume (µl)**
2	10X T4 DNA Ligase Buffer	2
3	Vector DNA	50 ng (0.020 pmol)
4	Insert DNA	37.5 ng (0.060 pmol)
5	Nuclease-free water	17
6	T4 DNA Ligase	1
7	Total	20

3. Gently mix the reaction by pipetting up and down and microfuge briefly.

4. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours.

5. Heat inactivate at 65 degrees C for 10 minutes.

00:10:00

6. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

    Use 25 uL DH5α cells, and add 2 uL of reaction mixture.

Nickel Column Purification

Introduction

Nickel columns are used to purify proteins that have a His-tag, as the string of histidine residues binds to immobilized nickel ions. This happens because electron donor groups on the histidine imidazole ring readily form coordination bonds with immobilized transition metals. The cells being purified will need to have been lysed before undergoing this protocol, and this can normally be done with Tris-HCl.

Materials

- HisPur Ni-NTA spin columns; 0.2 mL resin bed (25 / pack)
- HisPur Ni-NTA spin columns; 1 mL resin bed (5 / pack)
- HisPur Ni-NTA spin columns; 3 mL resin bed (5 / pack)
- Native Conditions Buffer:
  - Equilibration Buffer: 20 mM sodium phosphate, 300 mM sodium chloride (PBS) with 10 mM imidazole (pH 7.4)
  - Wash Buffer: PBS with 25 mM imidazole (pH 7.4)
  - Elution Buffer: PBS with 250 mM imidazole (pH 7.4)
- Denaturing Conditions Buffer:
  - Equilibration Buffer: PBS with 6M guanidine•HCl and 10mM imidazole (pH 7.4)
  - Wash Buffer: PBS with 6M guanidine•HCl and 25mM imidazole (pH 7.4)
  - Elution Buffer: PBS with 6M guanidine•HCl and 250mM imidazole (pH 7.4)
- Resin Regeneration Buffer:
  - MES Buffer: 20mM 2-(*N*-morpholine)-ethanesulfonic acid, 0.1M sodium chloride (pH 5.0)

Procedure

Spin Purification

1. Equilibrate column(s) to working temperature. Perform purifications at room temperature or at 4°C.
2. Prepare sample by mixing the protein extract with an equal volume of Equilibration Buffer. Use the Equilibration Buffer to adjust the total volume to be ≥ 2 resin-bed volumes.
3. Remove the bottom tab from the HisPur Ni-NTA Spin Column by gently twisting. Place column into a centrifuge tube.

   Note: Use 2.0, 15 or 50mL centrifuge tubes for the 0.2, 1 and 3mL spin columns, respectively.
4. Centrifuge column at 700 × *g* for 2 minutes to remove storage buffer.
5. Equilibrate column with two resin-bed volumes of Equilibration Buffer. Allow buffer to enter the resin bed.

6. Centrifuge column at 700 × *g* for 2 minutes to remove buffer.

7. Place the bottom plug in the column and add the prepared protein extract. Mix on an orbital shaker or end-over-end mixer for 30 minutes at room temperature or 4°C.

8. Remove the bottom plug. Centrifuge the column at 700 × *g* for 2 minutes and collect the flow-through in a centrifuge tube.

9. Wash resin with two resin-bed volumes of Wash Buffer. Centrifuge at 700 × *g* for 2 minutes and collect fraction in a centrifuge tube. Repeat this step two more times collecting each fraction in a separate centrifuge tube.

10. Elute His-tagged proteins from the resin by adding one resin-bed volume of Elution Buffer. Centrifuge at 700 × *g* for 2 minutes. Repeat this step two more times, collecting each fraction in a separate tube.

11. Monitor protein elution by measuring the absorbance of the fractions at 280nm or by Coomassie Plus (Bradford) Assay Reagent (Product No. 23238). The eluted protein can be directly analyzed by SDS-PAGE.

    Note: To remove imidazole for downstream applications, use gel filtration (e.g., Thermo Scientific Zeba Spin Desalting Columns) or dialysis (e.g., Thermo ScientificTM Slide-A-LyzerTM Dialysis Cassettes). Samples containing 6M guanidine•HCl must be dialyzed against a buffer containing 8 M urea before SDS-PAGE analysis. The Thermo ScientificTM PierceTM SDS-PAGE Sample Prep Kit (Product No. 89888) may also be used to remove guanidine.

Resin Regeneration

12. Wash resin with 10 resin-bed volumes of MES Buffer.

13. Wash resin with 10 resin-bed volumes of ultrapure water.

14. Store resin as a 50% slurry in 20% ethanol.

Troubleshooting

Problem	Possible Cause	Solution
Low protein yield	Poor expression of soluble protein	Optimize expression conditions
	His-tagged protein forms inclusion bodies	Alter growth conditions to minimize inclusion body formation and maximize soluble protein yield; alternatively, solubilize inclusion bodies and perform the purification with a compatible denaturant (e.g., Inclusion Body Solubilization Reagent, Product No. 78115)
	Insufficient cell lysis and extraction	Optimize cell lysis protocol
	Fusion protein does not bind to the column	Verify the sequence or perform an ELISA or Western blot using an antibody against the His tag to make sure the His tag is present
Poor protein purity	Insufficient washing	Wash resin additional times or modify imidazole concentration and pH of the Equilibration and/or Wash Buffer
Slow column flow	Column is overloaded	Apply less protein extract onto the column and make sure the extract is not too viscous or highly particulate

PCR

Introduction

Polymerase chain reaction (PCR) is used to amplify the amount of DNA through creating numerous copies of a given DNA sequence.

