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Lab Safety and Standard Operating Procedures

A) Safety

1. Read through the lab safety plan in the Safety Desk Book and ask Ishwar any questions pertaining to safety issues
2. Know the location of safety equipment: eye washes, showers, fire extinguisher, first aid kit, MSDS
3. Know whom to contact in case of an emergency (911, Ishwar, Office of Research Safety)
4. Know proper handling of organics and volatile liquids
5. Use proper chemical waste disposal (acetonitrile, organic solvents, Coomassie stain/destain, ethidium bromide, gel waste, TAE buffer)
6. Use biohazard waste disposal for all disposable materials that come in contact with *E. coli*. Use 10% bleach to decontaminate any non-disposable containers
7. Wear proper safety attire (coats, glasses, nitrile gloves, respirators)
   **** Always wear long pants and closed-toe shoes**
8. Know lab evacuation meeting point (in the grass on the northeast side of Cook Hall)

B) Administrative Issues

1. Ordering supplies: (Use clipboard request form on the 4 degree fridge). If you use the last of something or you see we are running low, place a reorder request.
2. Discuss any equipment maintenance needs with Ishwar
3. Isotope log: All labeled cultures must be approved by Ishwar. Usage of the ^{15}N and ^{13}C isotopes must be recorded in the isotope log book.
4. Services sign up: Log all mass spec submissions and results.
5. Refer to the "Lab Protocols" handbook for standard techniques in our lab
6. Use sign-up calendars to get priority for using various equipment
7. All DNA constructs and primers must be logged in RichMaker – ask Ishwar

C) General Lab Procedures

1. Keep the lab door closed and locked when no one is here (we have thefts!)
2. Computers: Keep your computer clean and dust-free. Back-up precious data. Keep food, drink, chemicals, and gloves away from the computers.
3. Clean your bench each week and consolidate freezer/fridge space once a month
4. Clean-up any spills or mess that you create – follow proper procedures
5. Autoclaving: Label, date and initial your media!
6. Dirty dishes:
   - Remove all labels/stickers
   - Bleach and rinse out all tubes and flasks containing cultures/biohazard
   - Leave the decontaminated large culture flasks to be hand washed
   - Everything else is rinsed and placed on the cart for the dishwasher

   Small items:
   - Wash off scoopulas after use – do not leave in the sink
   - Wash gel plates, stir bars, and columns in soap water; do not leave in sink
   - All other gel running items may be rinsed with water immediately after use
7. Communicate with others and always be courteous!

D) Basic Lab Equipment

-pH Meter- Our pH meter has two electrodes available for use. The narrow bore electrode is to be used only for NMR samples or samples with volumes too small to be measured by the standard electrode. Both are capable of measuring pH in Tris buffer solutions. Never use either electrode for measuring the pH of media or solutions containing reducing agents (DTT or BME), Urea, or Guanidine. Measure pH of buffer first and then add these components.

Always start by lifting the electrode out of the storage buffer, then opening the port in the top of the electrode. Either turning or sliding down the sleeve does this, depending on the electrode being used. Rinse the electrode extensively in UV/UF water. Gently blot dry with a Kimwipe. Place the electrode back into storage buffer when finished. Do not rest the tip of the electrode against the bottom of the storage tube. Never leave the electrodes hanging in air. Never touch the tip with your hands or gloves.

-Calibration of the pH electrodes- this should be done once per week. Place the electrode in the pH 7 buffer (yellow), allow reading to stabilize, press "std" twice. Remove the electrode from the buffer, rinse with UV/UF water, blot dry. Repeat with pH 4 buffer (red) if the buffer you are preparing needs to be at a pH in the 4-7 range or with the pH 10 buffer (blue) if your buffer is in the pH 7-10 range. The electrode is now ready for use. If the readout does not say GOOD ELECTRODE, pH readings may not be trustworthy – ask Ishwar for advice.

-Balance- Both balances in the lab are simple to use. They need only to be zeroed with weighing boat or paper before measuring and cleaned after use. Please do this every time. Turn off the balance by holding down the "on/off" button until the display turns off

-Pipette Use- There are multiple sets of four pipettes in the lab. Ideally each individual would have his or her own set ranging from 2 ul to 1 ml. However as the lab grows it will become necessary to share sets for a period of time. Improper usage can not only damage the pipettes (leading to volume errors), but contaminate your own and other's samples. Some general guidelines when using any pipette: tap firmly when seating a tip, press and release plunger smoothly, when expelling a sample go just past the first stop, always watch the amount being pulled into the tip and the amount expelled. Expel samples under the surface of the new solution to avoid splattering. Careful attention during pipetting can avoid many problems during experiments.

-Sonicator- The sonicator is located in the cold room. Before use, wash the tip with water then ethanol. Gently blot dry with a Kimwipe. Place sample (in ice bucket) under tip and slowly raise the platform so the tip is in the sample approximately 1 inch. Tighten the clamp to maintain this position. Heat is generated during sonicating, so monitor the level of the tip between cycles as the the sample tube might descend because of melting ice, raising the position of the tip. Turn on the power. Select the appropriate settings for your sample (e.g. use preset program #1 (1 second on and 5 seconds off) for lysing bacterial cells). The method is called by pressing the "recall" button and then inputting "01". Press "start". To limit sample heating, give at least a 10 minute break between back-to-back cycles. When finished sonicating, turn off the power to the unit and lower the platform to remove your sample. Rinse the tip with water and ethanol, then blot dry.

-Centrifuges- The Sorvall and Eppendorf centrifuges should always be used with the rotor lid on. Keep Sorvall centrifuge door closed at all times to maintain 4 degrees. The power to this unit should always stay on. Check for any ice build-up. If there is ice, defrost, wipe condensate with a paper towel and dry before operation. Operating the centrifuge when there is ice in the unit is unsafe.

Be aware of the rpm/rcf and the capacity of the tube you are spinning. The 15 and 50 ml conical tubes are rated to withstand 6000 g. Concentrator units also have limits – check documentation that comes with each unit for limits.

All tubes must fit into the rotors snugly using appropriate adapters. Sorvall centrifuges have carbon fiber rotors, so they are easy to carry. **Never drop these rotors as they damage easily and can be very expensive to fix!**

-Freezers- We have 3 freezers: an under-counter –20 °C freezer, a much larger –20 °C freezer and a –80 °C freezer. Each of these units need to be defrosted occasionally. In order to minimize the frost buildup and to maintain the cold temperatures, we need to keep the doors closed as much as possible. In addition, we will use an ice scraper to clean off the frost by hand without completely defrosting the freezers. Approximately once a year, we empty the –20 °C freezers and defrost it completely.

Each lab member has an assigned shelf space in the freezer. These areas should be kept clean by each owner and old samples, tubes, etc, should be discarded on a weekly basis. All cell pellets must be stored in Whirlpaks – be sure to label them with the name of the construct, the date it was expressed and your initials.

-Refrigerators- We have several refrigerators in the lab: the chromatography fridge (with the clear glass doors), under-counter refrigerators, a regular kitchen-type refrigerator (aka the 4 degree fridge), and the walk-in cold room. Each lab member is assigned some shelf space in under-counter refrigerators. As with the freezers, these areas should be kept clean by the owner and old samples discarded weekly.

Typically, the chromatography refrigerator holds columns and resins, the 4 degree fridge holds agar plates and chemicals (antibiotics, IPTG, TCEP, etc), and the cold room houses various equipment (FPLCs, incubator/shakers, gel equipment, nutators).

-Incubators- We have one stationary incubator for growing plates, one floor shaker incubator, and three bench-top shaker incubators for growing liquid cultures. The bench top incubators do not have a refrigeration unit and the growth temperature needs to be at least >5 degrees above ambient temperature. One of these incubators is located in the cold room, so that it can be used for growing cultures at 10 °C or higher.

