5.36 Biochemistry Laboratory
Spring 2009

SESSION 1

The following should be prepared by one or more TAs for session 1 and 2 experiments:

- 1 M IPTG. Dissolve 2.38 g of IPTG (MW 238.3) in 10 mL of water. Sterilize the solution by pushing through a 0.2 micron syringe-tip filter. Aliquot into 1-mL samples. Store in the -20 °C freezer.
- 1000X (50 mg/mL) kanamycin: Dissolve 500 mg of kanamycin in 10 mL of water. Sterilize the solution by pushing through a 0.2 micron syringe-tip filter. Aliquot into 1-mL samples and store at -20 °C.
- 1000X (50 mg/mL) streptomycin: Dissolve 500 mg of streptomycin in 10 mL of water. Pass through a 0.2 micron syringe-tip filter. Aliquot into 1-mL samples and store at -20 °C. Frozen stock solutions are stable for up to 3 months.
- LB/kan agar plates (20). Combine 10 g of LB, 6 g of bacto-agar and 400 mL of water in a 1 L bottle and autoclave* on a liquid setting for 20 min. Allow the solution to cool to approximately 55 ºC (until you can hold the bottle comfortably), then add 400 µL of 1000X kan (final concentration 50 µg/mL). Immediately transfer 25 mL-aliquots of the solution to 20 cell culture plates using 100-mL pipettes. Allow the LB/agar to solidify and store the plates at 4 ºC. Extra plates may be stored for use in session 10.
- LB/kan/strep agar plates (4). Combine 2.5 g of LB, 1.5 g of bacto-agar and 100 mL of water in a 200 mL flask, and autoclave* on a liquid setting for 20 min. Allow the solution to cool to approximately 55 ºC, and then add 100 µL of 1000X kan and 100 µL of 1000X strep (final concentration 50 µg/mL of each antibiotic). Immediately transfer 25 mL-aliquots of the solution to 4 cell culture plates. Allow the LB/agar to solidify and store the plates at 4 ºC
- Sterile LB media. Autoclave* 100 mL of LB media in a 200 mL bottle (2.5 g of LB in 100 mL water). Also, for each group prepare: 1) a 500-mL solution of LB in a 1-L flask and 2) a separate 100-mL solution in a 200-mL container. For sterilization, cover the containers with aluminum foil and make sure the cap is not screwed onto the 200-mL container. Stick a piece of autoclave indicator tape on the side of each flask. The tape will turn color upon reaching a sufficient sterilization temperature. Autoclave for 20 min.
- Sterile tips. Autoclave* enough tips so that each pair or group of students has their own set of each size.
- BL21-DE3 cells co-transformed with Yop and H396P Abl. These may already be available as a glycerol stock in the -80 freezer. If not, prepare as described below.

*Please not that only a trained TA may use an autoclave. Students should never use an autoclave during this class.

Co-transformation of Abl and Yop in BL21DE3 heat shock competent cells (if needed):

1) Thaw a single aliquot of BL21DE3 heat shock competent cells on ice. Also place the plasmid Abl and Yop DNA tubes on ice until ready for use.
2) Add 40-50 µL of the cells to a 1.5-mL eppendorf tube on ice.
3) Carefully mix 1 µL of the Yop plasmid DNA and 1 µL of the Abl plasmid DNA with the cells. (Note: the plasmid DNA was from a miniprep at ~ 100

ng/uL. The transformation should work well, however, for a range of concentrations.)

4) Move the tube from the ice bath to a 42 ºC water or oil bath for EXACTLY 45 seconds.
5) Immediately transfer the tube back onto ice, and incubate on ice for 2 minutes.
6) Rescue the cells with the addition of 600 µL of SOB media.
7) Incubate the tube at 37 ºC with end-over-end rotation for 1 hour.
8) Plate 100- 500 µL of the cell solution on pre-warmed kan/strep LB plates. (The plates should have kan and strep at 50 µg/mL each.)
9) Incubate overnight at 37 ºC.