Materials

- 10x standard Taq run buffer
- 10 mM dNTPs
- 10 µM forward primers
- 10 µM reverse primers
- template DNA
- Taq DNA polymerase
- nuclease-free H_2O
- PCR tubes
- Thermal cycler
- Gel supplies
  - Gel casting tray
  - Comb
  - Electrophoresis chamber
  - TAE
  - Agarose
  - SybrSafe
  - DNA ladder
  - Loading dye

Procedure

Reaction Mixture

1. Add the following to a tube in order:

   -If the amount of template DNA added is different, adjust the water so that the total volume is 25 µL

Table1

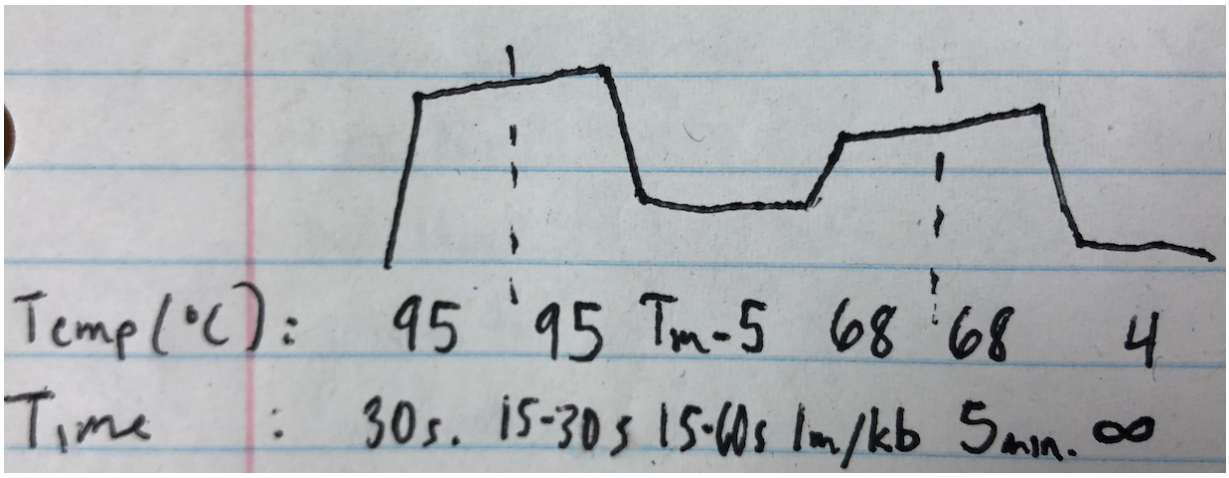
	Component	25 µL Reaction Amount
1	10x standard Taq rxn buffer	2.5 µL
2	10 mM dNTPs	0.5 µL
3	10 µM forward primer	0.5 µL
4	10 µM reverse primer	0.5 µL
5	template DNA	2.0 µL
6	Taq DNA polymerase	0.125 µL
7	nuclease-free H2O	18.875 µL

2. Spin down the sample before thermal cycling.

PCR Temperatures

3. Set up the PCR template in a thermal cycler according to the following diagram:

    -The Tm value is the temperature the primers should work at, and can be found in Benchling



4. Run a gel to confirm that the bands are in the correct location.

    -Put 2 µL of loading dye and 5 µL of nuclease-free water with each sample

5. If the bands appeared correct, proceed to perform a PCR clean-up.

pGEM

Introduction

pGEM acts as a cloning vector that has "AT" overhangs, allowing for the PCR product to ligate readily into the vector. This is used so PCR product is ready for any subsequent cloning.

Materials

- 1.5 mL tubes
- PCR product
- 2x Rapid Ligation Buffer
- pGEM T-Easy Vector
- Control Insert DNA
- T4 DNA Ligase
- diH_2O
- JM109 Competent Cells
- Room temperature SOC Medium
- LB/Amp plates
  - Will need to add diH_2O, IPTG, X-Gal
- Ice bath
- Heat bath

Procedure

Ligation

1. Vortex the tubes to mix, then centrifuge to collect all of the liquid at the bottom of the tubes.

2. Set up the ligation reactions as described below, with the total volume equaling 10 µL:

Table1

	Reagents	Standard Rxn	+ Control	- Control
1	2x Rapid Ligation Buffer	5 µL	5 µL	5 µL
2	pGEM T-Easy Vector	1 µL	1 µL	1 µL
3	PCR Product	3 µL	-	-
4	Control Insert DNA	-	2 µL	-
5	T4 DNA Ligase	1 µL	1 µL	1 µL
6	Deionized Water	total volume 10 µL	total volume 10 µL	total volume 10 µL

3. Mix the reactions by pipetting. Incubate the reactions for 1 hour at room temperature, or incubate overnight at 4 degrees Celsius.

