5.36 Biochemistry Laboratory
Fall 2007 - Fall 2008

Laboratory Manual for URIECA Modules 4 and 5
Course 5.36 U

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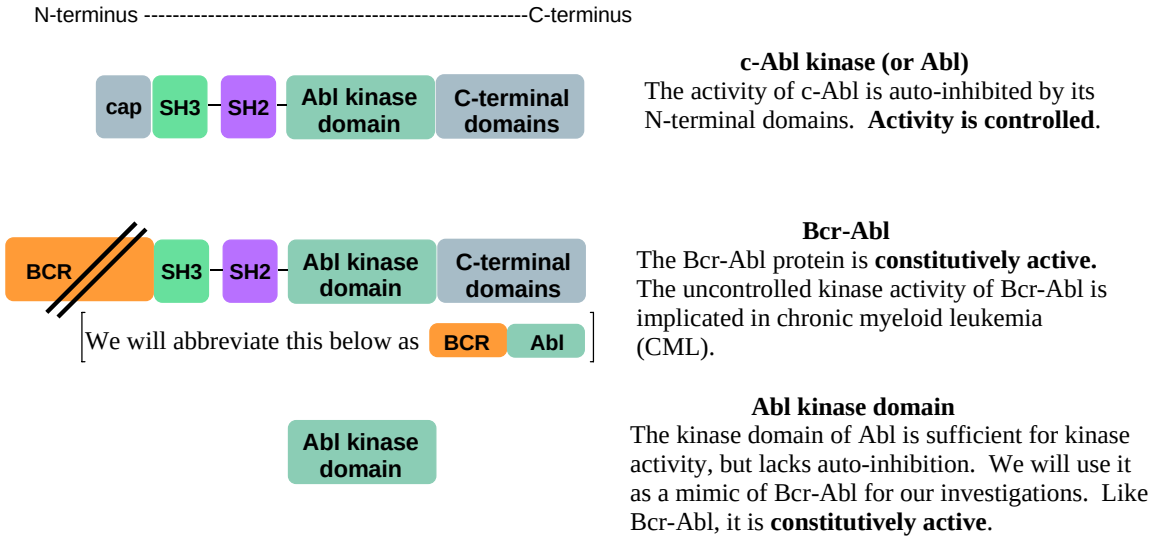
An Introduction and Background for URIECA Modules 4 and 5

Abl and Bcr-Abl Proteins

Abelson (c-Abl or Abl) is a protein tyrosine kinase that is involved in a number of highly-regulated cellular processes, including cell division, differentiation and adhesion. In healthy cells, c-Abl is auto-inhibited by domains at its amino (N)-terminus. This means that the kinase activity of c-Abl is tightly regulated, and the default activity setting is "off".

A chromosomal abnormality related to chronic myleloid leukemia (CML) causes the reciprocal translocation of genetic material from two different chromosomes, 9 and 22, which results in the formation of a mutant gene that contains part of the BCR (break cluster region) gene from chromosome 22 and part of the ABL gene from chromosome 9. This mutant gene is called BCR-ABL, and the protein it encodes, denoted Bcr-Abl, contains the kinase domain of c-Abl, but lacks the residues responsible for auto-inhibition. Bcr-Abl is therefore a constitutively active kinase, which means that the activity is permanently "on". This aberrant kinase activity is responsible for uncontrolled cell proliferation, which leads to cancer.



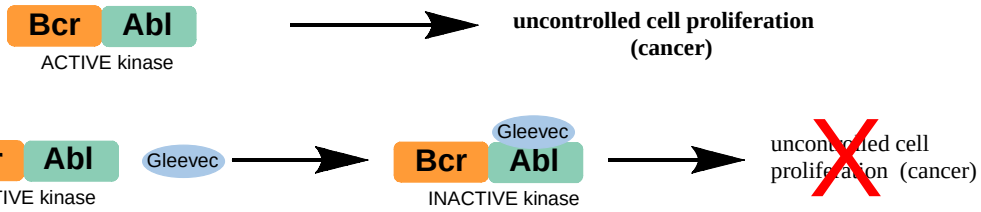
c-Abl kinase (or Abl)
The activity of c-Abl is auto-inhibited by its N-terminal domains. **Activity is controlled**.

Bcr-Abl
The Bcr-Abl protein is **constitutively active.** The uncontrolled kinase activity of Bcr-Abl is implicated in chronic myeloid leukemia (CML).

Abl kinase domain
The kinase domain of Abl is sufficient for kinase activity, but lacks auto-inhibition. We will use it as a mimic of Bcr-Abl for our investigations. Like Bcr-Abl, it is **constitutively active**.

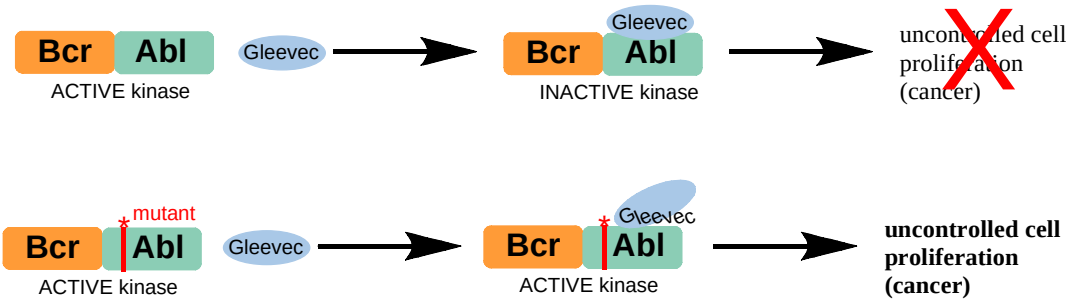
A Small Molecule Drug for CML Treatment

Bcr-Abl activity is the underlying cause for CML, and the identification of the Bcr-Abl oncoprotein led to high throughput screening and the "rational design" of potential small molecule inhibitors. These efforts culminated in the development of the drug Gleevec by chemists at the pharmaceutical company Novartis (a branch of which is a few doors down from us on Mass Ave). Gleevec (also known as Glivec, imatinib, and STI-571) showed excellent efficacy against CML and was approved by the FDA in 2001. This development was particularly thrilling to the scientific community because Gleevec is the first example of a small molecule tyrosine kinase inhibitor to treat human disease. Gleevec inhibits Bcr-Abl tyrosine kinase activity by competitively binding the ATP-binding site of the kinase domain and stabilizing the inactive conformation of the protein. Also exciting is the striking specificity of Gleevec for Abl. Gleevec only inhibits two other proteins at physiological levels, neither of which result in problematic side effects.