Make a glycerol stock of the transformed cells.
To make a glycerol stock:

1) Pick a colony from a fresh plate (no more than 2 days old), and inoculate a 1-mL culture of LB (with kan and strep).
2) Grow culture until OD_{600} = 0.5 (do NOT overgrow).
3) Transfer 600 µL of culture to a 1.5 µL sterile tube.
4) Add 600 µL of sterile 50% glycerol to the tube for a 25% final glycerol concentration.
5) Invert tube to mix. Make sure to carefully label the tube with the date, cell type, and plasmid(s).
6) Flash freeze in liquid nitrogen.
7) Store at -80. For easy identification, place the tube in a small plastic bag with a more detailed paper label inside.

During Session 1:

You should introduce the students to sterile technique by explaining the importance of autoclaving media and equipment and demonstrating the use of pipettes. To demonstrate, add 6 mL of sterile LB to a cell culture tube, since the students will have to do this at the end of class. Advise them to keep their bench-top area clean by wiping it down with 70% ethanol. Point out when to use sterilized tips and when it is ok to use non-sterile tips. Also explain that we must make the assumption that the air is sterile.

Since the safety lecture is now part of session 1, the TAs (rather than the students) will have prepared a 500-mL and a 100-mL solution of sterile LB. Please distribute the LB solutions to the students and explain that they are responsible for preventing contamination of their LB.

At some point during the lab period (before or after the students leave), the TA should prepare a fresh plate containing bacteria with Abl(229-511) and a plate with bacteria containing both the H396P Abl and Yop phosphatase. Accordingly, set out an LB/kan and an LB/kan/strep plate on the bench top to warm to room temperature. Take a scraping of the glycerol stock (from the Klibanov -80 ºC freezer) of DH5α cells with the Abl-encoding plasmid using a pipette tip and drop the tip into 1 mL of LB with 50 µg/mL kan in a cell culture tube. Work quickly to make sure that the cells do not thaw before you return them to the freezer. Place the tube in the 37 ºC shaker for 5 to 30 min, and then spread about 200 µL of the solution onto the LB/kan plate. Similarly, take a scraping of the glycerol stock of Yop-plasmid/H396P Abl-plasmid containing BL21-DE3 cells

with a pipette tip and drop the tip into 1 mL of LB with 50 μg/mL kan and 50 μg/mL strep in a cell culture tube. Place the tube in the 37 ºC shaker for 5 to 30 min, and then spread about 200 μL of the solution onto the LB/kan/strep plate. Place the plates in the 37 ºC incubator overnight.

SESSION 1B (lab open 1-2 pm)

Demonstrate to the students how to pick a colony from the plates. Inoculate 2 sets of culture tubes as described for the students in case some groups add the colonies to the media with the wrong set of antibiotics. Make sure the students check that their media is not cloudy (contaminated) before they add the antibiotics. Give them some of the extra media prepared by the TAs if their media is bad. Once everyone has picked their colonies, wrap the plates with parafilm and store them at 4 ºC.

SESSION 2 (lab open 1-5 pm)

Demonstrate use of the two shakers (37 °C and room temperature). The heated shaker should be set to 250 rpm. Please note that the room temperature shaker does not have an rpm speed setting, but instead is numbered 1-10. Set the speed to just above 5, which is close to 250 rpm. Advise students to start checking the OD of their media 2 hours after inoculation.

You should also demonstrate proper use of the UV/Vis spectrophotometer, although this can wait until after everyone has started protein expression. Remind students to use the quartz cuvettes for the DNA measurements and the plastic (disposable) cuvettes for the OD measurements. For the miniprep procedure, demonstrate proper use of the bench top centrifuges and explain the importance of keeping the samples balanced. Also show the students how to use the large centrifuges so they will be prepared for tomorrow to spin down their cell pellets from the 500 mL growth that they will induce today.