It is essential for all of the incubators to be kept clean so they will continue to function optimally. **All spills must be cleaned up immediately, as failure to do so can compromise the motor and drive mechanism.** For spills, use 70% ethanol to decontaminate the area (**do not use bleach, as it will corrode metal!**).

In addition, it is essential that the flask clips be assembled properly onto the rotating platform inside the incubator.

1) All of the slots for screws should be used to anchor the clip.
2) The clips for the 2800 ml flasks must have "bumpers" at the bottom.
3) The edge of the flasks must never extend over the edge of the rotating platform.

E) Other Operating Procedures

DNA Handling and Storage

We store all DNA in the –80 °C freezer. Each lab member is assigned their own freezer box which holds eppendorf tubes. All DNA such as primers and plasmid constructs should be stored in the box and the location should be logged in our online inventory system called Richmaker. You will need to be trained on how to use RichMaker by the lab manager.

Biohazard and Hazardous Chemicals

Any item that comes in contact with *E. coli* or *insect cells* is considered a **biohazard**. If it is disposable (such as serological pipets, cuvettes, conical tubes) the item can be bleached and then discarded in the Red Biohazard waste bin. If it is not disposable (culture flasks, centrifuge tubes, and bottles) the item must be soaked in 10% bleach until the solution is clear (i.e. all the bacteria are dead). Then the item must be rinsed with water and designated for hand wash or placed on the cart in a Nalgene for the dishwasher.

Any solution that becomes spontaneously contaminated (such as LB broth stored at room temperature) must also be considered a **biohazard** and must be bleached and cleaned as described above.

Hazardous chemicals include the following items (this is not a comprehensive list):

Acetonitrile
HPLC buffers
Methanol
Coomassie stain
Coomassie destain
Ethidium bromide
TAE running buffer
Lyophilizer waste (ice or melted ice)
Acrylamide gels
Agarose gels (with ethidium bromide)

Waste from these items MUST be collected **individually** in special containers and the lab manager MUST submit a request for ORS (Office of Research Safety) to collect the waste. The waste is characterized as either a solid or a liquid. Liquid waste goes into the white bottles or the big drums (HPLC waste). Solid waste goes into the clear bags. Each liquid waste container must be labeled with the percent composition of each buffer component and no abbreviations for chemicals are allowed.

Growing cultures, expressing protein, storing pellets

We have a finite number of culture flasks, centrifuge bottles, and Oakridge tubes that are used at different stages of protein expression and purification. Please be courteous and ask others

before you plan a large scale expression (4 liters or more) or a lengthy expression (longer than 24 hours). Combine pellets into a Whirlpak bag for storage at –80 °C.

Equipment use

Each person in the lab must be trained in the basic operation and trouble shooting of each piece of equipment before use. There are instruction manuals that can be referenced for each instrument. A trainer may be the lab manager or a senior lab member who is skilled in operating the instrument.

Equipment Maintenance

Only the lab manager or other authorized lab members may service an advanced piece of equipment such as the HPLC, FPLC, UV/UF water purifier, spectrophotometer, and lyophilizer. These pieces of equipment are very delicate and potentially dangerous and must be handled appropriately.

Stock Solutions for *E. coli* Growth and Protein Expression

Antibiotics (1000x stocks)

1) *Ampicillin/Carbenicillin (10 ml)*

   To 1 g of Ampicillin/Carbenicillin (stored at 4 °C), add UV/UF water up to 10 ml. Vortex, adjust volume to 10 ml, then filter solution using 0.22 μm filter. Dispense into 1 ml aliquots and store at -20 °C.

2) *Kanamycin (10 ml)*

   To 0.3 g of Kanamycin (stored at 4 °C), add UV/UF water up to 10 ml. Vortex, adjust volume to 10 ml, then filter solution using 0.22 μm filter. Dispense into 1 ml aliquots and store at -20 °C.

3) *Chloramphenicol (10 ml)*

   To 0.34 g of Chloramphenicol (stored at room temp), add ethanol up to 10 ml. Vortex, adjust volume to 10 ml, then filter solution using 0.22 μm filter. Dispense into 1 ml aliquots and store at -20 °C.

4) *Spectinomycin (10 ml)*

   To 0.5 g of Spectinomycin (stored at 4 °C), add UV/UF water up to 10 ml. Vortex, adjust volume to 10 ml, then filter solution using 0.22 μm filter. Dispense into 1 ml aliquots and store at -20 °C.

1 M IPTG (1000x stock reagent for inducing protein expression) (10 ml)

To 2.4 g of solid IPTG (stored at 4°C; Diagnostic Chemicals), add UV/UF water up to 10 ml. Vortex, adjust volume to 10 ml, then filter solution using 0.22 μm filter. Dispense into 1 ml aliquots and store at -20°C.

Protease Inhibitors:

1) *Leupeptin (10 mg/ml – 1000x stock)*

   Add 500 μl of H_2O to one 5 mg bottle. Vortex well. Use a gel loading tip to retrieve solution. Make 250μl aliquots

2) *Pepstatin A (1 mg/ml – 1000x stock)*

   Wash out one 5 mg bottle with 5 ml of ethanol. Make 500 μl aliquots

3) *PMSF (1.74 mg/ml – 100x stock; 10 mM)*

   Weigh out 0.0174 g PMSF per 10 ml of isopropanol. Make 1ml aliquots

USE EXTREME CAUTION WHEN WEIGHING PMSF!!!!
WEAR RESPIRATOR, COAT, GOGGLES, DOUBLE GLOVES

Media for Maintenance and Propagation of *E. coli*

LB Broth (1 liter) (for protein expression)

To 950 ml UV/UF water add 20 g of LB Broth Base powder. Adjust volume to 1000 ml. Autoclave at 121 °C for 15 minutes. Cool solution to around 45 °C and add appropriate antibiotic.

or

To 10 g Peptone powder, 5 g Yeast Extract powder and 5 g NaCl, add enough UV/UF water to bring volume to 1000 ml. Autoclave at 121 °C for 15 minutes. Cool solution to around 45 °C and add appropriate antibiotic.

SOC medium (1 liter) (for outgrowth after heat shock procedure during bacterial transformation)

To 20 g of Peptone powder, 5 g of Yeast Extract powder and 0.5 g NaCl, add 950 ml UV/UF water. Add 10 ml of 250 mM KCl solution. Adjust the pH to 7.0 with NaOH. Adjust the volume to 975 ml and sterilize by autoclaving at 121 °C for 15 minutes. Cool solution to room temperature. Add 5 ml of a sterile solution of 2 M $MgCl_2$ followed by 20 ml of a sterile solution of 18 % glucose. Store at -80 °C in 50 ml (or less) aliquots.

10X M9 solution (1 liter)

To 68 g anhydrous Na_2HPO_4, 30 g anhydrous KH_2PO_4 and 5 g NaCl, add 950 ml UV/UF water. Adjust volume to 1000 ml. Store solution at room temperature.

Minimal media (1 liter) (for expressing ^{15}N, ^{13}C-labeled proteins)

Solution A

To 100 ml of 10x M9 solution add 850 ml UV/UF water. Autoclave.

Solution B **Protect from light by covering solution with aluminum foil!**

10 ml 100X Vitamin solution (stored at -20 °C)
100 μl 1 M $CaCl_2$
500 μl 2 M $MgCl_2$
2 g glucose (source of ^{13}C-label if needed) (or 10 ml of 20% glucose)
1 g ammonium sulfate (source of ^{15}N-label if needed) (or 10 ml of 10% solution)

Add 1ml of the appropriate 1000x antibiotic stock solution, bring volume to 50 ml and filter through a 0.22 μm filter. Use sterile technique.