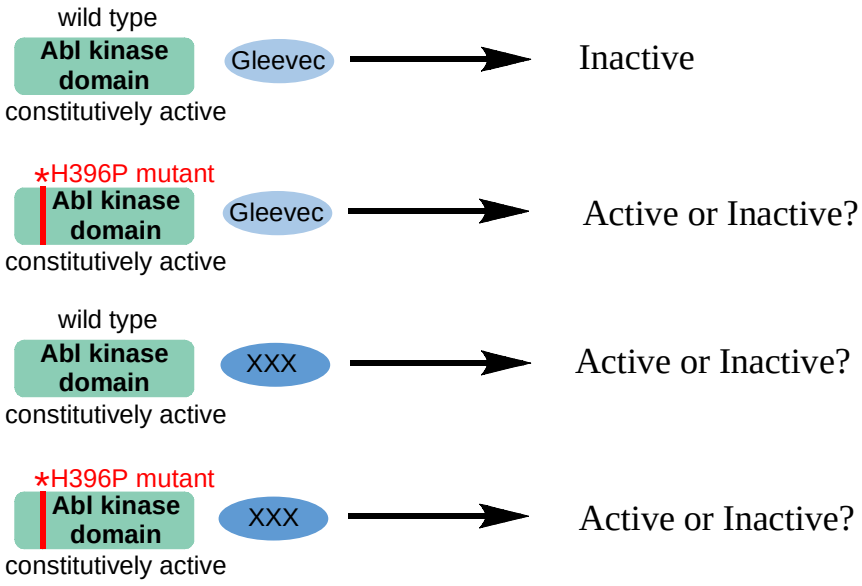


However….A PERCENTAGE OF CML PATIENTS DO NOT RESPOND TO GLEEVEC TREATMENT, AND OTHER PATIENTS THAT INITIALLY RESPOND TO TREATMENT EVENTUALLY DEVELOP GLEEVEC RESISTANCE.

The majority of these Gleevec-resistant cases can be linked to a single amino acid mutation in the Abl kinase domain of the Bcr-Abl protein. At least 30 *different* point mutations have been identified in Gleevec-resistant CML patients.



For in-vitro kinase activity experiments in this course, we will use the Abl kinase domain as a model for the full-length Bcr-Abl protein. The Abl kinase domain, similar to Bcr-Abl, lacks the N-terminal Abl regulation domains and is thus constitutively active. During this course you will express and purify an H396P mutant of the Abl kinase domain, a mutation that has been identified in patients with Gleevec-resistent CML. You will use this mutant along with (commercially available) wild-type Abl kinase domain in a coupled phosphorylation assay to determine kinase activity in the absence and presence of Gleevec and other kinase inhibitors. In addition, you will use site-directed mutagenesis to create a DNA expression vector for future expression of an Abl mutant of your choice.



Module 4 and 5 Overview: Gleevec is a blockbuster small molecule drug for chronic myelogenous leukemia (CML) that functions as a potent inhibitor of Bcr-Abl, an aberrant kinase implicated in the disease. While over 80% of patients treated with Gleevec in the chronic stage of CML experience remission, a significant population eventually develops resistance to the drug. A number of point mutations in the gene that encodes the Bcr-Abl protein have been identified in patients with Gleevec-resistant CML. Over the semester you will develop and execute a research plan to 1) determine whether a selected mutation (H396P) in the *BCR-ABL* gene confers Gleevec resistance using an in-vitro kinase assay, 2) explore the efficacy of alternative Bcr-Abl inhibitors on the wild-type and mutant kinases, 3) evaluate crystal structures to understand the mechanism(s) by which Bcr-Abl mutations block drug activity, and 4) use site-directed mutagenesis to create an Abl mutant of your choice. A brief description of the 15 lab sessions is provided below.

	H396P Abl protein expression/ kinase inhibition assays	***DNA site-directed mutagenesis***
Session 1	Grow a starter culture of cells with the H396P Abl and Yop-encoding vectors.	Grow a starter culture of cells with the wild type Abl vector.
Session 2	Express the H396P Abl protein. (Spin down cells on the following day.)	Isolate wt-Abl vector DNA through a miniprep. Quantify DNA concentration by UV-Vis.
Session 3		Digest isolated DNA to check for the wt Abl insert. Run DNA agarose gel. Design primers for an Abl kinase domain mutant.
Session 4	Prepare protein purification buffers. Create a BSA standard curve for future protein quantification.	
Session 5	Lyse cells and isolate the H396P Abl kinase domain. Dialyze protein into TBS.	
Session 6	Prepare an SDS-PAGE protein gel.	
Session 7/ Session 8	Run SDS protein gel. Concentrate protein and quantify final protein concentration.	
Session 9		Set up PCR for DNA mutagenesis.
Session 10		Complete the DPN digest and transform storage cells with mutant DNA. Pour LB/agar plates.
Session 11		Isolate (by miniprep) and quantify DNA. Prepare mutant DNA samples for sequencing.
Session 12	Prepare buffers and reagents for the coupled kinase activity assay.	
Session 13 and Session 14	Complete kinase assays: wt Abl kinase domain and the H396P mutant domain in the absence and presence of inhibitors.	
Session 15	Complete crystal structure viewing exercises.	Analyze DNA sequencing results.

Expanded Outline for URIECA Modules 4 and 5:
A per week average of 6 hr of laboratory, 5 hr of outside preparation and 1 hr of lecture.

Module 4 Protein Expression and Isolation of DNA

Sessions 1 and 2. *This week you will express the H396P Abl kinase domain. You will also isolate wild type (wt) Abl plasmid DNA for subsequent mutagenesis.*

Session 1

- Complete laboratory check-in.
- Autoclave LB for bacterial protein expression (TAs).
- Use sterile technique to transfer LB aliquots into three cell culture tubes.

Session 1- following day (~ 10 minutes of lab)

- Select a colony of bacteria containing plasmids for the H396P Abl kinase domain and Yop phosphatase (supplied by your TA) and inoculate 5 mL of LB/ kanamycin (kan) / streptomycin (strep).
- Select a colony of bacteria containing the Abl kinase domain plasmid and inoculate two 6-mL aliquots of LB/ kan.

Session 2

- Inoculate 500 mL of LB/ kan/ strep with your overnight H396P Abl/ Yop bacterial culture. Induce protein expression.
- Isolate the Abl plasmid DNA from the two 6-mL overnight cultures.
- Quantify the Abl plasmid DNA concentration by absorption at 260 nm.

Session 2- following day (< 1 hour of lab)

- Harvest cells by centrifugation. Record the pellet weight and store at -20 ºC.

Sessions 3 and 4: *In these sessions you will verify that the plasmid DNA you isolated contains a construct of the expected size for the Abl kinase domain. You will then design primers for subsequent site-directed mutagenesis. In preparation for purifying the H396P Abl kinase domain, you will prepare all the necessary buffers for the lysis and purification. You will also prepare a standard curve for future protein quantification.*

Session 3

- Digest your isolated wt Abl DNA with Xho1/Nde1 restriction enzymes.
- Analyze your digestion with an agarose gel and check for the ~6,000 bp insert.
- Select a mutant Abl kinase domain that you would like to prepare. Design your primers to create the mutant DNA. Primer proposals will be handed in at the beginning of session 4.

Session 4

- Prepare and pH lysis buffer, Ni-affinity column buffers, dialysis stock buffer solution, and protein gel buffers and solutions.

- Prepare the order form for your primers.
- Prepare bovine serum albumin (BSA) dilutions and create a standard curve for the Bio-Rad protein quantification assay.

Sessions 5 and 6 *In these sessions you will isolate the H396P Abl kinase domain using the amino-terminal hexahistidine tag. You will prepare an SDS gel for analyzing your protein elutions.*

Session 5 (4 hours of lab)

- Lyse your H396P Abl/Yop cell pellet.
- Isolate the H396P Abl kinase domain by hexahistidine-tag affinity purification.
- Combine the column elutions that contain detectable protein by UV/Vis. Dialyze the combined fractions to remove the imidazole.