Transformation

4. Prepare plates with LB/Amp/IPTG/X-Gal.

   -To prevent uneven application, first add 100 µL of diH_2O, then 40 µL of IPTG, then 40 µL of X-Gal

5. Centrifuge the reaction mixtures.

6. Add all of each ligation reaction to a sterile 1.5 mL tube on ice.

7. Allow the JM109 competent cells to thaw on ice, mixing by flicking.

8. Transfer 50 µL of the competent cells into the ligation reaction tubes. Gently flick the tubes and incubate on ice for 10-20 minutes.

9. Heat-shock the cells for 45-50 seconds in a 42 degree Celsius water bath. Immediately return the tubes to ice for 2 minutes.

10. Add 200 µL of room temperature SOC medium to each ligation reaction tube, and incubate for 1-1.5 hours at 37 degrees Celsius in the shaking incubator.

11. Plate all of each transformation culture onto separate plates. Incubate the plates overnight at 37 degrees Celsius; select white colonies.

Plasmid DNA Mini Prep

Project: iGEM 2019
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WEDNESDAY, 6/12/2019

Introduction

Miniprep allows for the isolation and purification of plasmid DNA from bacterial cell cultures.

Materials

- Cell culture
- Microcentrifuge tubes
- Spin columns
- Collection tubes
- Solutions 1, 2, 3
- HBC buffer
- DNA wash buffer
- Beaker, for discarded filtrate

Procedure

1. grow a cell culture (1-5 mL)
2. centrifuge culture at 10,000 g for 1 minute
    a. decant culture media
3. add 250 µL of Solution 1 from fridge
    a. vortex
4. add 250 µL of Solution 2
    a. invert tube until a clear lysate is seen
    b. incubate for 3 minutes
5. add 350 µL of Solution 3
    a. invert quickly, until white precipitate forms (after around 2 minutes)
    b. centriuge at max speed for 5 minutes
6. insert DNA mini column into 2 mL collection tube
7. transfer supernatant through aspiration into mini column
    a. centrifuge at max speed for 1 minute
    b. discard filtrate
8. add 500 µL HBC buffer
    a. centrifuge at max speed for 1 minute
    b. discard filtrate
9. add 700 µL DNA wash buffer diluted with ethanol
    a. centrifuge at max speed for 30 seconds
    b. discard filtrate
10. centrifuge column at maximum speed for 2 minutes
11. transfer column into a clean microcentrifuge tube
    a. add 50 µL of heated water
        I. water should be heated at 60 degrees Celsius for 10 minutes
    b. centrifuge at maximum speed for 60 seconds
12. store eluted DNA at -20 degrees Celsius

Prep of Chemically Competent E. coli (Rubidium Chloride Method)

Introduction

Cells need to be able to uptake plasmids and other genetic information when undergoing transformation, so this procedure allows for cells to become chemically competent. Cells are treated with calcium chloride to facilitate the attachment of plasmid DNA to the cell's membrane.

Materials

- Plate with colonies
- LB broth
- Spectrometer
  - For reading the cell density
- Test tubes
- $MgSO_4$
- 50 mL conical tubes
- TFBI
  - look at recipes below for different mL cultures
- TFB2
- Ice bath

Procedure

Preparing a Culture

1. Inoculate a single colony from a rich plate (Luria-Bertani agar) into 5 mL of rich LB broth in a test tube.

   -Shake overnight at 37 degrees Celsius

2. Subculture the overnight 1:100 in LB+20 mM $MgSO_4$ (typically 2.5 mL culture into 250 mL of LB+ 5 mL 1 M $MgSO_4$).

   -Grow to OD590=0.4-0.6 or Klett=60 (around 2-3 h)

3. Centrifuge at 3,220 g for 10 minutes at 4 degrees Celsius, using 50 mL conical tubes (divide the culture evenly into 8 tubes)

4. Gently resuspend pellet in 1/2.5 volume unit ice cold TFBI. For 300 mL subculture, use 120 mL TFBI.

   -Discard supernatant
   -Add 25 mL TFBI to 4 of the tubes
   -Resuspend using a 25 mL autopipetter

-Transfer cells to tubes without TFBI, and resuspend

Temperature-Sensitive Steps

5. Incubate on ice for 5 minutes.

   -Keep all steps on ice, and chill pipets/flasks

6. Centrifuge at 3,220 g for 10 minutes at 4 degrees Celsius.

7. Resuspend pellet in 1/25 original volume cold TFB2. For 300 mL of original subculture, use 12 mL TFB2.

   -Discard supernatant
   -Add 10 mL TFB2 to one of the tubes
   -Resuspend
   -Transfer cells to next tube and resuspend; repeat for all 4 tubes

8. Incubate on ice for 15-60 minutes.

   -While incubating, get a tray of ice, label the microfuge tubes, and let them chill

9. Aliquot 110 µL and 220 µL.

10. Quickfreeze the tubes using EtOH or a dry ice bath.

11. Store in a -80 degrees Celsius freezer.

Recipes for Media

Recipes

RB (Luria-Bertani medium)

per liter:
10 g Tryptone (Difco)
5 g Yeast Extract (Difco)
5 g NaCl
2 ml 1N NaOH

250mL Culture
0.294 g KOAc Potassium Acetate
1.2092 g RuCl
0.111 g $CaCl_2$
0.99 g $MnCl_2 \cdot 4H_2O$
15 mL Glucerol
opt to 100mL