Mix 19 parts of Solution A with 1 part of Solution B after Solution A has cooled to room temperature. Use sterile technique. **Always prepare fresh minimal media immediately before use**.

E. coli growth media containing agar (plates)

Luria Bertani (LB) agar (1 liter)

To 32 g of LB Agar powder, add 950 ml UV/UF water. Adjust volume to 1000 ml. Autoclave at 121 °C for 15 minutes. Cool solution to around 45 °C and add appropriate antibiotic. Pour 25-30 ml in each plate. Use sterile technique. Store plates at 4 °C after the agar has solidified.

Minimal media plates (1 liter)

Add 12 g of granulated agar to Solution A (in *Minimal media* protocol) and autoclave at 121 °C for 15 minutes. Cool Solution A to 45 °C and add Solution B (in *Minimal media* protocol). Pour 25-30 ml in each plate. Use sterile technique. Store plates at 4 °C after the agar has solidified.

Terrific Broth (TB) plates (for plasmid preps and for protein expression) (1 liter)

To 47 g of Terrific Broth powder and 12 g of granulated agar, add 950 ml UV/UF water. Add 4 ml glycerol (Fisher). Adjust volume to 1000 ml. Autoclave at 121 °C for 15 minutes. Cool solution to around 45 °C and add appropriate antibiotic. Pour 25-30 ml in each plate. Use sterile technique. Store plates at 4 °C after the agar has solidified.

Manipulation of *E. coli* cells

Transformation of Competent Cells

1) Carefully remove the required number of aliquots of competent cells from the -80 °C freezer, and place immediately on ice. Use one aliquot for each plasmid.

   Note: Competent cells are very delicate, so do not drop the tubes or agitate using a vortexer at any time

2) Allow the cells to thaw on ice for at least 15 minutes

3) Prepare appropriate dilution(s) of plasmid DNA. The desired concentration for transformation is typically 10-20 ng/μl.

4) Add 1 μl of the solution containing the plasmid to the competent cells, and stir contents gently with pipette tip

5) Incubate on ice for 30 minutes

6) Place the tubes in the heat block at 42 °C for 30 seconds

7) Put the tubes back on ice for 2 minutes

8) Add 150 μl of SOC medium (at room temperature) and stir gently

9) For cells to develop antibiotic resistance (aka the outgrowth period), shake at 225 rpm at 37 °C (place the 1.5 ml tubes into a 50 ml conical tube for shaking). For **ampicillin, carbenicillin**, **kanamycin or spectinomycin**, shake for **1 hour**; for **chloramphenicol or tetracycline** shake for **2 hours**.

10) Plate the cells using a spreader onto the appropriate antibiotic-containing agar plates.

11) Incubate at 37 °C with the plates **inverted**.

Making Glycerol Stocks

Grow culture to 0.2 OD_{600}. Add 700 μl of the culture to 1.5 ml tube. Add 300 μl sterile-filtered 50% glycerol. Store in the -80 °C freezer.

Streaking Plates from a Glycerol Stock

Scratch the surface of a frozen stock with a loop and streak a plate.

PCR Cloning

1. **Keep all samples on ice**!

Into each reaction tube add:

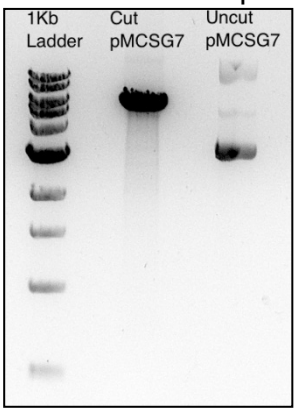
2. Template (use 20-50 ng of plasmid)
3. Forward Primer (use 1 μg)
4. Reverse Primer (use 1 μg)
5. Add thermopolymerase buffer (for 10x stock – add 5 μl per 50 μl reaction)
6. 0.5 μl polymerase (always use polymerase (e.g. *PfuTurbo*) with proofreading ability)
7. Including the 5 μl for dNTPs (to be added later) calculate and add the amount of sterile-filtered UV/UF water needed to bring total reaction volume to 50μl
8. Start up PCR machine
9. Choose an appropriate method (the most common parameter to optimize is the annealing temperature). **Anyone can change the methods stored in the PCR machine at any time.** Therefore, record all run parameters of the PCR reaction in your notebook.
10. When the temperature reaches 80 °C, pause the run **and then add dNTP mixture** (Use 5 μl of 2 mM dNTP stock)
11. When cycle is finished, the machine will maintain the tubes at 4 °C forever. However, remove the samples to the -20 °C freezer as soon as the run is complete and turn off the unit. Keep the samples at -20 °C until you are ready for the next step.

Cloning with pMCSG Vectors

1) Make sure the insert has the correct sequence complementary to the vector:
Forward primer should start with: 5'-TAC TTC CAA TCC AAT GCX -3'
Reverse primer should start with: 5'-TTA TCC ACT TCC AAT G tta tta-3'

2) Linearize vector with SspI (2.5 U/µl) by first diluting 25 U/µl Ssp1 stock to 2.5 U/µl with sterile water on ice. Then combine the following reagents:

pMCSG Vector (180 ng/µl)	20 µl
10x NEB 2.1 buffer	4 µl
Ssp1 (2.5 U/µl)	3.6 µl
UV/UF Water	12.4 µl



i. Incubate mixture at 37 °C for 2 hours
ii. Heat inactivate Ssp1 at 65 °C for 20 minutes
iii. Run on 1% agarose gel with uncut vector as comparison (Run loading dye to the end of gel ~1 hour)
Resulting gel should look like the one on right.

3) Gel purify linearized vector and PCR insert using Qiagen Gel Extraction Kit and measure DNA concentration after purification.

4) In separate tubes, treat linearized vector and insert with T4 DNA polymerase:

VECTOR	INSERT
14.5 µl purified pMCSG Vector	14.5 µl purified PCR product
2 µl 10X T4 DNA polymerase buffer	2 µl 10X T4 DNA polymerase buffer
2 µl 25 mM **dGTP**	2 µl 25 mM **dCTP**
1 µl 10 0mM DTT	1 µl 100 mM DTT
0.5 µl (2.5 U/µl) T4 DNA polymerase	0.5 µl (2.5 U/µl) T4 DNA polymerase

1. Incubate each tube for 30 minutes at room temperature
2. Heat inactivate T4 DNA polymerase by incubating for 20 minutes at 75 °C

5) Measure the concentrations (in ng/µl; 1 OD_{260} = 50 ng/µl double-stranded DNA) of the vector and insert using a NanoDrop; ideally, vector should be ~50 ng/µl concentration.

6) Annealing
i. Calculate the ratio, R, of the size of the vector (in base-pairs) and the size of the insert (also in base-pairs). If you have successfully measured the amount of the purified vector and insert (ng/µl), calculate the 1:1 molar ratio (in µl) of the two components. Example, if vector is 5 kb and insert is 0.5 kb, R=10. If [vector] is 50 ng/µl and [insert] is 15 ng/µl, then for a 1:1 molar ratio, you need ([vector]/([insert]xR)) = (50/(15x10)) or 0.33 µl of insert for every 1 µl of vector.
ii. Assemble different ratios of vector:insert (1:1, 1:3, 3:1). Use no more than 1 µl of 50 ng/µl vector (**linearized vector stock is precious!**) and the total volume of insert+vector should not exceed 2 µl.
iii. Incubate each mixture at room temp for 5 min and then add 1 µl of 25 mM EDTA.
iv. Mix by stirring with the pipet tip and incubate on ice for 30 minutes

7) Add no more than 2 µl of each annealing reaction to a separate aliquot of NovaBlue cells, transform and select for colonies on LB plate with the appropriate antibiotic.