Session 5- following day (~ 10 minutes of lab)

- Change the dialysis buffer.

Session 6 (2 hours of lab)

- Pour an SDS-PAGE gel for use in Session 7.
- Prepare your pre- and post-induction samples and Ni-NTA elutions for the SDS-PAGE gel analysis.

Sessions 7 and 8 *In these sessions you will analyze the purified H396P Abl kinase domain by SDS-PAGE gel electrophoresis. You will determine the concentration of the expressed protein after purification and dialysis.*

Session 7 (you may combine session 7 and session 8 into a single session)

- Run and stain the SDS-PAGE gel. Take a picture of the gel for your report.

Session 8

- Concentrate your dialyzed protein.
- Use the Bio-Rad quantification assay to determine the protein concentration of the H396P Abl kinase domain after purification and after dialysis.

Module 5: DNA Mutagenesis and Kinase Activity Assays

Sessions 9 and 10 *You will perform site-directed mutagenesis to construct the DNA for a mutant Abl kinase domain with a single base pair substitution. You will transform cells for subsequent isolation of your mutant DNA.*

Session 9 (2 hours of lab)

- Prepare your primers for the DNA mutagenesis.
- Set up and run the PCR reaction for the mutant DNA with your primers.

Session 9- following day (< 10 minutes of lab)

- Remove your pcr reaction from the thermal cycler and store at 4 ºC.

Session 10 (4 hours of lab)

- Set up the Dpn digestion of the QuikChange DNA.
- Pour LB/agar plates.
- Transform cells with your mutant DNA, and plate the transformed cells.

Session 10- following day (~ 10 minutes)

- Select 3 colonies from the plate and inoculate 3 separate 3-mL solutions of LB/ kan.

Sessions 11 and 12 *In these sessions you will isolate your mutant DNA and send off samples for DNA sequencing. You will prepare buffers for the coupled phosphorylation assays that will be carried out in sessions 13 and 14.*

Session 11

- Isolate the DNA from the selected colonies and quantify the DNA concentration.
- Prepare the DNA for sequencing and design sequencing primers.

Session 12

- Prepare the buffers and solutions for the coupled phosphorylation assay

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Sessions 13 and 14 *In these sessions you will analyze the activity of the (commercially available) wild type (wt) Abl and your purified H396P Abl mutant using a coupled phosphorylation assay. You will then probe for inhibition of the wt and H396P Abl kinase domains in the presence of Gleevec and other potential Abl inhibitors.*

Sessions 13 and 14

- Use the coupled phosphorylation assay to probe for wt Abl kinase activity in the absence of an inhibitor, in the presence of Gleevec, and in the presence of an alternative small-molecule Abl inhibitor.
- Use the coupled phosphorylation assay to probe for H396P Abl kinase activity in the absence of an inhibitor, in the presence of Gleevec, and in the presence of an alternative small-molecule Abl inhibitor.

Sessions 15 *In the final lab session you will discuss the class results from the inhibition assays and use a structure-viewing program to analyze the active site of Abl and a selected Abl mutant. You will also analyze the results from DNA sequencing to determine if your mutagenesis was successful.*

- Analyze your sequencing data from the site-directed mutagenesis. Print out a copy of the DNA analysis for your final report.
- Use the *PyMol* structure viewing program to view Abl crystal structures, and complete the structure viewing worksheet.

Journal Club presentations will take place during lecture periods at a time TBA.

SESSION 1

During laboratory Sessions 1 and 2 you will express the H396P Abl kinase domain. You will also isolate wild type (wt) Abl plasmid DNA for subsequent mutagenesis.

1) Preparation of LB media (the details in grey may be prepared by your TA)
LB (Luria-Bertani) media must be prepared and sterilized for use in the bacterial growth and protein expression you will carry out in the next few sessions. The media should contain a final concentration of 1% bacto-tryptone, 0.5% yeast extract and 1.0% NaCl in water. Using the premixed powder, add 25 g of LB powder/ L of water to achieve the desired concentrations. Prepare a 500-mL solution of LB in a 1-L flask and 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. Follow the TA's instructions for using the autoclave. Media is typically autoclaved for 20 min. Once the sterilized LB media has cooled enough for you to hold the flask comfortably, use sterile technique to add kanamycin (kan) and streptomycin (strep) (antibiotics) to a final concentration of 50 μg/mL each to the 500-mL solution. The TA's will provide you with a 50 mg/ mL stock of strep and a 50 mg/ mL stock of kan, so you should add 500 μL of strep and 500 μL of kan to the 500 mL of LB. Do not add antibiotics to the 200-mL bottle, and screw the cap on tightly once the solution has cooled completely.

Using sterile technique demonstrated by your TA, transfer two 6-mL and one 5-mL aliquots of LB from the 200-mL bottle into three cell culture tubes. Tomorrow you will check to confirm that your tubes have remained clear, indicating that you have used good sterile technique. If your media becomes cloudy (other than after intentional inoculation), it means the solution has been contaminated, and you must prepare fresh media.

SESSION 1B
1) Inoculation of LB/ kan with *E. coli* containing a plasmid encoding the wt Abl kinase domain

Confirm that your LB media is contamination-free by checking that the solutions are clear. Add 6 μL of the kan 50 mg/mL stock solution to each of the culture tubes containing 6 mL of LB to give a final concentration of 50 μg/mL kan. Using a sterile pipette tip, transfer half of a colony from a freshly-transformed bacterial plate (supplied by your TA) to a culture tube. You should drop the entire pipette tip into the solution. With a second pipette tip, transfer the other half of the colony to the second tube. The colonies on the plate are DH5-α *E. coli* cells, which are ideal bacterial storage cells to hold a vector of interest (in this case an Abl(229-511)-encoding plasmid). Place the culture tubes in a 37 °C shaker overnight. In session 2 you will isolate the Abl kinase domain plasmid DNA from the bacteria that grows up overnight.

2) Inoculation of LB/ kan/ strep with *E. coli* containing plasmids encoding the H396P Abl kinase domain and the Yop phosphatase

Add 5 µL of the 50 mg/mL kan stock solution and 5 µL of the 50 mg/mL strep stock solution to the culture tube containing 5 mL of LB to give a final concentration of 50 µg/mL of each antibiotic. Using a sterile pipet tip, transfer a colony of H396P Abl(229-511)/ Yop-containing bacteria from a plate (supplied by your TA) to the culture tube. The colonies on the plate are called BL21-DE3 cells, which are a cell-line commonly used for protein expression. Place the culture tube in a 37 °C shaker overnight. In session 2 you will use this "starter culture" to inoculate your 500-mL LB solution to express the H396P Abl(229-511) and Yop proteins.

SESSION 1B (lab open 1-2 pm) FOR THE MON/WED SECTION ONLY
Since your samples need to be inoculated the day before Session 2, you should only do the procedures in black today. Your TA will come in on Sunday to perform the inoculation (indicated in grey) so that your samples are ready for you on Monday.