TFBI

1.2g 30 mM KOAc (potassium acetate)
4.95g 100 mM RbCl
0.455g 10 mM $CaCl_2$
2.58g 50 mM $MnCl_2$
15% glycerol

$30\ \frac{mmol}{L} * \frac{1\ mol}{1000\ mmol} * 0.41L * 98.14\ g/mol = 1.2\ g\ KOAC$
$100mM * \frac{1\ mol}{1000\ mmol} * 6.41L * 120.92\ g/mol = 4.95\ g\ RbCl$
$10mM\ CaCl_2 * \frac{1}{1000} * 0.41L * 110.98\ g/mol = 0.455\ g\ CaCl_2$
$50mM\ MnCl_2 * \frac{1}{1000} * 0.41L * 125.84\ g/mol = 2.58\ g\ MnCl_2$

100mL culture
0.72 g KOAc
2.97 g RbCl
0.273 g $CaCl_2$
1.548 g $MnCl_2$
60 mL → 9 mL Glycerol

Adjust to pH 5.8 with acetic acid and filter (0.45 um, Nalgene units or Millipore filters) to sterilize.

It is convenient to make this as:

5 g	RbCl (Alfa)
12.3 ml	KOAc 1 M
4.1 ml	$CaCl_2$ 1 M
20.5 ml	$MnCl_2$ 1 M (this is pink)
61.5 ml	glycerol
pH to 5.8 with	8 ml HOAc 0.1 M

~~1.25g RbCl~~

make up to 410 ml; distribute in 100 ml sterile aliquots; and use 1 aliquot/250 ml culture.
60 100

TFBII

1.25g 10 mM MOPS or PIPES
0.18g 75 mM $CaCl_2$
10 mM RbCl
15% glycerol

$10 * \frac{1}{1000} * 0.15 * 209 = 0.31\ g\ MOPS$
$75\ \frac{mmol}{L} * \frac{1\ mol}{1000\ mmol} * 0.150L * 110.99\ g/mol = 1.25\ g\ CaCl_2$
$10mM\ RbCl * \frac{1}{1000} * 0.15 * 120.92\ g/mol = 0.18\ g\ RbCl$

Adjust pH to 6.5 with KOH and filter to sterilize

Make up as

1.5ml	MOPS 1 M pH 6.5 (this is yellow)
11.25ml	$CaCl_2$ 1 M
1.5ml	RbCl 1M
22.5 mL	glycerol
pH with	1 N KOH

make to 150 ml; filter; use 10 ml per original 250 ml culture.

Procedure adapted from the John Innes Institute (Norwich, England) via Joseph Utermohlen (Univ. of Arizona).

This procedure improves transformation with most strain backgrounds, when compared with $CaCl_2$ procedure.

Protein Solubilization

Introduction

The purpose of solubilizing proteins is to lyse the cells so that a sample of proteins can be collected for SDS-PAGE or other protein-related procedures. This will also show if the transformed cells express the desired protein. You will need to know if the desired protein is transmembrane, cytoplasmic, or periplasmic. For example, chitin synthase is a transmembrane protein.

Materials

- Colony of unmodified *E. coli* for negative control
  - Use positive control colony from transformations
- Colony of modified *E. coli*
- Luria Broth (LB) (5 ml per lysate sample)
- Phosphate-buffered Saline (PBS) (1 ml per lysate sample)
- 20 mM Tris-HCl, pH 7.5 (1 ml per lysate sample)
- Membrane protein lysis buffer (1 ml per lysate sample for transmembrane proteins)
  - 150 mM NaCl, 50 mM HEPES, 5% Triton X-100
  - To prepare 50 ml of membrane protein lysis buffer: 0.438 g NaCl, 2.5 ml 1M HEPES buffer, 2.5 ml Triton X-100, fill with ddH_2O until total volume is 50 ml
  - Chitin synthase specific buffer: 180 mL Protein Seperation Buffer A, 0.5% digitonin (w/v)
  - Source: http://www.jbc.org/content/268/3/1702.full.pdf
  - NodC should work with the regular protein lysis buffer
- Triton X-100 (50 ul per lysate sample for transmembrane proteins)
- 100 mm bacterial culture dish (1 per lysate sample)
- Large pipette tips
- Tubes for inoculation (1 per sample)
- Microcentrifuge tubes (2 per lysate sample)
- Incubator
- Sonicator
- Vortex machine
- Nanodrop

Procedure

Initial Colonies and Plating

1. Inoculate desired culture in 5 mL LB broth and grow overnight

2. Plate 100 µL on LB agar plate and allow to grow overnight until a thin layer of cells cover the agar.

3. In the -20 degree Celsius freezer, cool microcentrifuge tubes for lysates, along with sterile large pipette tips.

   -Make sure all solutions are in the 4 degrees Celsius fridge at least 24 hours in advance
   -Cool the plates at 4 degrees Celsius
   -Keep plate and all microfuge tubes on ice while working

4. Add 1 mL of cold phosphate-buffered saline (PBS) to plate and swirl.

5. Drain the PBS from the plate.

6. Using the wide end of a sterile large pipette tip, scrape all the cells from the culture plate into the cold microfuge tube.