For the miniprep procedure, check that RNaseA has been added to Buffer P1. If not, add the RNaseA. Buffer P1 should be stored at 4 ºC following RNaseA addition. Each group should receive a freezer box for storage of their isolated DNA in the -20 °C freezer.

SESSION 3

TAs should prepare the following buffers for week sessions 3 and 4:

- 10X TAE buffer. In a 1 L bottle, combine 48.4 g Tris base, 10.9 mL glacial acetic acid, 2.92 g EDTA (free acid, FW 292.25), and 1 L of water. The pH should be between 8.1 and 8.3 and should not require adjustment.
- Coomasie staining solution. 0.25 % Coomassie Brilliant Blue, 50 % methanol, and 10 % glacial acetic acid. Prepare 500 mL. Store at room temperature.
- Fast destain solution. 40% methanol and 10% glacial acetic acid. Prepare 1 L.
- Slow destain solution. 5% methanol and 10% glacial acetic acid. Prepare 1 L. Store both destain solutions at room temperature.
- 40% acrylamide/ bisacrylamide solution. Follow the instructions on the bottle. Store at 4 ºC.
- 0.5 µg/mL ethidium bromide (EtBr) solution. (Use extreme caution when working with EtBr. It is a suspected carcinogen and teratogen. Wear nitrile gloves and work with the powder form only in a hood.)

At the start of session 3, demonstrate setting up a DNA gel box to pour and run a gel. Stress that the DNA runs from the negative to the positive end of the gel, and show them which end to load their DNA on. Review what results should be expected on the gel from the four digestions. On the board, draw a diagram of the vector with the Abl insert and discuss the expected outcome after Xho1 digestion, Nde1 digestion and Xho1/ Nde1 digestion.

Advise the students to select a mutation from the list of Abl mutants found in Gleevec-resistant CML patients (Appendix B3) and record each pairs' target mutation. **Note that the Quikchange is NOT always successful, but that the students are encouraged to try any mutant of their choice. Mutations that have worked in the past are S417Y (this one is the most consistently successful) and G250E. Mutations that have failed in the past are F486S, M351T, and E255K. However, the unsuccessful mutations may have failed due to student error or may just require Quikchange optimization.**

SESSION 4

Prepare two to three 200-mL bottles of 1.0 M HCl for pH adjustments in session 4. Make sure that any concentrated HCl used for pHing by the students is also in a small container. Prior to session 4, check ALL of the pH meters and confirm that they are working properly.

Assign each group of students three class buffers to prepare: one of the Ni-NTA purification buffers (a, b, c) and two of the SDS-PAGE buffers (e and g, j and l, or k and m). Every group should make their own 10X dialysis buffer, solution d. You may end up having more than one group make a buffer, which is fine. Preparing buffers is an important skill for the students, so please still assign each group 3.

For the BSA protein assays, please review proper use of the UV/Vis spectrophotometer.

Today primer proposals are due for the DNA mutagenesis. Check the primer proposal for each group of students and help them enter in the primer information on the Invitrogen order form. Follow the primer ordering instructions that are included with the equipment/supply ordering sheets.

The students need to check their calculations with you prior to preparing the buffers. The correct values are included below.

Your TA will assign you to prepare one of the three Ni-NTA purification buffers described below:

a) Ni-NTA binding buffer (50 mM Tris, 300 mM NaCl, pH 7.8). In a 1 L bottle, combine 6.1 g of Tris base (FW 121.1), 17.5 g NaCl (FW 58.44), and 800 mL of cold water. Adjust the pH to 7.8 with HCl, and then adjust the final volume to 1L.

b) Ni-NTA washing buffer (50 mM Tris, 300 mM NaCl, 30 mM imidazole, pH 7.8). In a 500 mL or 1L bottle, combine 3.05 g Tris base (FW 121.1), 8.75 g NaCl (FW 58.44), 1.02 g imidazole (FW 68.08), and 400 mL of water. Adjust the pH to 7.8 with HCl, and then adjust the final volume to 500 mL.

c) Ni-NTA elution buffer (50 mM Tris, 300 mM NaCl, 200 mM imidazole, pH 7.8). In a 500 mL or 1L bottle, combine 3.05 g Tris base (FW 121.1), 8.75 g NaCl (FW 58.44), 6.8 g imidazole (FW 68.08), and 400 mL of water. Adjust the pH to 7.8 with HCl, and then adjust the final volume to 500 mL.