1) Inoculation of LB/ kan with *E. coli* containing a plasmid encoding the Abl kinase domain

Confirm that your LB media is contamination-free by checking that the solutions are clear. Add 6 µL of the kan 50 mg/mL stock solution to each of the culture tubes containing 6 mL of LB to give a final concentration of 50 µg/mL kan. Using a sterile pipette tip, transfer half of a colony from a freshly-transformed bacterial plate (supplied by your TA) to a culture tube. You should drop the entire pipette tip into the solution. With a second pipette tip, transfer the other half of the colony to the second tube. The colonies on the plate are DH5-α *E. coli* cells, which are ideal bacterial storage cells to hold a vector of interest (in this case an Abl(229-511)-encoding plasmid). Place the culture tubes in a 37 °C shaker overnight. In session 2 you will isolate the Abl kinase domain plasmid DNA from the bacteria that grows up overnight.

2) Inoculation of LB/ kan/ strep with *E. coli* containing plasmids encoding the H396P Abl kinase domain and the Yop phosphatase

Add 5 µL of the 50 mg/mL kan stock solution and 5 µL of the 50 mg/mL strep stock solution to the culture tube containing 5 mL of LB to give a final concentration of 50 µg/mL of each antibiotic. Using a sterile pipet tip, transfer a colony of H396P Abl(229-511)/ Yop-containing bacteria from a plate (supplied by your TA) to the culture tube. The colonies on the plate are called BL21-DE3 cells, which are a cell-line commonly used for protein expression. Place the culture tube in a 37 °C shaker overnight. In session 2 you will use this "starter culture" to inoculate your 500-mL LB solution to express the H396P Abl(229-511) and Yop proteins.

Both culture tubes should be cloudy with bacteria when you remove them from the shaker after overnight incubation in Session 2.

SESSION 2

1.) Expression of the H396P Abl kinase domain in the presence of Yop phosphatase

Inoculate 500 mL of LB/ kan/ strep with your 5-mL starter culture of bacteria containing the ABL and YOP genes. Place the flask in the 37 °C shaker and periodically check the OD_{600} (optical density or absorbance at 600 nm) using the UV/Vis spectrometer. Use (non-inoculated) LB as the sample blank. Review the spectrometer instructions in Appendix A2. Once the OD_{600} reaches 0.1, the density should double approximately every 20 minutes, which should help you plan when next to check the OD. When the media reaches an OD_{600} of 0.8 to 1.0, remove the flask from the 37 °C shaker and place it in the 24 °C shaker. At this point, transfer 1 mL of the culture to a 1.5-mL eppendorf tube and spin it down for 3 minutes in a bench top microcentrifuge. Remove the supernatant and store the pellet at -20 °C. This is your pre-induction sample for subsequent gel analysis. After the cell culture has been shaking at 24 °C for at least 10 minutes (or up to one hour), induce protein expression with the addition of IPTG. Add 100 μL of 1M IPTG to give a final concentration of 0.2 mM IPTG. Let the culture grow at 24 °C overnight.

2.) Isolation of the wt Abl plasmid DNA from the two 6-mL overnight cultures.

While you monitor the OD of your H396P Abl(229-511)/ Yop expression, you should concurrently work to isolate the plasmid DNA from your two 6-mL overnight bacterial cultures, which contain the wt Abl(229-511)-encoding plasmid. You will use the isolated plasmid DNA in later lab sessions for DNA mutagenesis to create a Bcr-Abl mutant implicated in Gleevec-resistant CML.

Today you will use a Qiagen miniprep kit to isolate the plasmid DNA. The following instructions are modified from the Qiagen miniprep handbook:

Miniprep Procedure:

a.) Harvest the bacterial cells from your overnight cultures (12 mL total) by transferring 1.5 mL of culture into each of four 1.5 mL eppendorf tubes and spinning down the cells in a microcentrifuge for 3 minutes. Discard the supernatant and add 1.5 mL of remaining culture to the four eppendorf tubes. Repeat the centrifugation and discard the supernatant. You should have a small bacterial pellet at the bottom of each tube.

b.) Check that RNaseA has been added to Buffer P1. Add 250 μL of Buffer P1 to each cell pellet and completely resuspend each pellet by vortexing.

c.) Add 250 μL of Buffer P2 to each tube, and mix by inverting the tubes 4-6 times. Do not vortex, since that can cause shearing of the DNA. If you have properly lysed the cells, the cell suspensions will turn blue after the addition of the P2 buffer. If there are colorless regions or brown clumps in the cells, continue mixing until a homogenous blue solution appears.

d.) Add 350 μL of Buffer N3 to each tube and mix immediately by inverting the tubes 4-6 times. The solutions should become colorless and cloudy.

e.) Centrifuge the tubes for 10 min at 13,000 rpm in you bench top microcentrifuge. A compact white pellet should form in each sample.
f.) Apply the resulting supernatant from each tube (which contains the plasmid DNA) to each of four QIAprep spin columns by decanting or pipetting.
g.) Centrifuge for 30-60 s. Discard the flow through.
h.) Wash the spin columns by adding 0.75 mL Buffer PE and centrifuge for 30-60 s. Discard the flow through.
i.) Centrifuge for an additional 1 min to remove residual wash buffer. This is essential for the success of any future enzymatic reactions.
j.) Place the QIAprep spin columns into clean and labeled 1.5-mL microcentrifuge tubes. To elute DNA, add 40 µL of Buffer EB warmed to 55 °C (10 mM Tris·Cl, pH 8.5) to the center of each spin column, let stand for 1 min, and centrifuge for 1 min.

Combine the DNA elution samples (160 µL total) in an eppendorf tube. For long-term storage, keep the purified DNA, labeled with your names and the date, at -20 °C.

3.) Quantification of the DNA concentration by absorption at 260 nm

Dissolve x µL (suggested amount is 3 µL) of DNA sample in 100 µL of water. Measure the absorbance at 260 nm in a quartz cuvette. See Section Appendix A2 for UV/Vis spectrophotometer instructions. To calculate the concentration in µg/µL of your double stranded DNA, multiply the Abs260 by (0.05)(100/x). Typical concentrations of miniprepped DNA are 0.1-2 µg/µL.

SESSION 2B

Preparation and storage of the H396P Abl(229-511)/Yop- containing cell pellet.
a.) Remove the 1-L culture flask from the shaker and transfer the contents into a 500-mL centrifuge tube.

b.) Prior to spinning down the culture, transfer 1 mL from the centrifuge tube into a 1.5-mL eppendorf tube and spin it down for 3 minutes in a bench-top microcentrifuge. Remove the supernatant and store the tiny pellet at -20 °C. This is your post-induction sample for subsequent gel analysis.

c.) Balance the mass of your centrifuge tube with a blank or with another group's tube. If needed, use a transfer pipette to adjust the amount of culture in each tube or to add buffer.

d.) Spin down the cell culture in the centrifuge at 6K rpm for 10 minutes at 4 °C. Make sure the centrifuge is balanced, and, when possible, combine your centrifuge run with that of other groups to minimize wait time.

e.) As the culture is spinning down, tare a 50-mL conical tube and record the weight on the tube and in your notebook.

f.) Once the centrifuge run is complete, decant the supernatant and carefully remove any residual liquid with a transfer pipette. To discard, treat the supernatant with 50% bleach, and then pour it down the drain with plenty of water. **The protein is in the pellet.**

g.) Carefully scrape the pellets into the tared 50-mL conical tube using a spatula.

h.) If needed (if the pellet is not transferring well from the 500 to the 50-mL tube), add some buffer to transfer any remaining pellet stuck in the 500-mL tube. Spin down the pellet in the 50-mL conical tube for 5 minutes using the centrifuge tube adaptors and making sure your sample is balanced with a blank. Discard the supernatant.