Solubilization

7. Add 1 mL of ice cold 20 mM Tris-HCl to the microfuge tube.

   -Incubate on ice for 10 minutes

8. Vortex tubes for 30 seconds, then incubate on ice for 30 seconds.

   -Repeat this step as many times as necessary to resuspend and lyse cells

9. Incubate tubes on ice for 1 minute.

10. Sonicate for 10 seconds at the lowest temperature option, then incubate on ice for 40 seconds.

    -Repeat this step 9 more times in order to lyse cells

11. Centrifuge for 20 minutes at 13,000 RPM in the cold room.

    -If the cold room isn't available, centrifuge in four 5 minute intervals, incubating on ice for 2 minutes in between each interval

12. Transfer the supernatant to a new, cold microfuge tube

    -If the protein is cytoplasmic or periplasmic, the pellet can be discarded
    -If the protein is transmembrane:
    -Add 1 mL membrane protein lysis buffer, vortex tubes for 30 seconds and then incubate on ice for 30 seconds (repeat as many times necessary for membrane resuspension)

13. Determine the concentration of the protein in the sample using a Nanodrop

    -Setting should be on A280
    -Cytoplasmic and periplasmic proteins use 20 mM Tris-HCl as blank
    -Transmembrane proteins use membrane protein lysis buffer as blank

14. The solubized enzyme can be concentrated through ultrafiltration through an Amicon YM-100 filter

Restriction Enzyme Digest

Introduction

Materials

- d_2H_2O
- DNA
- Restriction Enzyme(s)
- 10x Buffer

Procedure

Cloning

1. Add the following amounts:

Table1

	A	B
1	DNA	1-5 ug
2	10x Buffer	5 uL
3	RE 1	1 uL
4	RE 2	1 uL
5	d2H2O	X uL
6	**Total**	50 uL

2. Incubate @ 37 for an hour - overnight

3. Gel Electrophoresis

Checking

1. Add the following amounts:

Table2

	A	B
1	DNA	200 ng - 1ug
2	10x Buffer	1 uL
3	RE 1	0.4 uL
4	RE 2	0.4 uL
5	d2H2O	X uL
6	**Total**	10 uL

2. Incubate @ 37 for >30 minutes
3. Gel electrophoresis

SDS-PAGE Protein Gel

Introduction

SDS-PAGE is a method used to seperate and identify protein molecules. Sodium dodecyl sulfate (SDS) is used to denature the proteins, then they are isolated via electrophoresis.
This specific protocol will be used to validate the presence of chitin synthase in *Pseudomonas fluorescens*. The steps that may come before this include preparation of a cell culture, solubulization of the protein, and purification.

Materials

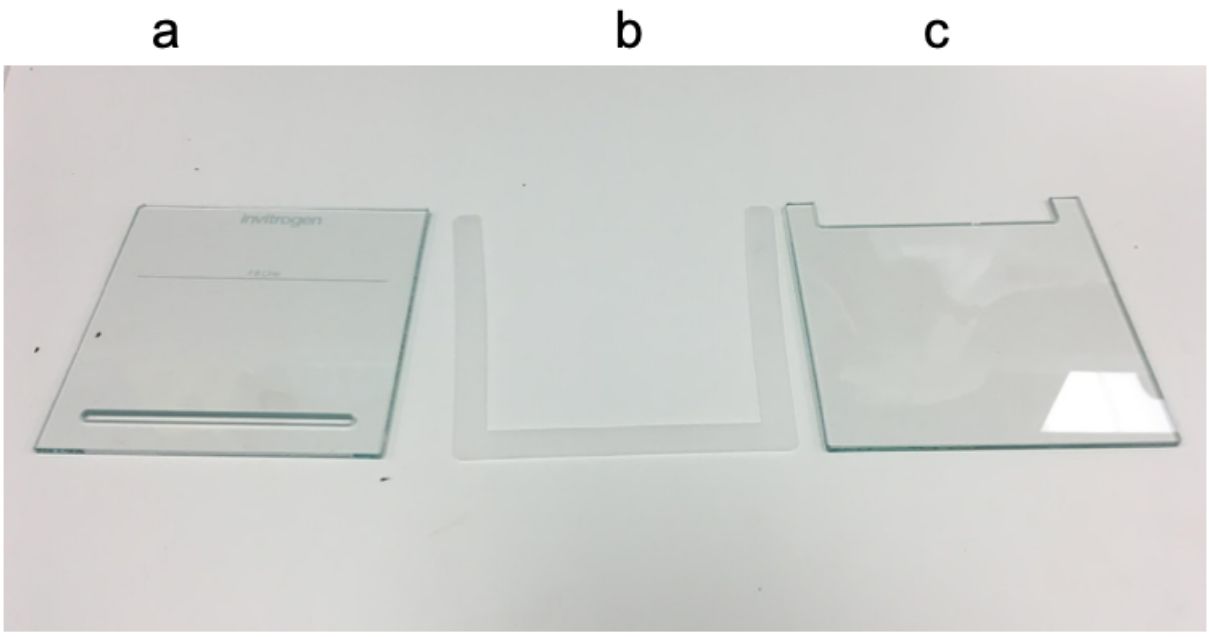
- 2 glass plates
- Gel Spacer
- Casting Support
- Conical tubes
- 40% Acrylamide
- Stacking Buffer
- Resolving Buffer
- d_2H_2O
- 10% Ammonium persulfate (APS) Solution
- Tetramethyleethylenediamine (TEMED)
- Butanol
- Well Comb
- SDS Sample Buffer
- Dithiothritol (DTT)
- Vertical Electrophoresis Cell
  - Electrophoresis Housing Unit
  - Electrophoresis Gel Cast Support with Electrodes
  - Lid
  - Tris-Glycine Running Buffer (1x concentration)
  - Power Supply (PowerPac Basic Power Supply)