EACH group must prepare a 10X stock of TBS for dialysis as indicated:

d) 10X TBS (dialysis buffer) (200 mM Tris, 1.37 M NaCl, pH 7.5). In a 1 L bottle, combine 24.2 g Tris base, 80.1 g NaCl, and 800 mL of water. Adjust the pH to 7.5 with HCl, and then adjust the final volume to 1 L.

You will be assigned two of the SDS-PAGE gel buffers below to prepare for the class to share. Several of the buffers will be prepared by the TAs, as indicated:

e) 10X electrophoresis buffer (Tank buffer). In a 2 L bottle, combine 60.4 g Tris base, 288 g glycine, and 10 g SDS. Add water to a final volume of 2 L.
f) Coomasie staining solution **(provided by TA)**. 0.25 % Coomassie Brilliant Blue, 50 % methanol, and 10 % glacial acetic acid.
g) 6X reducing protein loading buffer. For 15 mL of solution, combine 3.5 mL water, 3.5 mL of 1 M Tris-HCl, pH 6.8, 3.6 mL glycerol (use a positive displacement pipette for this viscous liquid), 1.0 g SDS, 930 mg DTT, and 1.2 mg bromophenol blue. Store this solution at – 20 ºC.
h) Fast destain solution **(provided by TA)**. 40% methanol and 10% glacial acetic acid. This solution can destain gels in 1-3 hours.
i) Slow destain solution **(provided by TA)**. 5% methanol and 10% glacial acetic acid. This solution is for destaining overnight.
j) 1.0 M Tris-HCl, pH 8.8. In a 1 L bottle, add 121.1 g of Tris base and 800 mL of water. Adjust the pH of the solution to 8.8. Add water to a final volume of 1 L.
k) 0.38 M Tris-HCl, pH 6.8. In a 1 L bottle, add 46 g of Tris base and 800 mL of water. Adjust the pH with HCl to 6.8. Add water to a final volume of 1 L.
l) 50 % sucrose, 100 mL
m) 10 % ammonium persulfate (APS), 10 mL. store at 4 °C.
n) 40% acrylamide/ bisacrylamide solution **(provided by TA)**

SESSION 5

For cell lysis, stress the importance of fully homogenizing the pellet in the B-PER detergent. For the purification, review the concept of an affinity tag on the board. Remind the students not to disturb the resin bed in the purification procedure. Label a container "used Ni/NTA resin". The used resin should be stored in the binding buffer at 4 °C for subsequent regeneration.

For the protein dialysis, have the students make up 1X TBS from their 10X solutions early in the lab period, and store the TBS (dialysis buffer) in the fridge so it is cold for the protein dialysis.

Prepare 20 mL of a 10% SDS solution in water. Store in a 50-mL eppendorf tube at room temperature.

SESSION 6

Demonstrate the set up for pouring an SDS gel and placement of the poured gel in the gel box. The gel combs should face inward and either two gels or a gel and a blank cassette must be inserted. Point out how far to fill the gel with the resolving gel solution to leave adequate room for the stacking gel and gel comb (up to the highest line on the gel cassette).

SESSION 7 and 8

Students can combine these sessions into a single day or have two very short lab days.

One TA should prepare the following for session 7:

- Protein ladder: Prepare 20 µL aliquots and store at -20 ºC.

Review setting up a gel box and loading protein samples. Demonstrate how to scan a gel (or take a picture) for the report. Designate and label a bottle for used Coomassie blue stain. The stain can be reused. Label a waste bottle for used destain solution. Remind the students that they must not pour these solutions down the sink. Provide the students with an aliquot of protein ladder from the -20 ºC freezer for the SDS-PAGE gels. If time is running short, you can speed up the staining and destaining process by heating the gels and stain (or destain) in the microwave for 15 seconds at a time for 3 to 4 times total. If you do this, be careful not to inhale deeply when you open the microwave, since both the stain and destain solutions contain acetic acid.