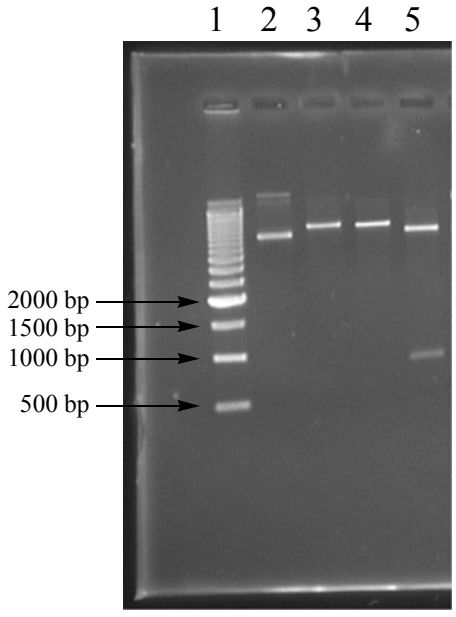
i.) Weigh the tube and record the pellet weight.

j.) Store the H396P Abl(229-511)/Yop-containing cell pellet in the -20 °C freezer until needed for protein purification. Please make sure to write your names and the date on your tube!

SESSION 3

During sessions 3 and 4 you will verify that the plasmid DNA you isolated contains a construct of the expected size for the Abl kinase domain. You will then design primers for subsequent site-directed mutagenesis. In preparation for purifying the H396P Abl kinase domain, you will prepare all the necessary buffers for the lysis and purification. In session 4, you will also prepare a standard curve for future protein quantification.

Restriction enzymes (RE's) are enzymes that selectively cleave double stranded DNA based on recognition of specific, short DNA sequences called recognition sites. In DNA engineering, restriction enzymes are used to cut open plasmids for the ligation of a complementarily-cut DNA insert into the plasmid for protein expression. In Session 2, you isolated a DNA plasmid (the pET-28a vector) containing an insert encoding residues 229-511 of the Abl kinase domain. Since the gene encoding the Abl kinase domain is positioned in the pET-28a vector between Xho1 and an Nde1 recognition sites, treatment of the plasmid with those two restriction enzymes should result in excision of the ABL insert. Today you will excise the insert by digestion of the isolated plamid DNA with Xho1 and Nde and check the size of the digestion products on an agarose gel to confirm that your vector and insert are the expected size in base pairs (bp). As denoted in the vector map, the pET28a vector (minus the segment between the Nde1 and Xho1 sites) is **5,289 bp**, and the ABL insert should be **849 bp**. As experimental controls, you will simultaneously prepare a no-enzyme "mock" digest and digests containing only one of the two restriction enzymes required to excise the ABL insert. By gel analysis of the four reactions, you will expect to see a band for the no-enzyme "mock" reaction at approximately **6,138 bp** (consistent with the size of the insert plus vector. The one-enzyme reactions should linearize the plasmid DNA without excising any segment. Since coiled plasmid DNA travels further by electrophoresis than equivalent linearized DNA, these reactions should result in bands with an apparent size slightly larger than the uncut plasmid. A sample gel is provided below.



lane 1 500-bp DNA ladder

lane 2 no-enzyme "digestion"

lane 3 Xho1-only digestion

lane 4 Nde1-only digestion

lane 5 Xho1/Nde1 digestion

1.) DNA digestion with Xho1 and Nde1 restriction enzymes

Set up analytical-scale digests of your plasmid DNA using 1 μg of DNA and final reaction volumes of 20 μL each. In each of four sterile 0.65 mL tubes, add the following components in the order listed, where *x* = the volume of miniprep elution added (in μL) to give 1 μg of DNA:

	no enzyme	**Xho1 only**	**Nde1 only**	**Xho1/Nde1**
Sterile water	17.8 – *x* μL	17.3 – *x* μL	17.3 – *x* μL	16.8 – *x* μL
RE 10X Buffer D	2 μL	2 μL	2 μL	2 μL
BSA, 10 μg/ μL	0.2 μL	0.2 μL	0.2 μL	0.2 μL
Plasmid DNA, 1 μg	*x* μL	*x* μL	*x* μL	*x* μL
Mix by pipetting, then add:				
Xho1, 10 units/ μL	0 μL	0.5 μL	0 μL	0.5 μL
Nde1, 10 units/ μL	0 μL	0 μL	0.5 μL	0.5 μL
Final volume	20 μL	20 μL	20 μL	20 μL

Mix the reactions gently by pipetting, close the tubes, and centrifuge for several seconds in a bench-top microcentrifuge. Incubate the digestions for 1 hour in the 37 ºC incubator. Meanwhile, prepare an agarose gel (described below) to analyze the digested DNA by electrophoresis. When the digestions are complete, remove the tubes from the incubator and add 4 μL of 6X nucleic acid loading buffer to each of the reactions.

DNA electrophoresis enables size-based DNA separation. Due to the negatively charged phosphate backbone of DNA, DNA molecules migrate from the negatively-charged to the positively-charged end of the gel. The smaller molecule travel faster than the larger molecules. The size (in bp) of each separated DNA fragment is determined by comparison to a DNA ladder that has a band at given intervals of bp. Here we will use a 500 bp ladder that includes bands of DNA that are 500 bp, 1000 bp, 1500 bp, 2000 bp, and so on. Depending on the size of the DNA fragments you wish to analyze, more of less agarose can be incorporated into a DNA gel. The higher the percentage of agarose, the smaller the effective range of separation will be. For analyzing the **849-bp** insert and the **6.1 kb** Abl-containing vector, you will prepare a 1.2% (w/v) agarose gel.

2.) Preparation of a DNA 1.2 % agarose gel

Set up the gel box as demonstrated by the TA. Insert the gel comb at the negatively-charged (black) end of the box. In a 200-mL Erlenmeyer flask, weigh out 1.2 g agarose and add 100 mL of 1X Tris acetate electrophoresis (TAE) buffer. To prepare the 1X buffer, dilute 10 mL of 10X TAE (provided by your TA) with 90 mL of DI water. Dissolve the agarose by heating the flask in a microwave on medium heat until the solution just boils. Prior to microwaving, plug the top of the flask with a paper towel to help prevent boiling over. All of the agarose solid should be dissolved and the solution should be clear. Cool the solution to approximately 60 ºC (cool enough to hold the flask

comfortably), and pour the solution into a prepared gel box. Allow the gel to set for approximately 30 minutes before loading. The colorless gel solution will have a bluish tint when the gel has solidified.

Once the gel has solidified, carefully remove the comb by pulling strait upwards. Pour 1X TAE into the gel box so that it just covers the gel. Using a gel-loading tip that fits the 20-µL pipette, load 3 µL of 500-bp DNA ladder and 10 µL of each of your four DNA digestion samples. Remember, DNA will migrate toward the positive (red) electrode. Loading the samples at the wrong end will cause the samples to run off the gel! Place the top on the DNA gel box and run the gel at 100 Volts for 1 hour.