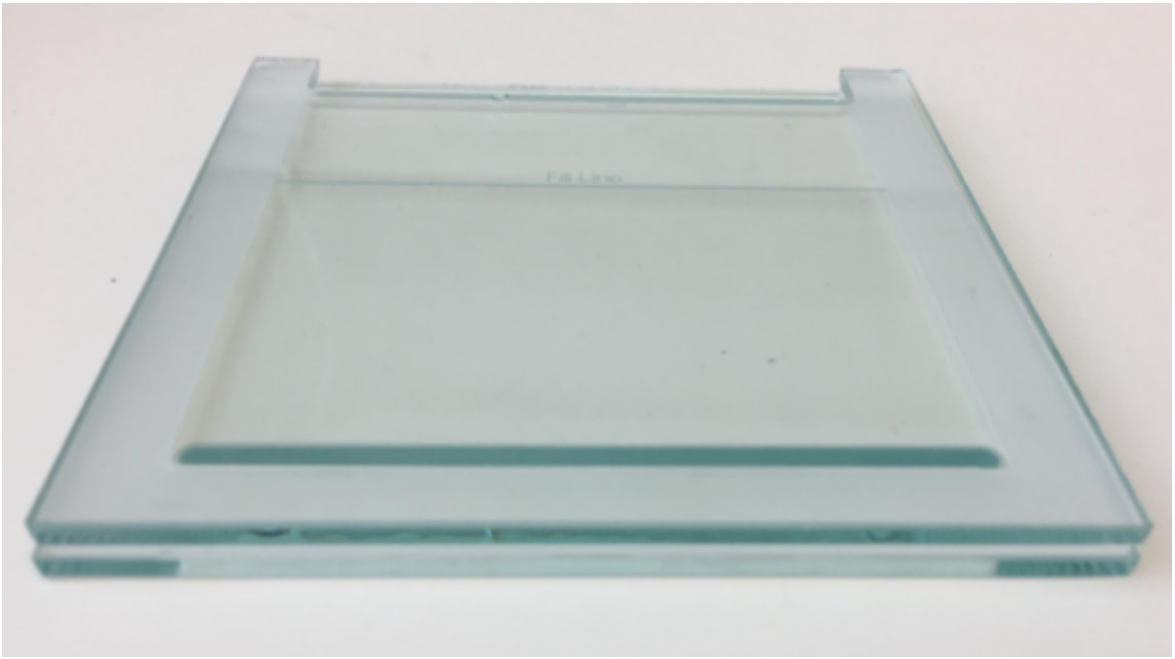
Procedure

Preparation of Gel Cast

1. Set up the vertical loading tray.

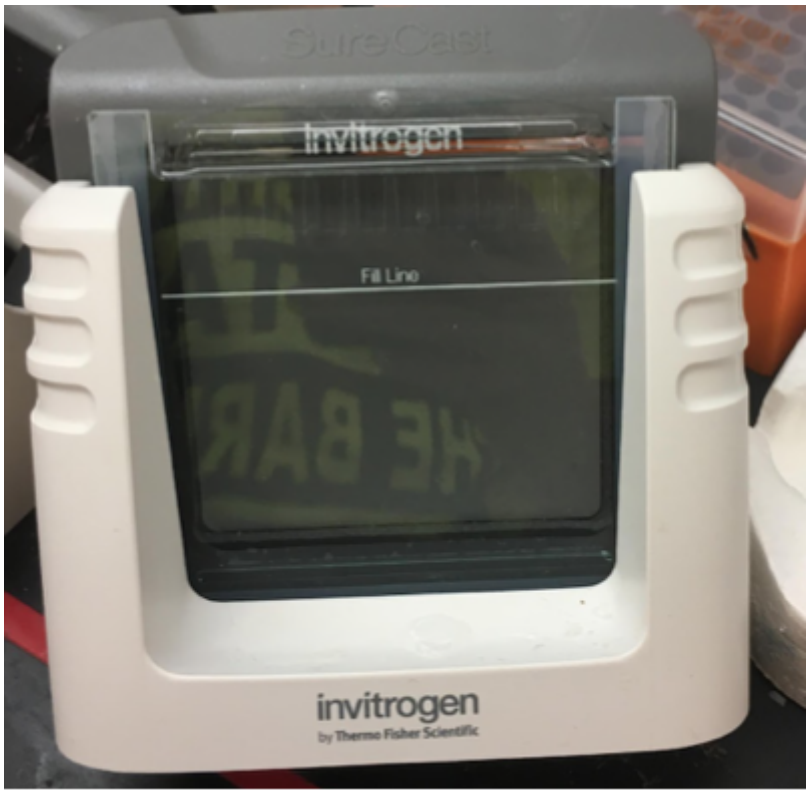
    -The gel spacer (b) is placed on top of the Invitrogen labeled glass plate (a), with the edges aligning.
    -The final glass place (c) is placed on top of the gel spacer.