Optimized Site-Directed Mutagenesis Protocol

1. Primer Design:

Principles:

- T_m must be greater than 78, optimal T_m ~80
- Optimal length is 25-45 base-pairs, can go up to 55 base-pairs
- Center mutant codon to the extent possible
- Primers should terminate in at least 2 GC base-pairs
- Primer should contain at least 40% GC base-pairs
  (For help with primer design, use the following URL - http://www.genomics.agilent.com/primerDesignProgram.jsp?_requestid=69269)

T_m Calculation:

$T_m = 81.5 + 0.41(\%GC) – (675/N) – (\%Mismatch)$

Where N = primer length in base-pairs
%GC = 100 x (number of GC base-pairs / N)
%Mismatch = 100 x (number of mutated base-pairs / N)

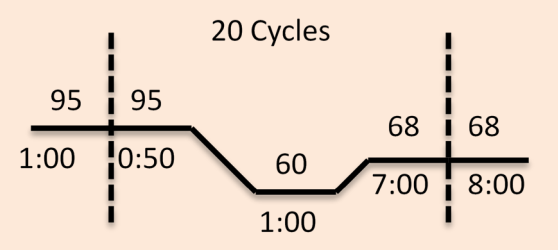
2. Mutagenesis PCR

Combine in a thin-walled PCR tube:
0.5 μg Sense Primer
0.5 μg Antisense
10 ng dsDNA Template
5 μl 10X thermopolymerase reaction buffer (Pfu Turbo buffer)
1 μl pfu turbo DNA polymerase (with proofreading ability)
N μl sterile-filtered UV/UF H_2O to bring total volume to 45 μl

Once thermocycler heats up to 95 °C, pause program and add 5 μl 2.5mM dNTPs

3. Thermocycler parameters:

Run protocol “quick-dbd” under user Tanya with annealing temp 60 °C



(If further optimization is required, increase annealing temperature)

4. Dpn I Treatment

Add 1 μL Dpn I enzyme (10 U/μl) to each 50 μl PCR reaction
Incubate in thermocycler at 37 °C for 1 hr

Add another 1 μl Dpn I enzyme (10 U/μl)
Heat in thermocycler at 37 °C for an additional hour

4. Isopropanol Precipitation of DNA

Purpose: Increase DNA concentration and number of transformed colonies

1. To the ~52 μl Dpn I-treated sample above, add 25 μl of 7.5 M NH_4OAc solution to adjust salt concentration
2. Transfer to larger 1.5 ml tube, preferably clear, not frosted for maximum visibility
3. Add 150 μl isopropanol
4. Incubate at room temp for 10 minutes
5. Centrifuge 10 minutes, 14,000 rpm in tabletop centrifuge
6. Carefully pipette off supernatant, discard
7. Add 1 ml of 80% ethanol to pellet to wash, vortex gently to dislodge pellet
8. Centrifuge 10 min, 14,000 RPM
9. Very carefully pipette off 80-90% of supernatant, <u>but do not disturb the pellet</u>. Pellet should be extremely small but visible
10. Gently air-dry remaining supernatant using Argon gas, without blasting away pellet. Alternative is to let the pellet air-dry overnight
11. Resuspend dry pellet in 10 μl sterile-filtered UV/UF H_2O, transform NovaBlue cells with 1.5 μl solution

Colony PCR

1) Pick healthy looking individual colonies from the agar plate with sterilized pipette tips and transfer the bacteria into a 1.5 ml tube containing 20 μl of sterile-filtered UV/UF water. If colonies are not at least 1 mm in diameter, wait longer before doing PCR, otherwise, you might get false positives.
2) Mix gently (vortex slowly or use a pipette tip)
3) Spot 1 μl of solution onto a fresh plate in a grid layout and incubate the plate(s) at 37 °C overnight.
4) Boil the 1.5 ml tubes for 10 minutes.
5) Centrifuge for 1 minute at 12,000 rpm.
6) Turn on the thermocycler and allow it to warm up for at least 5 minutes. To each reaction tube add:

   2 μl T7 promoter primer (in the -80 °C freezer; 1 μg of primer)
   2 μl T7 terminator primer (in the -80 °C freezer; 1 μg of primer)
   5 μl 10x Taq buffer
   0.5 μl Taq polymerase (in the -20 °C freezer)
   25.5 μl of sterile UV/UF water
   10 μl of supernatant from the boiled samples above

   Notes: Polymerase with proofreading function not required (Taq is fine for these experiments). T7 primers are commonly used but since pMCSG23 vectors have two T7 promoters, use insert-specific forward primer or the MBP-specific primer along with T7 terminator primer
7) Run Kurt's “exp 001” protocol on the thermocycler
8) Pause program and wait 5 minutes when temperature reaches 80 °C.
9) Add 5 μl of 2 mM dNTP mix to each reaction (stir gently when adding).
10) Resume program.
11) When the program is complete, run 20 μl samples from each reaction on a 2% agarose gel along with a DNA ladder.
12) Amplify plasmid from those colonies with the correct size insert.

Vector	T7 Promoter	T7 Terminator	Size
pMCSG7	383	64	320 bp +insert
pMCSG10	1058	64	995 bp +insert
pMCSG21	208	542	335 bp +insert
pMCSG23	1488^	1661	174 bp +insert
pMCSG23	1291*	1661	371 bp +insert

^insert-specific forward primer, not T7 promoter primer
*for MBP-specific primer, not T7 promoter primer
#for pMCSG9 and pMCSG24 vectors, expected sizes approximately track those for the pMCSG23 and pMCSG10 vectors, respectively, above

General Stock Solutions

0.5 M EDTA, pH 8.0 (50 ml)

Add 9.3 g of disodium EDTA dihydrate to 45 ml of UV/UF water. Stir vigorously on a magnetic stirrer. Adjust pH to 8.0 with 5 N NaOH. Adjust volume to 50 ml. Check pH. Sterile filter using a 0.22 μm filter. Store at 4 °C.

3 M Sodium acetate, pH 5.2 (10 ml)

To 4.08 g sodium acetate trihydrate, add 9 ml UV/UF water. Adjust pH to 5.2 with 5 N HCl. Adjust volume to 10 ml. Store at 4 °C.

10 mM Tris-HCl, pH 8.5 (200 ml)

To 0.24 g of Trizma base (Sigma), add 190 ml UV/UF water. Adjust pH to 8.5 using HCl. Adjust volume to 200 ml. Store at 4 °C.

1 M $CaCl_2$ solution (10 ml)

To 2.2 g of solid $CaCl_2$ hexahydrate, add UV/UF water up to 10 ml. Vortex, adjust volume to 10 ml, then filter solution using 0.22 μm filter. Store at 4 °C.

2 M $MgCl_2$ solution (10 ml)

To 4.1 g of solid $MgCl_2$ hexahydrate, add UV/UF water up to 10 ml. Vortex, adjust volume to 10 ml. Filter solution using 0.22 μm filter. Store at 4 °C.

X-Gal solution (5 ml)

Work in the fume hood!!! DMF is toxic
X-Gal is hazardous and light sensitive – cover in foil

To 100 mg of X-Gal (stored at -20°C), add 5 ml dimethylformamide (DMF). Cover tube in aluminum foil and store at -20 °C.

250 mM KCl (50 ml)

To 0.93 g of KCl, add 45 ml UV/UF water. Adjust volume to 50 ml. Store at 4°C.

