

## **Rutgers Biomod Laboratory Manual**

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Contact:

### **Rutgers Biomod**

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**1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).** SDS-PAGE is the technique used to separate proteins based upon molecular weight. Polyacrylamide is a polymer that is used to separate proteins and small DNA and RNA fragments (<100bp). While polyacrylamide is nontoxic, acrylamide is very toxic, and the process by which acrylamide forms polyacrylamide is dangerous to perform. Therefore, we use pre-cast gels. SDS is a chemical that denatures proteins and makes them negatively charged. Therefore, the proteins will separate based upon molecular weight.

Ref: Mini-PROTEAN Tetra Cell Instruction Manual, BIORAD.

<http://www.bio-rad.com/en-us/product/mini-protean-tetra-vertical-electrophoresis-cell>

1. Mix 6uL 4X protein loading dye with 10uL protein sample in PCR tube. Inc (incubate) mixture at 99C for 5min.
2. Take pre-cast gel out of packaging. Do not remove plastic case. Remove green tape at bottom of gel case and green comb at top of gel case.
3. Refer to BIORAD PAGE chamber instructions on how to place gels into gel frame, and how to assemble gel chamber (p 10).
4. Pour 1X Running Buffer into Gel Chamber until it reaches appropriate lines (2 gels, 4 gels). Flood gel frame completely with 1X Running Buffer.
5. Load gel lanes with 12uL of sample.
6. Load gel lane with 10uL of BioRAD Precision Plus Protein Unstained Protein Standards.
7. Run gel at 110V for 30-45min until bands reach ends of the gel.
8. Remove gel from plastic casing and place onto GelDoc Unstained (Green) Imaging Tray. Image with GelDoc.

**2. Standard 500mL “Large Scale” Protein Expression & Purification.** *E. coli* BL21(DE3) (genotype: *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHI ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*) is a special strain of *E. coli* that only produces protein from a T7 promoter when IPTG (Isopropyl β-D-1-thiogalactopyranoside) is added into the culture (a process called induction). Therefore, a large scale culture should only be induced when cell density has reached the middle of log phase growth, about OD = 0.6-0.7. Proteins are purified using affinity chromatography. Only the protein with the His tag will bind to the column during the flow through and wash step. Imidazole will remove protein from the column during the elution step, as imidazole will bind to the metal at a higher affinity than his tag will.

For AtzC and AtzC, the wash buffer was 0.1M Na<sub>2</sub>HPO<sub>4</sub>, 30mM imidazole, pH 7.6; the elution buffer was 0.1M Na<sub>2</sub>HPO<sub>4</sub>, 500mM imidazole, pH 7.6.

Note 1: During purification, keep all proteins on ice or in the cold room. Room temperature (RT) can be damaging to proteins in solution.

Note 2: The day before purification, prepare and filter wash, elution buffers, see protocol 4. Also prepare columns, see protocol 5.

Note 3: **WARNING:** For use of large centrifuge, ALWAYS BALANCE SAMPLES WITH DI H<sub>2</sub>O USING A SCALE BEFORE LOADING INTO THE ROTOR. Centrifugation of large weights (~500g) is very dangerous and can lead to complete destruction of the **\*\*very expensive\*\*** centrifuge.

Note 4: Protein concentration cannot be determined of the elution since imidazole absorbs light at 280nm. To remove imidazole, you must dialyze protein in a buffer without imidazole, such as 1X PBS. See protocol 7.