2. Set up the glass casting apparatus.

    -Plate a is placed withthe open groove at the bottom against the back, and the blank c plate is held against the front



Making the Gel

3. Determine the size of the pores needed for the resolving gel, depending on the size of the protein you want to seperate.

   -Chitin synthase is around 180 kDa
   -Acrylamide is considered a health hazard, as it is a carcinogen and mutagen. Handle it in a fume hood, and take extra precautions to prevent the acrylamide from making any contact with skin.

Typical Pore Size for SDS Gel

	% Acrylamide	MW Range (kDa)
1	7	25-500
2	10	15-200
3	12	10-100
4	15	3-75

4. To make a 10% resolving gel (bottom layer) solution, add the following in order to a conical tube:

   -2.0 mL of 40% acrylamide
   -2.0 mL resolving buffer
   -3.9 mL distilled water
   -80 µL of 10% APS
   -8 µL TEMED (add quickly, since the gel will begin to polymerize)

5. Agitate the solution lightly to mix.

    -Avoid bubbles if possible

6. Pour the solution into the casting apparatus, to the. first fill line.

7. Add butanol to cover the top, and then let the gel set.

    -This is done to prevent the gel from reacting with oxygen, and so that the gel settles evenly.

8. Once the gel has set, remove the butanol by absorbing with a paper towel.

    -Avoid touching the gel with the paper towel

9. To prepare the stacking gel (top layer) solution, add the following in order:

    -300 µL of 40% acrylamide
    -750 µL stacking buffer
    -1.92 mL distilled water
    -30 µL of 10% APS
    -3 µL TEMED

10. Agitate the solution lightly to mix.

11. Pour on top of the resolving gel, to the top of the divot.

    -Overflow out of the cast is okay

12. Place the comb snuggly into the gel so that the flat, smooth side faces the back and is pressed against the glass.

    -Allow the gel to set

Sample Preparation

13. For each sample, add the following into a 1 mL centrifuge tube:

    -20 µL SDS sample buffer
    -2 µL DTT
    -18 µL of 10-20 µg of the desired protein sample

14. Heat at 95 degrees Celsius for 2 minutes

Running the SDS-PAGE Gel

15. Place the electrodes in the housing unit.

    -Do not put both electrodes in, unless you are using both sides

16. Once the gel has completely solidified, remove the comb and place the glass casting plates into the housing unti with the gel.

    -Use the grey/black locking support with the electrode on it to lock the glass plates in place by pulling the grey handle towards the glass plate

17. Fill the unit with Tris-Glycine buffer until it slightly overflows.

18. Load 5 µL of the PAGE ladder.

    -Use protein gel loading tips

19. Load the rest of the protein samples.

20. Place the lid on top of the housing unit, and connect to the power source.

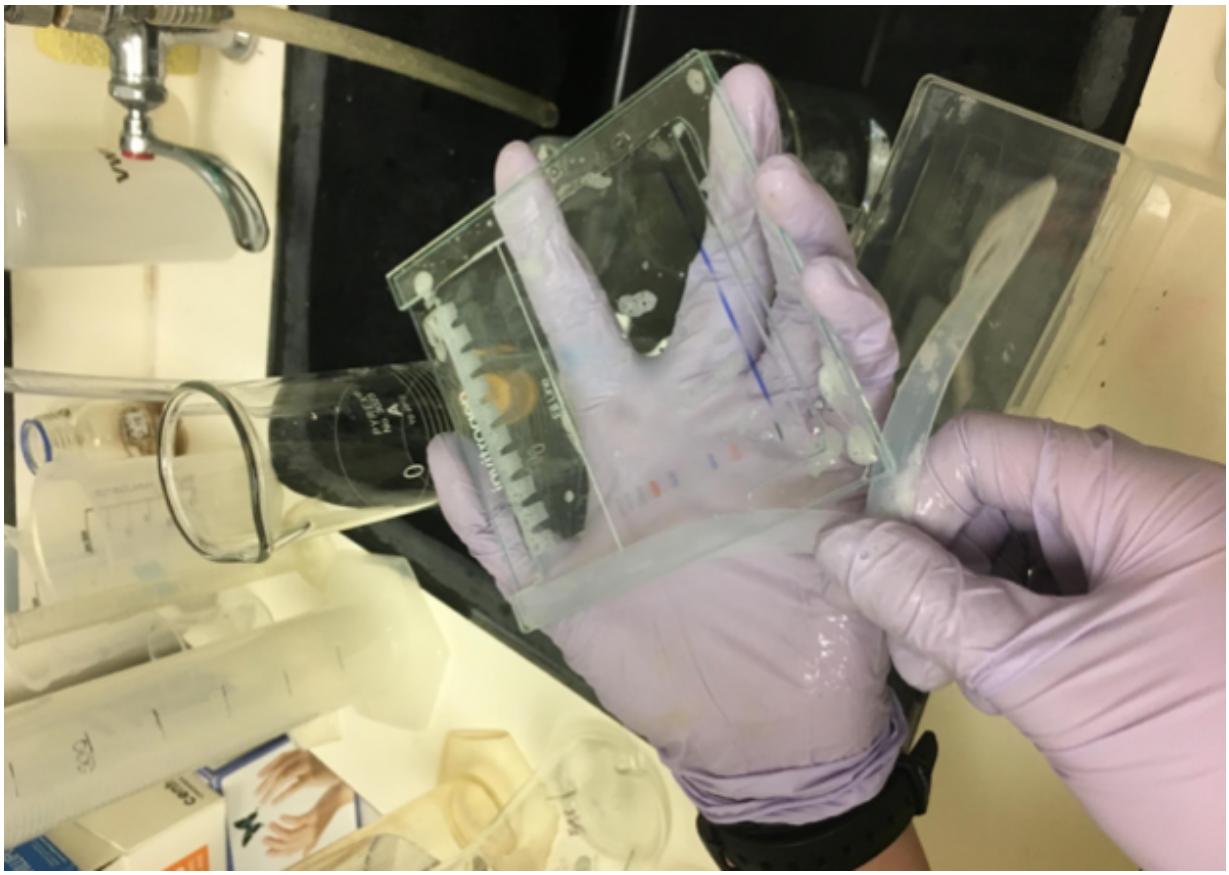
21. Run the gel at 90 V through the stacking gel (should take around 30 minutes), then change the voltage to 130 V and run for 30 more minutes.