Solutions for DNA Agarose Gel Electrophoresis

50X TAE (200 ml)

Dissolve 48.4 g of Trizma base (Sigma) into approximately 100 ml water, add 11.4 ml glacial acetic acid and 20 ml 0.5 M EDTA (pH 8.0). Adjust volume to 200 ml. Filter solution through a 0.22 μm filter. Store at room temperature.

Dilute to 1X TAE to pour and run gels.

Ethidium bromide solution (10 ml)

Ethidum bromide is toxic and a mutagen – wear gloves!!!
EtBr is light-sensitive – use foil

To 100 mg of ethidium bromide, add 10 ml UV/UF water. Vortex solution to ensure that the dye has dissolved. Wrap the container in aluminum foil and store at 4 °C.

6X Agarose gel-loading buffer (10 ml)

Dyes are hazardous and light sensitive – use foil

To 25 mg bromophenol and 25 mg xylene cyanol FF, add 7 ml UV/UF water. Add 3 ml glycerol. Vortex solution to dissolve dye. Cover tube with aluminum foil and store at 4 °C.

Enzyme Stocks

Lysozyme

50 mg/ml in 20 mM Tris, pH 8.
Dissolve 250 mg of dry lyophilized powder in 5 ml of Tris, pH 8 buffer. Dispense in 500 µl aliquots and store at -20 °C.

DNase I

5 mg/ml in 20 mM Tris, pH 8, 0.15 M NaCl.
Dissolve 25 mg of dry lyophilized powder in 5 ml of Tris, pH 8, 0.15 M NaCl buffer containing 10% glycerol. Dispense in 500 µl aliquots and store at -20 °C.

Cleaning Procedures

Cleaning frits

1. Make 25 ml of a 40% (*v/v*) solution of formic acid (**work in the fume hood!**).
2. Put dirty frits in a 150 ml Pyrex beaker and completely cover frits with the formic acid solution. Sonicate for 30 minutes using the Labline sonicator.
3. Replace formic acid solution with 100 ml of UV/UF water and sonicate for 30 minutes.

Cleaning NMR tubes

1. After removing NMR sample, soak the NMR tube overnight in the RBS35 NMR cleaning solution. If it is a new NMR tube, soak the tube overnight in 0.1 M EDTA solution.
2. Set-up the 5 mm NMR tube cleaning assembly. Make sure there's a trap between the vacuum line and the cleaning assembly.
3. Using a Kimwipe soaked in acetone, gently wipe the outer surface of the tube to remove any labels.
4. Invert and insert the NMR tube into the receptacle. Secure the tube with a stopper.
5. Turn on the vacuum and rinse the NMR tube extensively with water (5 squirts from a wash bottle; pause for a few seconds between squirts).
6. Rinse the tube again with RBS35 solution (3 squirts).
7. Rinse tube extensively with water (5 squirts).
8. Rinse tube with methanol (5 squirts).
9. Wait for 10 minutes for tube to dry completely before removing from assembly. Leave tube to air-dry for about an hour before capping and storing.

Cleaning NMR tube caps

1. Collect NMR tube caps in a 50 ml conical tube and soak overnight in RBS35 NMR cleaning solution.
2. Rinse with water carefully and extensively.
3. Place the caps on a paper towel and blot dry as much as possible. Let them air-dry overnight.

Solutions for Tris.HCl SDS-PAGE

1.5 M Tris.HCl, pH 8.8

To 18 g of Trizma base, add 95 ml UV/UF water. Adjust pH to 8.8 using 2 N HCl. Adjust volume to 100 ml. Store at room temperature.

0.5 M Tris.HCl, pH 6.8

To 6 g of Trizma base, add 95 ml UV/UF water. Adjust pH to 6.8 using 5 N HCl. Adjust volume to 100 ml. Store at room temperature.

10% SDS solution

Heat 180 ml of UV/UF water to 68°C to assist dissolution. Add 20 g of electrophoresis-grade SDS (Bio-Rad; weigh carefully without spilling) and stir. When the SDS is dissolved, adjust the pH to 7.2 with 5 N HCl. Adjust the volume to 200 ml. Store at room temperature.

Resolving gel (Bottom part – poured first) **Toxic chemicals - wear gloves!**

	12%	**15%**	**18%**
UV/UF Water	4.15 ml	3.35 ml	2.55 ml
1.5 M Tris.HCl (pH 8.8)	2.5 ml	2.5 ml	2.5 ml
10% SDS	0.1 ml	0.1 ml	0.1 ml
Acrylamide:bis-Acrylamide	3.2 ml	4.0 ml	4.8 ml
10% APS (**freshly pp'd**)	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl

Stacking gel (Top part – poured last) **Toxic chemicals - wear gloves!**

UV/UF Water	6.1 ml
0.5 M Tris.HCl (pH 6.8)	2.5 ml
10% SDS	0.1 ml
Acrylamide:bis-Acrylamide (38.5:1)	1.3 ml
10% APS (**freshly prepared**)	50 µl
TEMED	10 µl

5X SDS gel-loading buffer

To 5 ml 0.5 M Tris.HCl, pH 6.8, add 5 ml glycerol, 1 g SDS, 0.775 g DTT and 0.25 g bromophenol blue (BioRad). Mix thoroughly, heat to 60 °C, vortex and dispense into 500 µl aliquots and store at -20 °C.

Tris.HCl electrode buffer

Add 3 g of Trizma base and 14 g of glycine to 950 ml of UV/UF water. Mix thoroughly till it dissolves and then add 10 ml 10% SDS solution and adjust volume to 1000 ml. Always prepare solution fresh before using.

Solutions for Making Tris-Tricine Gels (original low-throughput recipe; but see next page)

The following recipe makes 2 gels:

	13% Resolving	**15% Resolving**	**4% Stacking**
H_2O	2.64 ml	2.08 ml	6.1 ml
40% Acrylamide:bis-Acrylamide^	3.66 ml	4.22 ml	0.9 ml
3 M Tris/0.3% SDS*	3.75 ml	3.75 ml	2.34 ml
Glycerol	1.2 ml	1.2 ml	----
TEMED	9 μL	9 μL	18 μL
10% APS (**freshly pp'd**)#	50 μL	50 μL	50 μL

^Acrylamide and bis-acrylamide mixture in 37.5:1 molar ratio

Solutions for 3-layer gel (resolution of small proteins)

	16.5% Resolving	**10% Spacer**	**4% Stacking**
H_2O	1.96 ml	5.06 ml	6.1 ml
40% Acrylamide:bis-Acrylamide^	4.9 ml	3.0 ml	0.9 ml
3 M Tris/0.3% SDS*	3.75 ml	3.75 ml	2.34 ml
Glycerol	1.2 ml	----	----
TEMED	9 μL	9 μL	18 μL
10% APS (**freshly pp'd**)#	50 μL	50 μL	50 μL

^Acrylamide and bis-acrylamide mixture in 37.5:1 molar ratio

When pouring a 3-layer gel, pour spacer immediately on top of resolving gel slowly and carefully to avoid mixing. Top off spacer with methanol as normal and wait for polymerization to occur.

**3 M Tris/0.3 % SDS (500 ml)*

To 182 g Trizma base, bring volume up to 500 ml, heat and stir. Adjust pH to 8.45. Add 1.5 g SDS, stir until dissolved.