1. Start a 5mL culture in appropriate antibiotic and inc overnight (O/N) 250RPM 37C.
2. Use entire 5mL culture to inoculate 500mL sterile (autoclaved) media containing the appropriate antibiotic. Inc at 150RPM 37C until optical density (OD) at 600nm = 0.6-0.7. For *E. coli* BL21(DE3) in LB, this takes approx. 2hr. Measure OD by analyzing a 1mL aliquot on a spectrophotometer.
3. Add 500uL of 1M IPTG for final concentration of 1mM IPTG. Inc 150RPM 16-18C O/N.
4. Centrifuge large cell culture in 1L centrifugation tube at 4000RPM, 20min, 4C. Decant and discard supernatant.
5. Resuspend cell pellet in 30mL wash buffer. Transfer to 50mL Falcon tube and add nails worth of DNase I, lysozyme. Add 300uL 1M PMSF (protease inhibitor). Sonicate cells at amplitude = 50, time = 3:30, pulse = 2s ON; 10s OFF. To use sonicator:
  - a. Fill plastic bucket with ice water.
  - b. Wash sonication probe with ethanol, diH<sub>2</sub>O.
  - c. Position Falcon tube into plastic bucket and remove cap.
  - d. Place sonication probe into middle of Falcon tube. Be careful not to let edge of probe touch Falcon tube. (if the probe touches any part of the falcon tube - it can break) **\*very expensive\***
  - e. Turn on sonication console and adjust sonication settings.
  - f. Allow sonication to occur. Process is automatic.
6. Re-centrifuge at 20,000RPM, 4C, 20min in large centrifuge (BALANCE TUBES\*very important\*)
7. Collect 20uL of supernatant for SDSPAGE. Run the rest of the supernatant on Ni-NTA column. Collect nails-worth of pellet and resuspend in 100uL 1X PBS for SDSPAGE.
8. Collect flow through for SDSPAGE. Wash column with 60mL wash buffer. Collect wash for SDSPAGE.
9. Elute in 15mL elution buffer. Collect elution.
10. Run supernatant, pellet, flow through, wash, elution on SDSPAGE, see protocol 2.

**3. Preparation of Ni-NTA Affinity Columns.** This process can be done at RT, but the steps of protein purification must be done at 4C (in the cold room). Note that columns should be labeled with your NAME, DATE OF PREPARATION, RESIN (i.e. Ni-NTA or Co-NTA), and NUMBER OF TIMES USED (in tally marks). Ni-NTA sepharose can be reused up to 5 times.

1. Shake Ni-NTA resin solution before use (keep at 4C), as the resin tends to sink to the bottom of the jar. Pipette 4mL Ni-NTA resin into column.
2. Wash column with 20mL H<sub>2</sub>O. Allow H<sub>2</sub>O to flow through column.
3. Store column in %20 EtOH to prevent bacterial growth. Make sure to thoroughly wash column with H<sub>2</sub>O before use, to prevent damage to proteins from residual EtOH.

**4. Regeneration of Ni-NTA Affinity Columns.** After purification, Ni-NTA columns can be regenerated into additional Ni-NTA columns or Co-NTA columns. This process can be done at RT.

1. *Optional:* I wash the column with soap and water to prevent gunk from collecting in the membrane of the column and slowing down flow through rate. This process is controversial, as some people don't do this, and don't seem to have any issues with flow rate. You can skip this section, but my recommendation is to do this.
  - a. Resuspend resin in 20% EtOH and place in glass beaker. Make sure to use a different glass beaker for each column, so that each column gets back the original amount of resin it started with.
  - b. Add soap into column and wash with water. Be sure to completely remove all soap from column as soap will denature protein.
  - c. Rinse column with ddH<sub>2</sub>O until it flows easily through column. Pipette resin back into column.
2. Wash column with 20mL elution buffer. This removes proteins bound to the metal.
3. Wash column with 20mL ddH<sub>2</sub>O.
4. Wash column with 20mL 100mM EDTA (to remove metal).
5. Wash column with 20mL ddH<sub>2</sub>O.
6. Wash column with 20mL 100mM NiSO<sub>4</sub> (use CoSO<sub>4</sub> if you want to prepare a Co-NTA column).
7. Wash column with 20mL ddH<sub>2</sub>O.
8. Store column in 20% EtOH.

**5. Dialysis of Proteins.** Dialysis is a technique where the solution a protein is dissolved in is removed and replaced with the dialysis buffer. This may result in protein precipitation if the protein cannot tolerate the dialysis buffer. Our standard dialysis buffer is 1X PBS. This process should be carried out at 4C (in cold room).

1. Cut 3-4 inch strips of 3.5kD dialysis tubing. Inc tubing in 1L diH<sub>2</sub>O to remove toxic preservative agent.
2. Fold dialysis tubing three times and clamp with dialysis clamp at one end.
3. Pipette protein solution into dialysis tubing. Allow in some air so that dialysis bag will float. Fold top of tubing three times and clamp with dialysis camp at the other end.
4. Place into 2L dialysis buffer. Cover with foil and stir on stir plate O/N 4C.

**6. Concentration of Proteins.** Proteins can be concentrated through ultrafiltration. This process takes about 2-4hr to complete.

1. Place protein solution into 10kD or 3.5kD (depending on protein MW) ultrafiltration tube.
2. Centrifuge at 3500RPM for 10min, 4C. Each 10min, gently mix protein concentrate to prevent precipitation along ultrafiltration membrane. Continue until desired concentration is reached.