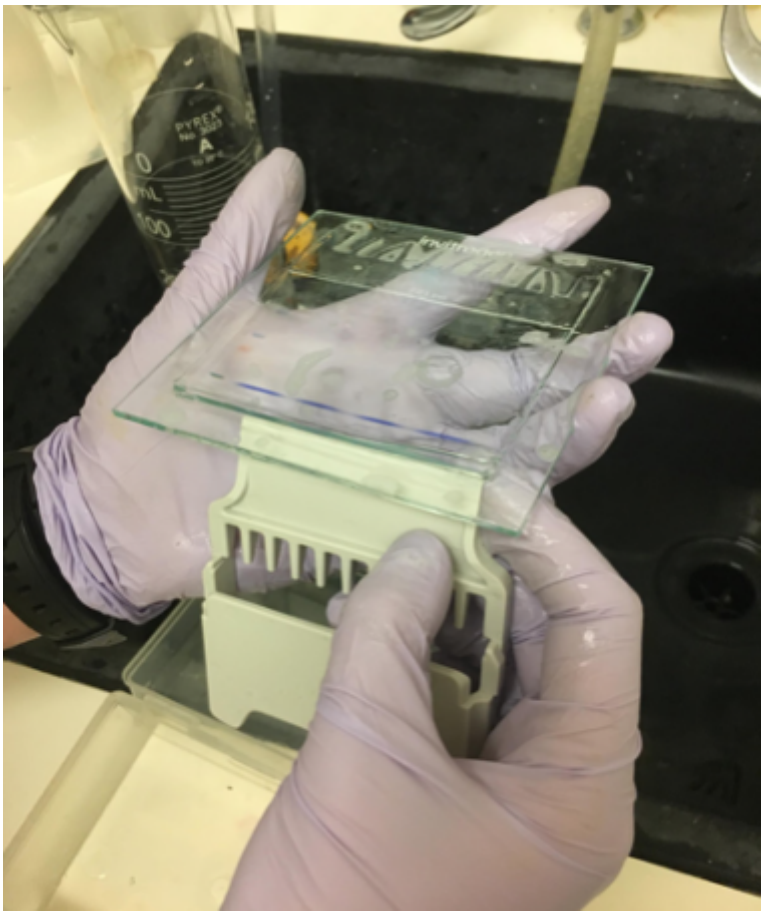
    -The gel should run until the samples have reached the area just above the groove thats on the bottom of the plate in the back of the casting apparatus

22. Turn off the power source when completed, then remove the lid.

23. Remove the casting apparatus from the housing unit by unlocking the cast support.

24. Use the edge of the well guide to pry away at the gel spacer, lifting the top plate from the bottom plate. Push the gel up through the groove using the well guide.





-Be very careful because the gel is thin, and tears easily
-Place the gel into an empty storage box

Staining the Gel

25. Fill the storage box with Commassie G stain, until it covers the gel.

26. Place the box on a shaker plate, and shake at room temperature for 45 minutes to an hour.

27. Drain the stain into the bottle labeled "reused Commassie Stain".

28. Cover the gel with destain solution.

    -Place on the shaker, let it run overnight
    -This step may need to be repeated until the bands appear against a relatively clear background

29. Pour the destain solution down the drain with water

Imaging the Gel

30. Place the gel on the orange plate in the gel imaging machine.

31. Use the visible light setting to image the gel.

-Auto exposure should wotk for the time setting

-Press capture to create an image, then save and print it