Once the gel running is complete, carefully remove the gel from the box and place it in a small plastic container, such as the top of a gel-tip box. Stain the gel with approximately 0.5 µg/ mL ethidium bromide (EtBr) solution for 10 minutes by agitating on a gel rocker. EtBr is a fluorescent DNA intercalating agent, which means it inserts itself between DNA bases. Intercalated EtBr fluoresces under UV light with an intensity that is approximately 20 fold brighter than EtBr that is not DNA-associated. This fluorescence increase results from the exclusion of water molecules (a common fluorescence quencher) associated with EtBr in solution. As an intercalating agent, it is not surprising that EtBr is highly mutagenic and a suspected carcinogen and terratogen. While EtBr is less dangerous in solution than in the powder form, you should always wear nitrile gloves (not latex) and use extreme caution when handling any EtBr solution. Any gloves, paper towels, etc. that touch EtBr solutions should be discarded as solid EtBr hazardous waste.

After incubation, dispose of the EtBr solution in the designated waste container. Rinse the gel with water. Place the gel on the DNA transilluminator for visualization of your bands, using appropriate UV shielding. Take a picture of your gel with the Polaroid camera box for your lab report. Once you have a clear picture, discard the gel in the designated solid EtBr waste container.

To enable studies on additional point mutations found in the *BCR-ABL* gene of Gleevec-resistant cancer patients, you will introduce a point mutation in the wt DNA that you isolated and verified. Your resulting mutant DNA may be used in future iterations of the course for expression and use in kinase assays (as we are doing this year with the H396P mutant). Mutant DNA can be made from the wt DNA using site-directed mutagenesis. From the list of identified mutations in Appendix B3, select a mutant that interests you. While your DNA gel is running, you should begin designing primers to create the desired mutant DNA. Primer proposals should be written in the form of the proposal worksheet in Appendix C2 and turned in to your TA at the beginning of Session 4.

Overview of DNA mutagenesis

You will use the QuikChange® (http://www.stratagene.com/manuals/200518.pdf) site-directed mutagenesis method to create your desired point mutation in the wt plasmid DNA that you have previously isolated by miniprep and analyzed by Xho1/Nde1 digestion. This mutagenesis method relies on a high-fidelity DNA polymerase and mutant oligonucleotide primers, which you will design and that include the base pair substitution of interest. A DNA polymerase is an enzyme that catalyzes the polymerization of

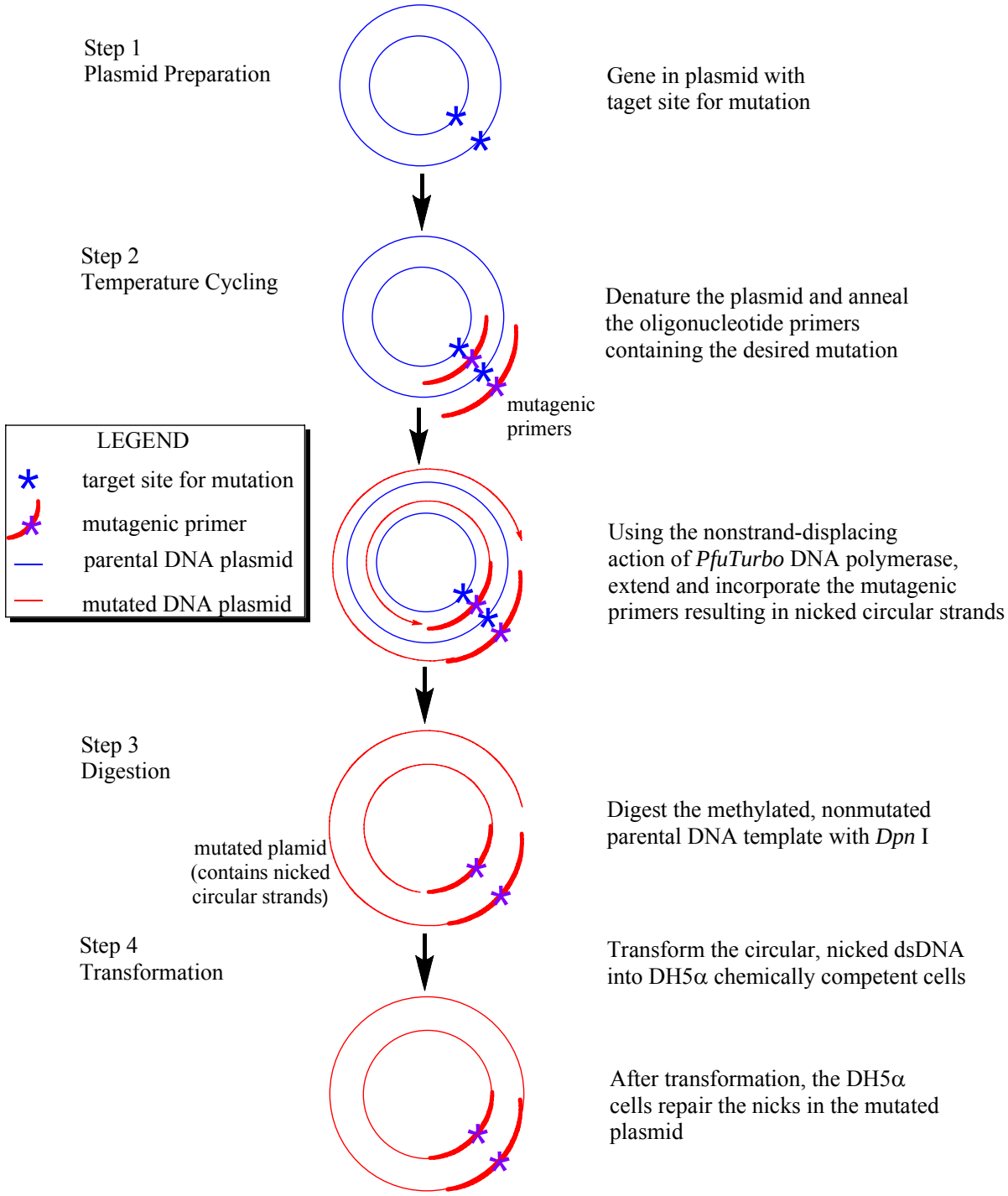


Figure 1. Overview of QuikChange site-directed mutagenesis. Modified from Figure 1 of the QuikChange mutagensis handbook (http://www.stratagene.com/manuals/200518.pdf).

polymerase through temperature cycling. Specifically, the plasmid is denatured and the primers containing the desired mutation are annealed to the complementary strand. The polymerase then uses the plasmid DNA as a template to extend the primers, resulting in a mutated plasmid with staggered nicks (breaks in the DNA where there are no connecting nucleotides against a strand of template DNA to make a new complimentary strand of DNA. The polymerase chain reaction (PCR) utilizes thermostable DNA polymerases, which enable the high temperature denaturation of DNA plamids to create single-stranded templates without simultaneously denaturing the polymerase enzyme. In the standard mutagenesis procedure (**Figure 1**), the mutant primers are extended by the phosphodiester bond between nucleotides). In order to remove the wt template DNA from the PCR product mixture, the mixture is then treated with *Dpn* I endonuclease, an enzyme that specifically digests methylated and hemimethylated DNA. Since DNA isolated from most *E. coli* strands is methylated, treatment of the PCR product mixture with *Dpn* 1 selectively digests the parent template DNA, leaving only mutant DNA assembled in vitro. The nicked vector DNA (containing the desired mutation) is then transformed into DH5α cells. The cell machinery repairs the nicked DNA, and the repaired mutant plasmid can be isolated by miniprep.