#10% APS (200 μl)

To 20 mg ammonium persulfate add 200 μl H_2O. **Use immediately! Aqueous ammonium persulfate is unstable – do not make stock solutions!**

Solutions for Making Tris-Tricine Gels (High-Throughput Recipe)

The following recipe makes 9 gels:

	12% Resolving	**15% Resolving**	**4% Stacking**
H_2O	16 ml	11.25 ml	16.5 ml
40% Acrylamide:bis-Acrylamide^	18 ml	22.5 ml	3 ml
3 M Tris/0.3% SDS[$]	20 ml	20 ml	10 ml
Glycerol	6 ml	6 ml	--
TEMED	45 μL	45 μL	45 μL
10% APS (**freshly pp'd**)[#]	250 μL	250 μL	125 μL

^Acrylamide and bis-acrylamide mixture in 37.5:1 molar ratio

[$]*3 M Tris/ 0.3 % SDS (500 ml):*

To 182 g Trizma base, bring volume up to 500 ml, heat and stir. Adjust pH to 8.45. Add 1.5 g SDS, stir until dissolved.

[#]*10% APS (300 μl)*

To 30 mg ammonium persulfate add 300 μl H_2O. **Use immediately!** Aqueous ammonium persulfate is **unstable** – do not make stock solutions!

Tips for making gels in high-throughput mode:

- When preparing the gel cassette, make sure that there is a plastic cover between the short plate and spacer plate (makes it easier to separate prepared gels)
- When making gels, after pouring the resolving gel, add a layer of methanol or ethanol and wait for polymerization to occur. This will help ensure the top of the gel is smooth
- After pouring the stacking gel, following polymerization of the resolving gel, be sure to insert teeth right away to allow for lanes to form
- Once gels are prepared, separate carefully, wrap each in a paper towel, wet just until all the gels are covered in water, and place in the 4 °C fridge for use later

Solutions for Tris-Tricine SDS-PAGE

10X Cathode buffer- inner chamber (1000 ml recipe)

121.04 g Trizma base
179.2 g Tricine10 g SDS
Add water to 900 ml, heat and stir to 60 ^{o}C. QS with water to 1000 ml
Dilute to 1X when ready to use (100ml 5x buffer to 900mL H_2O)

10X Anode buffer- outer chamber (1000 ml)

121 g Trizma base
Bring volume up to 950 ml with water, stir. QS with water to 1000 ml
Dilute to 1X when ready to use (100 ml 10X buffer + 900 ml H_2O)

5X Tris-Tricine sample loading buffer:

5 ml 0.5 M Tris.HCl, pH 6.8
6 ml glycerol
2 g SDS
0.775 g DTT
5 mg Coomassie G-250
Mix, bring volume to 10 ml and heat to 60 ^{o}C, vortex. Aliquot into 500 μl amounts, store at -20 ^{o}C

Molecular weight markers

Resuspend 1 vial of Molecular Weight Markers (SeeBlue protein marker) in 100 μl UV/UF water. Centrifuge vial within a 50 ml conical tube to pool all of the marker at the bottom of the vial. Dispense into 25 μl aliquots and store at -20°C. Use 5-10 μl for a mini-gel.

Coomassie Staining solution (1 liter)

Work in the fume hood!
Prepare 1000 ml of **destaining solution below**. Add 1.25 g of Coomassie Brilliant Blue R-250 (**always wear gloves**). Vigorously stir solution using a magnetic stirrer for 30 minutes. Filter solution through a Whatman No. 1 filter paper to remove any particulate matter. Store solution at room temperature in the flammable cabinet under the hood.

Coomassie Destaining solution (1 liter)

Work in the fume hood!
Using a 1 l graduated cylinder, add 500 ml UV/UF water, 400 ml methanol, and 100 ml glacial acetic acid. Mix thoroughly by stirring. Pour solution into suitable destain bottles. Store at room temperature in the flammable cabinet under the hood.

Protein Gel Assembly and Sample Loading

Mini-PROTEAN 3 Electrophoresis Module Assembly and Sample loading:

1. Mini-PROTEAN 3 Electrophoresis Module Assembly

1.1. Remove casting frames from the casting stand and remove the gel cassettes from the casting frames.

1.2. Place gel cassettes into the slots at the bottom of each slide of the electrode assembly. Face short plate of the gel cassette inward toward the notches of the U-shaped gaskets.

1.3. Lift gel cassettes into place against the green gaskets and slide into the clamping frame.

1.4. Press down on the electrode assembly while closing the cam levers of the clamping frame.

1.5. Lower the inner chamber assembly into the mini tank. Fill with ~150 ml of Cathode Buffer (until the level is half way between the taller and shorter plate of the gel cassettes)

1.6. Add 650 ml of running buffer to the mini tank (lower buffer chamber).

2. Sample Loading

2.1. Carefully remove any loose stacking gel debris in the wells. Since the debris might be hard to see when the wells are filled with cathode buffer, check for any obstruction by rapidly dispensing 5-10 µl of cathode buffer into the wells.

2.2. Load samples using gel loading pipette tips

2.3. Do not forget to run molecular weight standards!

NOTE: Load samples slowly to allow them to settle evenly on the bottom of well and don't puncture the bottom of the well with the pipette tip.

3. Gel Electrophoresis

3.1. Place lid on mini tank. Align color coded banana plugs and jacks. Lid prevents incorrect orientation.

3.2. Insert electrical leads into power supply with the *correct polarity*.

3.3. Apply power and begin electrophoresis; start at 40 volts until samples are in gel, then 100 volts constant. Run time determined by phenol red migration.

4. Gel Removal

4.1. Turn off power and disconnect leads.

4.2. Carefully remove lid, lift out the inner chamber assembly, and pour off running buffer

4.3. Open cams of clamping frame and remove electrode assembly and gel cassettes.

4.4. Remove gel by gently separating the two plates of the gel cassette.

4.5. Lift the gel off the plate and place it directly into Coomassie or Western transfer buffer

4.6. Rinse the Mini-Protean 3 cell apparatus with DI water after use.

Expressing Labeled Proteins in Minimal Media

Here are two protocols differing slightly in how you get started:

Starting from a new transformation:

1. Transform bacteria in LB plate.
2. Pick a colony (~1 mm in diameter) from the plate and streak a MM plate.
3. Pick a colony (at least ~0.5 mm in diameter) from MM plate and inoculate liquid MM culture.

Starting from a glycerol stock:

1. Streak a MM plate with a glycerol stock of already transformed bacteria.
2. Pick a colony (at least ~0.5 mm in diameter) from MM plate and inoculate liquid MM culture.

Practical tips:

It takes about 36-48 hours for discrete colonies to appear on MM plates when grown at 37 °C. Inoculate 100 ml MM cultures around 8 or 9 PM and let them grow for 12 hours. The OD_{600} should be ~0.8 ~8 or 9 AM. Inoculate 1 liter cultures. The doubling time should be ~1 hour. The key to getting high levels of expression in MM is to (a) keep bugs growing constantly (i.e. do not transfer plates/liquid cultures to the refrigerator at any stage) and (b) ensure that the OD_{600} is ideally below 0.8 and definitely below 1.0. The latter is crucial because after the exponential growth phase, bacteria enter a state of stasis and are "too old" to make much protein. Growth kinetics (i.e. doubling times) are an excellent indicator of the health and happiness level of bacteria (like your pulse rate and other vital signs), so be sure to monitor this closely.

Sample Preparation for Affinity Chromatography

Keep samples on ice at all times!