SESSION 9

One TA should prepare the following aliquots for Sessions 9 and 10:

- PfuTurbo: Prepare 10 µL aliquots and store at -20 ºC. One aliquot should be sufficient for 3 pairs of students.
- SOB media. In a 1-L bottle, combine 20 g tryptone (2%), 5 g yeast extract (0.5%), 584 mg NaCl (10 mM), 186 mg KCl (2.5 mM), and 1.2 g MgSO4 (FW 120.37, 10 mM). Add water to a final volume of 1 L. Dissolve the powder, aliquot the resulting solution into 100 mL bottles and autoclave for 40 min in the liquid cycle. Meanwhile, prepare a $MgCl_2$ solution: Dissolve 2.03 g $MgCl_2 \cdot 6H_2O$ (FW 203.31) in 20 mL of water and filter through a 0.2 micron filter into a sterile container. Into each 100-mL bottle of autoclaved media, add 2 mL of the filtered $MgCl_2$ solution. Store the SOB media at room temperature.
- A "Used Magic Bead" container (If you decide to use magic beads instead of spreaders). A small container (50-200 mL) should be filled ¼ full with ethanol and labeled for used magic beads.
- Sterile 1.5-mL eppendorf tubes. Check that there is a sufficient number of sterile eppendorf tubes for the DH5α cell aliquots and for the class transformation procedure. If not, autoclave at least 200 tubes in a glass beaker covered with aluminum foil on the dry cycle (40 minutes for sterilization and 20 minutes for drying).
- DH5α cell aliquots. Thaw a tube of DH5α cells on ice. Prepare 30-uL aliquots in sterile 1.5-mL eppendorf tubes. Flash freeze the cells with liquid nitrogen by pouring the tubes in a dewer and pouring in enough liquid nitrogen to cover the tubes. Either wait for the liquid nitrogen to evaporate or remove the tubes with tweezers and immediately freeze the cell aliquots at -80 °C. Do not submit the cells to repeated freeze-thaw cycles. If an aliquot is thawed, it should be used or discarded.

At the start of session 9, demonstrate programming and running the PCR thermal cycler and load the following program for the class: 95 °C for 30 sec; [20 cycles of 95 °C for 30 sec: 55 °C for 1 min: 65 °C for 12 min]; 10 °C for infinity. The class will do a single run (depending on the tube capacity of the PCR), so all the samples should be loaded at roughly the same time. Advise the students to wait until the entire class is ready for the run before they add the PfuTurbo polymerase. Once all the samples are loaded, start the run.

SESSION 10

For plate pouring, each group will need to autoclave a 100-mL solution of media for a 20-minute liquid cycle. You should do the actual autoclaving once the students have prepared the solutions. Organize the timing such that the class can accomplish their autoclaving in one or two runs. In addition, you should advise the students to start immediately with their DpnI digestions, since they will need to incubate the digestions for at least 1 hour before beginning the transformations.

SESSION 10B

If there are any students that were unsuccessful in generating a single colony, have them pick three colonies from another student's plate. Since some of the colonies may contain wild-type DNA or other undesired mutations, this doubles the chance that a desired mutant will be isolated from that plate. **Remind the students that they are therefore still making an important contribution, even if their own mutant failed to produce colonies.**

SESSIONS 11 and 12

Prior to session 12: TA's should prepare 1 M NaOH for adjusting the pH of the ATP solution. Please also check ALL of the pH meters and confirm that they are working properly.

TAs should remind the students that mutagenesis is not always successful on the first try and occasionally extensive optimization in necessary. Students should realize that they are doing new, untested experiments, so they should not be discouraged if their mutagenesis was not successful on this first attempt. Remind them that we still have the primers and another group of students can try for that mutation next year.