**7. Polymerase Chain Reaction.** PCR (amplification) is used to make linear double stranded (ds) DNA with particular overlaps that can be encoded by primers. Below is a standard PCR protocol. You should modify this protocol based upon your template length and primer melting temperature.

Ref: PCR using Q5 High-fidelity DNA polymerase, New England BioLabs.

<https://www.neb.com/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491>

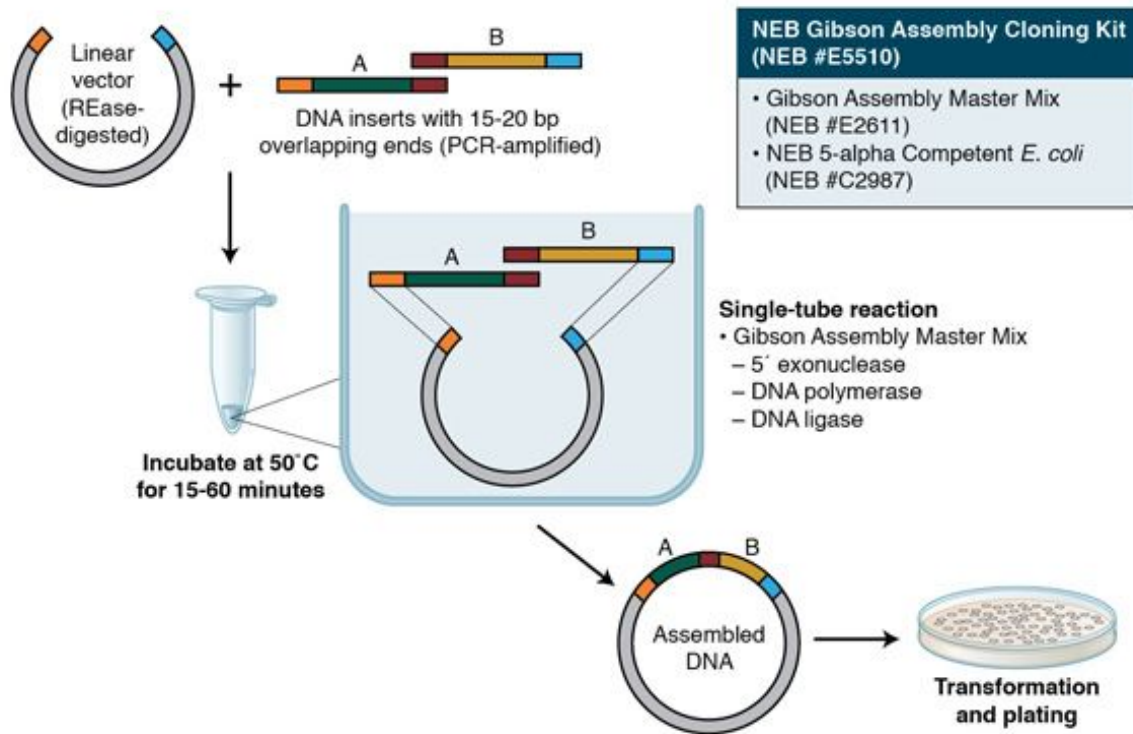
1. Primers (from IDT) arrive as DNA salts. Prepare 100mM primer solutions by adding ...
2. Dilute to 10mM by 10-fold dilution. Mix 100uL of 100mM primer stock in 900uL of ddH<sub>2</sub>O.
3. Mix the following in a PCR tube:
  - a. 10uL 5X Q5 Rxn Buffer
  - b. 1uL 12.5mM dNTPs
  - c. 2.5uL 10mM Forward Primer
  - d. 2.5uL 10mM Reverse Primer
  - e. 0.5uL Q5 Polymerase
  - f. 50ng Template DNA
  - g. Water to dilute to final volume of 50uL
4. Run on thermocycler:

\*Use NEB T<sup>m</sup> calculator to determine optimal temperature.

  - a. 1 cycle of T = 98C, 30s
  - b. 30 cycles of T = 98C, 5s; T = 50-72C\*, 30s; T = 72C, 30s/kb template
  - c. 1 cycle of T = 72C
  - d. Hold = 4C
5. Validate PCR rxn by 1 or 2% agarose gel electrophoresis (depending on insert size). Use NEB Melting Temp Calculator for third temperature (max is 72C). Mix 3uL PCR sample in 7uL H<sub>2</sub>O and 4uL 6X DNA Loading Dye, run on gel. See protocol 1.