1. Remove the centrifuge bottle(s) or Whirl-Paks containing the cell pellet from the -80 °C freezer and allow the pellet to thaw on ice.
2. Add approximately 30 ml of Lysis or Equilibration buffer (the composition of buffers is dependent on the type of column you will be using) to the cell pellet. Note: Use a minimum of 30 ml of buffer for cells derived from every liter of culture; if you have cells from multiple liters of culture, scale the volume of lysis buffer proportionately.
3. Add protease inhibitors to the solution. Pepstatin A and leupeptin are at 1000X concentration while PMSF is at 100X concentration in the respective stock solutions (30 or 300 µl of each). Be sure to vortex each stock solution once thawed.
4. Add Triton X-100 to the solution (300 µl).
5. Re-suspend the cells by gently drawing the buffer and the cells up and down using a serological pipette.
6. Carefully transfer suspension to a clean Oakridge (30 ml) centrifuge tube.
7. Add DNase I (180 µl) to the suspension and add 150 µl of 1 M $MgCl_2$ to activate enzyme.
8. Optionally, add lysozyme (150 µl) to the suspension.
9. Place the centrifuge tube in an ice bucket. Sonicate the cells in the cold room (see sonication protocol elsewhere). Lysis should be complete between 1 and 3 cycles of sonication (each cycle takes 30 minutes) depending on the size of the original cell pellet (which in turns depends on the final OD_{600} of the culture).
10. Centrifuge the lysate for 30 minutes at 15,000 x g (about 12,000 rpm in the F-21S rotor).
11. Decant the supernatant carefully into a 50 ml conical tube.
12. If the protein you are purifying is in the soluble fraction, incubate supernatant with appropriate resin if you plan to purify in batch mode on your bench, or load the supernatant into the superloop of the FPLC. If the protein is not in the soluble fraction, but is in the inclusion bodies, continue with this protocol.
13. Re-suspend the inclusion body pellet in 30 ml of equilibration buffer containing 6 M guanidinium hydrochloride or 8 M urea. Initially vortex the pellet and use a glass stir rod, if necessary, to break it up. Sonicate between 1 and 3 cycles, depending on the size of the pellet.
14. Centrifuge the solution for 30 minutes at 15,000 x g (about 12,000 rpm in the F-21S rotor).
15. Decant the supernatant into a 50 ml conical tube and incubate with appropriate resin if you plan to purify in batch mode on your bench, or load the supernatant into the superloop of the FPLC.

Western Blotting Protocol

DAY 1

1. Put ice pack in the freezer!
2. Run gel with molecular weight markers (or western blot markers) and the samples. Do not stain with Commassie.
3. Transfer the proteins from gel to nitrocellulose as follows.
    a. Prepare 1 liter of transfer buffer (Transfer buffer can be reused up to 3 times):
        3.03 g Tris
        11.26 g Glycine
        200 ml methanol
        Adjust to 1 liter with UV/UF H_2O
    b. Using transfer buffer equilibrate the gel.
    c. Make gel sandwich by bottom to top: Grey side of cassette, fiber pad, filter paper, gel, membrane, filter paper, fiber pad. Do this submersed in transfer buffer (you can use the container that holds syringe filters). Make sure to carefully get all of the bubbles out.
    d. Add ice pack from freezer to help keep it cold and fill module with transfer buffer in cold room on top of stir plate. Start stirring solution.
    e. Insert gel sandwich, grey side towards black (negative electrode) Clear side to red. The gel should be closest to the black electrode.
    f. Run using optimized transfer times. (For example, proteins with molecular weight ~55 KDa, transfer 1.5 hours at 250A; use shorter transfer times for smaller proteins and longer times for larger proteins).
4. Wash the blotted nitrocellulose 2 times for 5 min each with UV/UF H_2O
5. Stain with Ponceau S stain (will stain in ~30 seconds)
6. Rinse 3 times with UV/UF water.
7. Scan blot using the Canon Scanner in the lab.
8. Wash 2 times for 10 min each with PBS to remove Ponceau S stain.
9. Make PBS:
    8 g NaCl
    0.2 g KCl
    1.44 g Na_2HPO_4
    0.24 g KH_2PO_4
    adjust to 800 ml with UV/UF H_2O
    pH to 7.4 with HCl
    adjust to 1 liter with UV/UF H_2O
10. Make 25 ml 5% milk/0.1% NP-40 in PBS
    1.25 g non-fat dry milk
    25 μl Nonidet-P 40
    25 ml PBS

11. Block the blotted nitrocellulose in 12 ml fresh 5% milk/0.1% NP-40 in PBS for 1 hour at room temperature with constant agitation (150 rpm)
12. In remaining 12 ml of 5% milk/0.1% NP-40 in PBS add an appropriate dilution of primary antibody. Check antibody specifications for proper dilution, most primary antibodies are optimized for 1:500 – 1:1000 dilution (antibody solution can be reused for a month by storing at 4 °C)
13. Incubate the nitrocellulose with dilution of primary antibody in fresh 5% milk/0.1% NP-40 in PBS overnight with agitation at 4 °C (150 RPM)

DAY 2

14. Make 90 ml 5% milk/0.1% NP-40 in PBS

    4.5 g non-fat dry milk
    90 μl NP-40
    90 ml PBS

15. Wash the nitrocellulose 2 times for 5 min each with 5% milk/0.1% NP-40 in PBS (each wash should use ~10 ml)
16. Make dilution of secondary antibody. Check antibody specifications for the proper dilution, most secondary antibodies are used at a 1:1000 dilution
17. Incubate with secondary antibody in 5% milk/0.1% NP-40 in PBS at room temperature with agitation at 150 rpm for 1 hour
18. Wash the nitrocellulose 3 times for 5 minutes with 5% milk/0.1% NP-40 in PBS (each wash should use ~10 ml)
19. Wash the nitrocellulose 2 times for 5 minutes each with PBS (each wash should use ~10 ml)

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20. Prepare chemiluminescent substrate by combining 1 part Peroxide solution to 1 part Enhancer solution (2 ml of each)
21. Incubate blot with solution for 5 minutes
22. Remove blot and read using Pxi chemiluminescence imaging system in the Keck Facility

Detection Kit: (Pierce 34080) SuperSignal West Pico Chemiluminescent Substrate

Measuring Protein Concentration

Gill & von Hippel Assay

1. Analyze your protein sequence to determine the number of tryptophans (one letter amino acid code, W), tyrosines (one letter code, Y) and cystines (oxidized cysteines) in the protein. Since we do not work with extracellular disulfide bridged proteins, we only need to consider tryptophans and tyrosines (extinction coefficients ε_{280nm} are 5.69 and 1.28 mM^{-1} cm^{-1}, respectively). Calculate ε for protein = **a** x ε_W + **b** x ε_Y = (**a** x 5.69) + (**b** x 1.28), where **a** and **b** are the number of tryptophans and tyrosines, respectively, in the protein.
2. Record the baseline for a UV spectrum (300-230 nm) using the same buffer that the protein is in. To 5 µl of this buffer, add 295 µl of 6 M guanidine hydrochloride in 20 mM sodium phosphate buffer, pH 6.5
3. To 5 µl of protein, add 295 µl of 6 M guanidine hydrochloride in 20 mM sodium phosphate buffer, pH 6.5. Calculate absorbance at 280 nm (subtract any baseline offset at 300 nm). Repeat measurements diluting the protein solution to 400 and 500 µl. Use a NanoDrop for proteins with high $\varepsilon_{protein}$ (>10 mM^{-1} cm^{-1}).

BCA Assay

1. Standard Curve Preparation ***(must make a new standard curve every time!)***
    1) Label eight 3 ml tubes 1-7 and Blank
    2) Follow the table below to make the appropriate BSA standard concentrations for the BCA assay. Making each concentration directly instead of making serial dilutions leads to more accurate standard concentrations.