3.) Design of forward and reverse mutant oligonucleotide primers

You will design two primers, each 25-45 nucleotides in length, which are the reverse complement (A to T and C to G) of each other and incorporate your desired mutation. The following guidelines are from the Quikchange® instruction manual and should be adhered to exactly for successful mutagenesis.

- The designed mutation should be in the middle of each primer with 10-15 bases of correct sequence on both sides.
- Both mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.
- Primers should be between 25 and 45 bases in length, with a melting temperature (T_m) greater than or equal to 78 ºC. Use the formula below to estimate the T_m of your proposed primers:
    - $T_m = 81.5 + 0.41(\%GC) – 675/N - \%$ mismatch
      where, **N** is the primer length in bases and the values for **%GC** and **% mismatch** are in whole numbers.
- If possible, the primers should have a minimum GC content of 40% and should terminate in one or more C or G bases.
- Primers must be purified by either fast polynucleotide liquid chromatography (FPLC) or polyacrylamide gel electrophoresis (PAGE). Failure to purify the primers results in a significant decrease in mutation efficiency. This purification can be performed by the primer synthesis facility and should be selected on the order form.

(See section C1 of the appendix for an example of primer design)

SESSION 4

Laboratory Procedures

1.) Preparation of Ni-affinity column buffers, dialysis stock buffer solution, and SDS-PAGE buffers: solutions for isolation and analysis of the H396P Abl kinase domain.

The Ni-NTA buffers and SDS-PAGE buffers will be shared by the class. Your TA will therefore assign each group a total of three class buffers to prepare. Some of the buffer preparation protocols contain blanks, and for these you are must to calculate the required amount of each reagent (in grams) to achieve the specified concentration. CONFIRM THAT YOUR CALCULATIONS ARE CORRECT WITH YOUR TA BEFORE YOU START TO PREPARE YOUR BUFFERS.

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 _____ g of Tris base (FW 121.1), _____ 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 _____ g Tris base (FW 121.1), _____ g NaCl (FW 58.44), _____ 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 _____ g Tris base (FW 121.1), _____ g NaCl (FW 58.44), _____ 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 _____ g Tris base, _____ 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 _____ 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 _____ 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)**

2.) Preparation of BSA solutions and creation of a standard curve for the Bio-Rad protein quantification assay. (*This can be completed anytime before session 6. At a minimum, you should prepare the 1 mg/mL BSA stock solution today.*)

Prepare 5 mL of a 1 mg/mL aqueous solution of bovine serum albumin (BSA). To do this, weigh out 5 mg of BSA into a 15-mL conical tube on the balance. Add 5 mL of water and invert or vortex the tube to completely dissolve the BSA. Divide the stock solution into 1-mL aliquots in 1.5-ml eppedorf tubes and store at -20 °C for use in protein quantification assays throughout Modules 4 and 5. Prior to each assay, you must prepare five dilutions of your BSA stock solution to create a standard curve. The linear range of the Bio-Rad protein assay is 0.2 to 0.9 mg/mL. Therefore in six separate 0.65-mL eppendorf tubes, label and add the following: 270 µL of the BSA stock and 30 µL of deionized water (to give 0.9 mg/mL), 210 µL of BSA and 90 µL of water (to give 0.7 mg/mL), 165 µL of BSA and 135 µL of water (to give 0.55 mg/mL), 120 µL of BSA and 180 µL of water (to give 0.4 mg/mL), 60 µL of BSA and 240 µL of water (to give 0.2 mg/mL), and no BSA and 300 uL of water (to use as your "blank").

Prepare 20 mL of the assay dye reagent by diluting 1 part Bio-Rad dye concentrate with 4 parts DI water. Filter the resulting solution and store any unused reagent for up to two weeks at room temperature. To create a standard curve using your BSA standards, pipette 20 µL of each standard into an eppendorf tube. Add 980 µL of the prepared dye reagent to each tube and vortex briefly. Incubate the tubes at room temperature for 5 to 50 minutes. Measure the absorbance of each solution at 595 nm by UV/Vis spectrometry using disposable polystyrene cuvettes. See Appendix A2 for instructions on using the Varian Cary 100 spectrophotometer. The sample with 20 µL of water in 980 µL of assay reagent should be used as your "blank" in the spectrophotometer. Plot the absorbance vs. concentration for the BSA standards using

Microsoft Excel or another graphing program and determine the R-squared value and slope of the best-fit line to establish that you can obtain a strait line with your standards. Print a copy for your lab report. If your R-squared value is less than 0.9, repeat the exercise until you achieve an acceptable line. Good laboratory practice calls for the creation of a new standard curve each time you run a protein assay.

3.) Hand in the order form for your primers. See your TA about entering your primer information onto an Invitrogen order form.

SESSION 5

While we wait for the DNA primers to arrive for the mutagenesis, we will focus on purifying the H396P Abl kinase domain. Today you will lyse your H396P Abl(229-511)/ Yop-containing pellet, and isolate the H396P Abl kinase domain via its amino-terminal hexahistidine tag.

Affinity tag purification of recombinant proteins:

A common strategy for isolating a recombinant protein from cell lysate is the use of an affinity tag, which is a small peptide or protein fragment that can reversibly bind to a specifically-functionalized solid support. In affinity-based purification, the protein of interest is expressed with a tag immediately amino-terminal or carboxy-terminal to the protein. After cell lysis, the tagged protein is pulled out of the crude lysate through the binding of the tag to affinity resin. The resin is copiously washed to remove any non-specific binders, and the protein of interest is then eluted from the column by changing the pH to reduce binding affinity or by introducing a competitive binder. Common affinity tags include the GST tag (a 27-kDa protein that binds glutathione coated beads), the FLAG tag (an 8-residue peptide, DYKDDDDK, that binds a FLAG-antibody functionalized resin), and the hexahistidine tag (HHHHHH, which binds to Ni/NTA functionalized resin). Here we will utilize the hexahistidine tag appended to the amino-terminus of the Abl kinase domain. Since no other proteins in the bacteria have this tag, you should be able to selectively isolate the hexahistidine-tagged H396P Abl(229-511) protein. Elution of the target protein is accomplished by the addition of 100 to 200 mM imidazole, which competes for the Ni binding. The structure of imidazole and histidine are shown in **Fig. 2** to demonstrate their identical Ni-coordination sites. In addition to purification applications, affinity tags can also be used for protein visualization on gels. For example, an anti-hexahistine antibody conjugated with a fluorescent marker can be used to visualize which bands on a gel contain the hexahistidine tag.

Figure 2. Structures of histidine and imidazole

Laboratory

1.) Cell lysis

I. Thaw the cell pellet on ice or at room temperature.
II. Add 10 mL of B-PER detergent and 100 µL of a 100X protease inhibitor cocktail solution to the pellet in a 50-mL conical tube. Pipette up and down using a 10-mL pipette until the cell suspension is homogenous. In order to maximize your protein yield, it is essential that the suspension is free of lumps and completely homogenous before moving on to the next step. This may mean pipetting up and down 40 or more times.