**8. The Gibson Reaction.** *Gibson Assembly employs three enzymatic activities in a single-tube reaction: 5' exonuclease, the 3' extension activity of a DNA polymerase and DNA ligase activity. The 5' exonuclease activity chews back the 5' end sequences and exposes the complementary sequence for annealing. The polymerase activity then fills in the gaps on the annealed regions. A DNA ligase then seals the nick and covalently links the DNA fragments together. The overlapping sequence of adjoining fragments is much longer than those used in Golden Gate Assembly, and therefore results in a higher percentage of correct assemblies.* -- "Gibson Assembly", New England Biolabs.

<https://www.neb.com/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/gibson-assembly>



We currently make a “homemade” mixture of the three enzymes required for the Gibson Assembly Master Mix. Gibson Assembly was first described (although some people did it before him) by D.G. Gibson *et al.* in *Nat Methods* 6: 343-345, 2009.

1. Prepare a 10:1 ratio of inserts to linear vector (i.e. mix 100ng of insert with 10ng of linear vector). The minimum insert to vector ratio is 3:1, but 10:1 prevents issues arising from difficulties with low insert concentration. You can maximize insert concentration by preparing 3x PCR reactions and pooling into 1 PCR purification column.
2. Mix into 8uL of Homemade Gibson Master Mix. Final volume should not exceed 15uL.
3. Incubate at 50C for 50min.
4. Transform into *E. coli* XL10Gold using Heat Shock Protocol.
5. The next day, pick 4 colonies / insert. If 2 inserts are used, pick 8 colonies. If there are no or very few (<5) colonies on your plate, this indicates failed Gibson reaction.
6. The next day, miniprep colonies. Collect 8uL of plasmid and send for sequencing. Use sequencing to validate successful cloning.

**9. Transformation of *E. coli* via Heat Shock.** Transform cloning reaction products into *E. coli* XL10Gold for plasmid replication. Transform plasmids for protein production into *E. coli* BL21(DE3).

1. Retrieve 100uL competent cells (CC) from -80C and place on to ice.
2. Thaw (~5min) and prepare 50uL cell aliquots.
3. Add DNA onto CCs and inc. 20min on ice. For plasmids, use 1uL. For Gibson Rxn, use entire Gibson reaction mixture.

4. Inc CC 42C 45s.
5. Inc CC 2min ice.
6. Add 800uL LB (rescue). Inc 1hr, 250RPM, 37C.
7. For plasmids, plate 200uL onto LB-agar. For Gibson, pellet cells by centrifugation at 4000RPM for 2min. Remove 600uL media and resuspend cell pellet in 200uL. Plate resuspended cells.
8. Inc plate 37C O/N.

**10. Minipreparation of Plasmid DNA.** Miniprep is the technique used to purify plasmid DNA from a culture of *E. coli*.

NOTE: All centrifugation steps should be carried out at max speed (13-14,000rcf) unless stated otherwise.

Ref: Thermo Fisher GeneJET Miniprep Kit Manual.

<https://www.thermofisher.com/order/catalog/product/K0502>

1. Start 5mL culture of bacteria from glycerol stock or picked colony. Inc O/N 37C 250RPM.
2. Transfer culture to 15mL Falcon Tube. Pellet cells by centrifugation at 6000RPM for 5min. NOTE: if pelleting more than 6 cultures, use the medium-sized centrifuge at max speed for 15min.
3. Resuspend cell pellet in 250uL resuspension solution. Transfer to Eppendorf Tube.
4. Add 250uL lysis solution and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. Note. Do not vortex to avoid shearing of chromosomal DNA. Do not incubate for more than 5 min to avoid denaturation of supercoiled plasmid DNA.
5. Add 350  $\mu$ L of the Neutralization Solution and mix immediately and thoroughly by inverting the tube 4-6 times. Note. It is important to mix thoroughly and gently after the addition of the Neutralization Solution to avoid localized precipitation of bacterial cell debris. The neutralized bacterial lysate should become cloudy.
6. Centrifuge for 10 min to pellet cell debris and chromosomal DNA.
7. Transfer 700uL of supernatant to the supplied GeneJET spin column by pipetting. Avoid disturbing or transferring the white precipitate. Note. Close the bag with GeneJET Spin Columns tightly after each use!
8. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
9. Add 500  $\mu$ L of the Wash Solution (diluted with ethanol prior to first use as described on p.3) to the GeneJET spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube.
10. Repeat the wash procedure (step 8) using 500  $\mu$ L of the Wash Solution.
11. Discard the flow-through and centrifuge for an additional 10 min to remove residual Wash Solution. This step is essential to avoid residual ethanol in plasmid preps.
12. Transfer the GeneJET spin column into a fresh 1.5 mL microcentrifuge tube. Add 30  $\mu$ L of the Elution Buffer to the center of GeneJET spin column membrane to elute the plasmid DNA. Take care not to contact the membrane with the pipette tip. Incubate for 2 min at room temperature and centrifuge for 2 min. Note. An additional elution step (optional) with Elution Buffer or water will recover residual DNA from the membrane and increase the overall yield by 10-20%. For elution of plasmids or cosmids >20 kb, prewarm Elution Buffer to 70°C before applying to silica membrane.
13. Discard column and store DNA at -20C.