Tube	Volume of PBS	Volume of 10 mg/ml BSA Stock	BSA Concentration
Blank	500 µl	0 ml	0 µg/ml
1	495 µl	5 µl	100 µg/ml
2	487.5 µl	12.5 µl	250 µg/ml
3	475 µl	25 µl	500 µg/ml
4	462.5 µl	37.5 µl	750 µg/ml
5	450 µl	50 µl	1000 µg/ml
6	425 µl	75 µl	1500 µg/ml
7	400 µl	100 µl	2000 µg/ml

2. Performing the BCA Assay
    1) Make Working Reagent by combining 50 parts of Reagent A with 1 part Reagent B (25 ml A + 500 µl B)
    2) Aliquot 100 µl protein buffer into “Blank” test tube
    3) Dilute unknown protein as appropriate
    4) Add 2 ml of Working Reagent to each test tube and incubate 30 min at 37 °C
    5) Cool all tubes to room temperature
    6) Measure the absorbance at 562 nm vs. water as the reference
    7) Subtract the absorbance of the blank from the value found

PEG Precipitation of DNA

This protocol works for PCR products >160 bp.

1) Make a stock solution of 40% (w/v) PEG8000, 30 mM $MgCl_2$. Store stock solution at 4 °C.

2) Add 0.5 volume of this stock solution to 1 volume of PCR reaction (typical volumes are 25 ul of stock solution for 50 ul of PCR reaction) and mix well. Spin resulting solution at 12000 rpm in the table top centrifuge for 30 minutes.

3) Decant and blot with Kimwipe to remove the supernatant. Take care not to disturb the pellet (tiny and sometimes may not be readily visible).

4) Add 1 ml of 70% ethanol and gently rock the tube a few times. Spin resulting solution at 12000 rpm for 10 minutes, then decant and blot with Kimwipe. Let the tube air-dry overnight. Re-suspend DNA in suitable buffer or sterile-filtered water.

Reference

Paithankar & Prasad (1991) *Nucleic Acids Research* **19**, 1346.

Large-scale Plasmid DNA Preparation from *E.coli* (for transfections)

1. Spin 1 liter of transformed *E. coli* cells containing the plasmid of interest at 6000 rpm for 20 minutes at 4 ºC.
2. Re-suspend the cell pellet in 15 ml of 25 mM Tris/HCl pH 8.0, 50 mM glucose, 10 mM EDTA.
3. Split into two 50 ml centrifuge tubes. Add 2.5 mg lysozyme per tube. Incubate at room temperature for 5 minutes.
4. Add 15 ml 0.2 M NaOH 1% SDS. Mix by inverting the tube. Incubate at room temperature for 5 minutes.
5. Add 11.25 ml of 3 M potassium acetate pH 5.5 into each tube. Mix by inverting the tube. Incubate at room temperature for 5 minutes.
6. Spin at 4000 rpm for 15 minutes at 4 ºC.
7. Filter supernatant through miracloth into a clean 50 ml centrifuge tube.
8. Add 0.6 volumes ispropanol. Incubate at room temperature for 5 minutes.
9. Spin at 4000 rpm for 15 minutes at 4 ºC.
10. Re-suspend each pellet in 15 ml of 10 mM Tris/HCl (pH 8.0), 10 mM EDTA.
11. Add 1 volume of 5M LiCl (pre-chilled at -20ºC). This precipitates RNA and proteins. Incubate on ice for 5 minutes.
12. Spin 4000 rpm at 4 ºC for 15 minutes. Transfer supernatant to new 50 ml tube.
13. Add 0.6 volumes of isopropanol to precipitate the supernatant. Incubate at room temperature for 5 minutes, spin at 4000 rpm for 15 minutes at 4 ºC.
14. Re-suspend and combine the 2 pellets in 10 ml Tris/HCl, 1 mM EDTA. Vortex vigorously.
15. Add 100 µl of 5 mg/ml heat-treated RNase A. Incubate at room temperature for 15 minutes.
16. Add 4 ml of 30% PEG 6000, 2.5 M NaCl. Incubate on ice for 30 minutes.
17. Spin at 4000 rpm for 30 minutes at 4 ºC.
18. Re-suspend the pellet in 10 ml of 10 mM Tris/HCl pH 8.0, 1.0 mM EDTA.

Chloroform-extract PEG:

19. Add 2 ml of chloroform, vortex briefly and spin at 4000rpm for 1 minute at 4ºC. Retain the top aqueous layer with a pipette. ***Be careful to only remove the aqueous layer***

20. Ethanol DNA precipitation:

    a. Add 0.1 volumes of 5 M NaCl and 3 volumes of absolute ethanol. Mix by inverting the tube a few times.

    b. Spin at 4000 rpm for 15 minutes at 4 ºC.

    c. Wash with 70% EtOH and spin at 4000 rpm for 15 minutes at 4 ºC.

    d. Leave the tube upside down to dry overnight.

21. Re-suspend into an appropriate volume of filter sterilized UV/UF H_2O, commercial DNA elution buffer or 10 mM Tris/HCl pH 8.0, 0.1 mM EDTA (**note that EDTA can inhibit enzymes that require metal (e.g. Mg^{2+}) for catalysis**).

Ligation-dependent Cloning Protocol

Amplify vector that will be used for cloning (pcDNA3.1, pRM2, etc.)

PCR amplify and gel purify insert

Perform restriction enzyme digestion in separate tubes for each vector and insert:

(Actual restriction enzymes, buffers, and heat inactivation temperature will vary and is determined by the primer design)

1. Prepare reaction by adding 1 µg DNA and 5 µl of appropriate buffer (NEB2.1, cutsmart, etc.) to microcentrifuge tube
2. Add sterile filtered water to a total volume of 48 µl
3. Add 1 µl of restriction enzyme #1
4. Incubate 1 hr at 37 °C
5. Heat inactivate 30 min at heat inactivation temperature of enzyme #1
6. Incubate on ice 5 min
7. Add 1 µl restriction enzyme #2
8. Incubate 1 hr at 37 °C
9. Heat inactivate 30 min at heat inactivation temperature of enzyme #2
10. Run on agarose gel sufficiently long to separate cut and uncut plasmid
11. Gel extract
12. Measure concentration
13. Prepare 3 ligation reactions using the following setup:

	Vector Control	**Rxn 1**	**Rxn 2**
Vector	50ng	50ng	50ng
Vector:Insert molar ratio	-	1:1	1:4
T4 ligase buffer	2µl	2µl	2µl
Total Volume	20µl	20µl	20µl

Use the URL (http://nebiocalculator.neb.com/#!/) to calculate molar ratios

14. Incubate at 16 °C for 4 hrs or overnight
15. Heat inactive the T4 ligase 30 min at 75 °C
16. Transform into DH5α

Competent Cell Preparation

TB buffer

- 10 mM HEPES
- 15 mM $CaCl_2$
- 55 mM $MnCl_2$
- 250 mM KCl

Mix all the components above except $MnCl_2$ and adjust the pH to 6.7 with KOH. Then add the $MnCl_2$ and filter sterilize the mixture using a 0.44-µm filter.

Procedure

1. Inoculate 3 ml LB medium with the DH5Alpha/Bl21DE3 and incubate the culture overnight at 37°C.
2. Add the overnight culture to 500 ml LB medium and incubate the culture at30°C until the absorbance at 600 nm was approx. 0.5 (between 0.4 and 0.6).
3. Chill the culture for at least 10 min on ice.
   In the following steps, the cell suspension should always be kept on ice.
4. Centrifuge the cell suspension for 10 min at 6000g.
5. Gently resuspend the pellet in 100 ml ice-cold TB buffer.
6. Incubate the cell suspension on ice for 10 min.
7. Centrifuge for 5 min at 6000g at 4 °C.
8. Gently resuspend the pellet in 18.6 ml ice-cold TB buffer and add 1.4 ml DMSO.
9. Incubate the cell suspension on wet ice for at least 10 min.
10. Aliquot the cell suspension at 100 µl per tube.
11. Shock-freeze the cell suspension in liquid nitrogen and store the tubes at -80 °C.

Reference

Inoue *et al*. (1990) *Gene 96*, 23-28.