Fill out the form for DNA sequencing and have the students enter their constructs. The students should each include 3 correctly labeled samples. Make a copy of the sequencing form before submitting, so you can match the numbers with the correct students, in case anyone forgot to copy down that information.

SESSION 12 (lab open 1-4 pm)

TAs should prepare the NADH stocks, peptide stocks, and PEP, if there are insufficient stocks in the freezer. It is essential that the peptide stock is at neutral pH. Divide the remaining solutions among the group of students. Check their calculations before they start the buffer preparation.

Prepare inhibitor solutions: 100 μM of Gleevec in DMSO and a 100 μM of Dasatinib in DMSO. You will need less than 50 μL of each solution for the class assays, but make large enough solutions that you are able to measure the inhibitors accurately. Split the solutions into 10-μL aliquots to avoid excessive handling by the students. Inhibitor solutions should be made fresh each semester and should be stored at -20 °C.

For Sessions 13 and 14 (kinase assays) the class period will be split up into two time slots: 1-3 and 3-5. Have half of the class sign up for each of these time slots prior to those sessions.

Calculations for 10x kinase buffer and ATP solution (full instructions are in the manual):

- **10 x Kinase Buffer**: 1 M Tris base (FW 121.14) pH 7.5, 100 mM $MgCl_2$.This should be prepared in several steps:
    1. First prepare 1 L of a 1M solution of Tris: In a 1 L bottle, combine 121.1 g Tris, and 0.8 L of room temperature DI water. Adjust the pH to 7.5 with HCl (it is recommended to start with concentrated HCl and then switch to 1.0 M HCl as you get close to the desired pH.) Add water to a final volume of 1 L.

- **100 mM ATP, pH 7.5** (ε_{259} = 15400 $M^{-1}cm^{-1}$). ATP FW = 551.14 (anhydrous). To prepare 9 ml of a 100 mM ATP solution, add 0.5 g of ATP to 7 mL water.

Adjust the pH of the solution to 7.5 using 1 M NaOH (approximately 1.3 mL will be needed for the pH adjustment).

Calculations for the assay buffer:

For 2 mL* of 1x assay buffer, combine the following in a 2 mL eppendorf tube:

- 200 µL of 10x kinase buffer
- 57 µL of 35 mM PEP for a final concentration of 1 mM
- 40 µL of 100 mM ATP for a final concentration of 2 mM
- 25.8 µL of 12 mM NADH for a final concentration of 0.11 mg/mL or 155 µM. (Make sure the frozen stock that you use is recent.)
- 1,117.2 µL of distilled water to give a "final" volume of 1440 µL (This leaves 560 µL of spare volume for the enzymes and peptide.)

Store your 1x assay buffer in 400-µL aliquots in the -20 C freezer. Label your tubes well so they are not confused with the 10x kinase buffer!

SESSION 13 and 14

Since the students may need a lot of individualized attention during the assays, split each session into two time slots to run the assays. Half of the class should come to lab from 1-3 pm, and the remaining half should come to lab from 3-5 pm.

Double check that there is sufficient commercial wt Abl kinase for all of the groups to run the assay in the absence of an inhibitor and in the presence of Gleevec and Dasatinib. This is a total of 3 assay runs with wt Abl, requiring 9 uL of Abl enzyme per group. Order more enzyme at least two workdays ahead of time if required.

Prior to Session 13, do a test run of all assays to make sure that the reagents are good and the UV spec. is working properly. Remake reagent and inhibitor aliquots as needed. Inhibitor aliquots should be made fresh each semester. The expected specific activity for the wt Abl kinase domain in the absence of inhibitor is 3-4 U/mg. The specific activity for the purified H396P mutant is typically 10 fold lower.

SESSION 15

This session is held in a computer room (typically room 1-115). Bring the session 15 answer key to class to help answer any questions.

Since this is the final lab class, please remind the students about their upcoming journal club presentation and have them check out of the lab.