**11. Gel Extraction of DNA.** Gel extraction is the technique used to purify DNA from a agarose gel. This method is useful if you wish to isolate specific bands of DNA.

NOTE: All centrifugation steps should be carried out at max speed (13-14,000rcf) unless stated otherwise.

Ref: Omega BioTek EZNA Gel Extraction Kit Manual.

<http://omegabiotek.com/store/product/e-z-n-a-gel-extraction-kit/>

1. Perform agarose gel/ethidium bromide electrophoresis to fractionate DNA fragments. Any type or grade of agarose may be used. However, it is strongly recommended that fresh TAE buffer or TBE buffer be used as running buffer. Do not reuse running buffer as its pH will increase and reduce yields.
2. When adequate separation of bands has occurred, carefully excise the DNA fragment of interest using a wide, clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
3. Determine the appropriate volume of the gel slice by weighing it in a clean 1.5 mL microcentrifuge tube. Assuming a density of 1 g/mL, the volume of gel is derived as follows: a gel slice of mass 0.3 g will have a volume of 0.3 mL.
4. Add 1 volume Binding Buffer (XP2).
5. Incubate at 60°C for 7 minutes or until the gel has completely melted. Vortex or shake the tube every 2-3 minutes. Important: Monitor the pH of the Gel/Binding Buffer mixture after the gel has completely dissolved. DNA yields will significantly decrease when the pH > 8.0. If the color of the mixture becomes orange or red, add 5 µL 5M sodium acetate (pH 5.2) to bring the pH down. After this adjustment, the color of the Gel/Binding Buffer mixture should be light yellow.
6. Insert a HiBind® DNA Mini Column in a 2 mL Collection Tube.
7. Add no more than 700 µL DNA/agarose solution from Step 5 to the HiBind® DNA Mini Column.
8. Centrifuge at 10,000 x g for 1 minute at room temperature.
9. Discard the filtrate and reuse collection tube
10. Repeat Steps 7-9 until all of the sample has been transferred to the column.
11. Add 300 µL Binding Buffer (XP2). Centrifuge at maximum speed ( $\geq 13,000 \times g$ ) for 1 minute at room temperature. Discard the filtrate and reuse collection tube.
12. Add 700 µL SPW Wash Buffer. Note: SPW Wash Buffer must be diluted with 100% ethanol prior to use. Centrifuge at maximum speed for 1 minute at room temperature. Discard the filtrate and reuse collection tube.
13. Centrifuge the empty HiBind® DNA Mini Column for 10 minutes at maximum speed to dry the column matrix.
14. Transfer the HiBind® DNA Mini Column to a clean 1.5 mL microcentrifuge tube. Add 30-50 µL Elution Buffer or deionized water directly to the center of the column membrane. Let sit at room temperature for 2 minutes. Centrifuge at maximum speed for 1 minute.
15. Store DNA at -20°C.

## 12. Large Scale Expression and Purification of AtzA Proteins with Chaperones

In previous AtzA studies most of the enzyme expressed was found in inclusion bodies and largely inactive. This rendered studies of metal content and activation difficult to interpret. To overcome these problems, I describe here the use of a double chaperone system which provides consistent folding of AtzA in vivo and the ability to produce AtzA in high yields and activity. This protocol results in a yield of ~6.5mg/1L culture.

1. Transform BL21(DE3) with pAG (containing the chaperone system GroEL, GroES, CmR, gift from Larry Wackett, University of Minnesota), and *atzA::pET15b+* using the heat shock protocol. Plate onto LB-agar-Cm-Carb and inc O/N 37C.
2. Pick a colony and culture in 5mL LB-Cm-Carb. Inc O/N 37C, 250RPM.

3. Inoculate 2.5mL of O/N culture in 500mL of LB-cm-carb in a 2L flask. Inc 37C, 150RPM until OD600=0.5-0.6.
4. Cool flask by inc. 16C, 150RPM, 30min.
5. Induce expression of chaperone by adding 5mL 10% L-arabinose to flask for a final concentration of 0.1% L-arabinose. Inc 2hr 16C, 150RPM.
6. Induce expression of AtzA by adding 50uL 1M IPTG. Inc o/n 16C.
7. Follow protein purification protocol.

### 13. Site Directed Mutagenesis:

Using 1µl of Pfu-Ultra High-Fidelity Polymerase, 1 µl of dNTPs, and the reaction buffer from the QuikChange Site-Directed Mutagenesis kit from Agilent, 125 ng of the forward and reverse primers and 50 ng of the double stranded DNA template with distilled water added to a total reaction volume of 50 µl were mutated using polymerase chain reaction. Each primer was designed to have at least 15 base pairs of overlap with the other one in the pair and at least 15 base pairs of overlap with the template, an annealing temperature below 70°C and above 55°C, no hairpins, and a maximum length of 60 base pairs. In the PCR cyclor program it was denatured for 30 seconds at 95°C then 16 cycles of denaturing at 95°C for 30 seconds, annealing at whatever temperature the primer pair had for 1 minute, and elongating at 68°C for six minutes. The PCR product was then digested by adding 1µl DpnI and incubating at 37°C for six hours in order to destroy the unmodified plasmids. The remaining product was transformed into the chemically competent *E. coli* XL-10 Gold or DH5α cells.

**14. Transmission Electron Microscopy (TEM)** -- TEM is a microscopy technique in which electrons are transmitted through an ultra-thin specimen. Electrons are beamed into a specimen using magnets and focusing lenses. When preparing samples for TEM, recognize that some optimization is required. Different buffers, staining reagents, and protein concentrations should be prepared to determine optimal imaging. Literature reviews should be done prior to TEM to determine optimal conditions. Additionally, the grid should be kept extremely clean, to prevent imaging contaminants. Common stains used are 1% PTA or 1% (10mg/mL) uranyl acetate.

Working Principle (from [http://www.hk-phy.org/atomic\\_world/tem/tem02\\_e.html](http://www.hk-phy.org/atomic_world/tem/tem02_e.html)):

TEM works like a slide projector. A projector shines a beam of light which transmits through the slide. The patterns painted on the slide only allow certain parts of the light beam to pass through. Thus the transmitted beam replicates the patterns on the slide, forming an enlarged image of the slide when falling on the screen.

TEMs work the same way except that they shine a beam of electrons (like the light in a slide projector) through the specimen (like the slide). However, in TEM, the transmission of electron beam is highly dependent on the properties of material being examined. Such properties include density, composition, etc. For example, porous material will allow more electrons to pass through while dense material will allow less. As a result, a specimen with a non-uniform density can be examined by this technique. Whatever part is transmitted is projected onto a phosphor screen for the user to see.

Sample Preparation Protocol:

1. Incubate the grid with the copper side down and the dark side (the grid side) facing upwards under a UV lamp O/N. I recommend you do this by placing the grids on filter paper in a Petri dish. Additionally, the Petri dish retains some negative charge that will interfere with sample adhesion -- the filter paper will insulate the grid from this effect. The UV exposure will glow discharge the TEM grid and allow proteins to better bind to the TEM grid surface.
2. Use the special EM forceps to hold the grid and place on a fulcrum such that the grid side is facing up (see figure).



3. Place 10uL of sample onto grid side. Inc 1-5min. It is recommended that the sample be ultrapure by running it through a gel filtration column.
4. Wick sample liquid off of grid until dry. Do this by holding the filter paper perpendicular to the grid. You never want to allow the filter paper to come in contact with the grid surface.
5. Repeat steps 3-4 for stain. It is recommended that the stain be prepared fresh, or that the stain is aliquoted and kept frozen. Common stains for TEM are 1% uranyl acetate and 1% PTA.
6. The grid is now ready for imaging. The technician will load the grid for you onto the microscope.