III. Add an additional 10 mL of B-PER and 100 µL of 100X protease inhibitor cocktail and pipette up and down 10 to 15 times to achieve a homogenous suspension.
IV. Gently agitate (by shaking or rocking) the suspension for 10 min at 4 ºC.
V. Centrifuge the tube at 10,000 rpm for 15 min at 4 °C. Remember to balance the centrifuge with a tube of equal mass, such as another group's cell lysis mixture.
VI. Decant the solution (containing your soluble protein) into a fresh 50-mL conical tube. Add Ni-NTA binding buffer to a final volume of approximately 46 mL and immediately continue on to the Ni-NTA purification. If necessary, you can store the lysate on ice if you are waiting for Ni-NTA resin.
VII. To discard of the cell pellet, add 30% bleach to the pellet in the conical tube. Let the pellet sit in the bleach solution for at least 30 min, then dispose of the pellet and solution down the drain using plenty of water.

2.) Purification of the H396P Abl kinase domain by Ni-NTA affinity chromatography

While the purification will be carried out at room temperature on the benchtop, all buffers should be stored at 4 ºC and all elutions should be stored in an ice bucket.

I. To your solution of crude peptide in binding buffer, add a total of 1 mL Ni-NTA pre-activated resin. Note: 1 mL of resin is equivalent to 2 mL of a 50% resin slurry. The slurry can be transferred directly from the resin bottle to your 50-mL conical tube using a pipette.
II. Gently agitate the resin with your protein solution for 20 min at 4 °C.
III. Carefully add the cell lysate/resin mixture to a 20-mL plastic column, and collect the flow through in a 50-mL conical tube. **It is very important that you never allow the resin to go dry!** Make sure that there is always at least a small amount of buffer above the resin bed.
IV. Once the cell lysate reaches the level of the filled resin, wash the resin with 10 mL of binding buffer and then with 30 mL of washing buffer. Collect the flow through in a 50 mL conical tube.
V. Elute the hexahistidine-tagged Abl protein from the column by adding 1.2-mL aliquots of elution buffer. Collect each 1.2-mL aliquot in a separate, labeled 1.5-mL eppendorf tube. Collect 7 elution fractions in total. Fractions containing your protein may be light blue from Ni that has eluted with the protein.
VI. For subsequent gel analysis (in session 7) remove a 40-uL aliquot from each elution fraction. Carefully label these and store at 4 ºC.
VII. Combine your elution fractions and dialyze the protein into TBS as described (in section 4.)) below.
VIII. Wash the column with 10 mL of washing buffer, and deposit your used resin in the designated container for regeneration.

3.) Optional: Absorption measurements of Ni/NTA elution fractions

Measure the A_{280} of all fractions. See Appendix A2 for spectrophotometer instructions. Absorption at 280 is a less sensitive detection method than visualization on an SDS-PAGE gel, so you may find that fractions with no detectible protein by UV/Vis

have protein present on a gel. In your lab report, you should compare the results for detection by absorption at 280 nm with those for SDS-PAGE gel analysis.

4.) Dialysis of the purified H396P Abl(229-511) protein

Since the activity of some proteins can be damaged by prolonged storage in buffers with high concentration of imidazole, you will dialyze your eluted H396P Abl(229-511) fractions into TBS buffer. Consider the sizes (molecular weights) of the components in you elutions: the hexahistidine tagged Abl kinase domain is approximately **32 kDa**, and the imidazole is **68 Da** (0.068 kDa). Therefore using a 10 kDa MWCO (molecular weight cut off) dialysis device should easily enable removal of imidazole from the protein solution.

Based on the Ni-NTA elutions that contain detectable protein by absorption at 280 nm, combine all elutions with a significant concentration of the Abl domain. (Alternatively, you can combine all 7 elution fractions if you did not measure the A_{280} values.) Soak a 10-kDa MWCO dialysis cassette in 1X TBS or water for at least ten minutes. Using a syringe, carefully load the protein solution into the dialysis cassette. Be careful not to pierce through the side of the cassette. **Please have your TA observe you during this step to ensure the correct protocol is being followed and that no protein is lost.** Using the Styrofoam floatation clips, float the cassette in 2 L of cold TBS. Dialyze your protein at 4 °C overnight. In preparation for buffer exchange, chill another 2 L of TBS at 4 °C overnight. Tomorrow you will exchange the dialysis buffer with the fresh pre-chilled TBS.

SESSION 5B

Exchange the dialysis buffer with fresh pre-chilled TBS, and continue dialysis at 4 °C.
The used TBS can be poured down the drain to dispose.

SESSION 6

1.) Preparation of an SDS page gel

SDS-PAGE gel analysis will allow you to check the identity (by molecular weight) and purity of the H396P Abl kinase domain. In an SDS gel, proteins are separated based on their molecular weights. This occurs because the SDS (a detergent) binds to all denatured proteins in an amount approximately proportional to the size of the protein. This results in all proteins having an equal charge to mass ratios, since the negatively-charged SDS masks any charges from the protein side chains. The protein size is thus the sole factor that affects its migration speed through the pores of the polymerized gel. The molecular weight of each protein can be deduced by comparing the migration distance of the protein bands to that of a protein ladder, which is comprised of protein samples with known molecular weights.

Assembly of your SDS-PAGE gel

I. Make up the 12 % running gel. In a 15-mL conical tube, combine 3 mL 40% acrylamide/ bisacrylamide solution, 2.4 mL 1.0 M Tris-HCl (pH 8.8), 1.2 mL 50% sucrose, 3 mL water, and 100 µL 10% SDS. **Wait until a TA is by your side and watching before you add the APS or TEMED**. Once you are ready to pour the gel and your TA is watching you, add 250 µL of 10% APS solution and 2.5 µL of TEMED.
II. Mix the solution by inverting 4-6 times, and then immediately transfer enough solution to fill the gel cassette part way using a Pasteur pipette. Your TA will demonstrate how high the running gel should be to leave sufficient room for the stacking gel and the gel comb. Once the gel is poured, add a thin layer of water to the top of the running gel to keep the top level.
III. Allow the gel to polymerize, which can take up to 30 minutes. While the gel polymerizes, you can prepare the stacking solution (step IV) minus the APS and TEMED, which should not be added until just before the stacking gel is poured. Once the running gel has solidified, decant the water layer and use filter paper to absorb any residual water.
IV. Pour the stacking gel. In a 15 mL conical tube, combine 500 µL 40% acrylamide/ bisacrylamide solution, 1.7 mL 0.38 M Tris-HCl (pH 6.8), 2.5 mL water, and 50 µL 10% SDS. Next add 400 µL 10% APS solution and 2.5 µL TEMED. Mix the solution by inverting 4-6 times, and pour the solution on top of the running gel to the top of each cassette.
V. Quickly insert a comb into the stacking gel. The gel may take up to 30 minutes to polymerize. The purpose of the stacking gel is to concentrate the protein samples as they enter the running gel.
VI. Once the gels have polymerized, place the cassettes in a plastic container with 1X Tank buffer. Wrap the container in saran wrap to prevent the gel from drying out and store the gel at 4 ºC. You will use your gel in Session 7.

2.) Preparation of SDS-PAGE samples

You should prepare the following gel samples: pre-induction, post-induction, and all seven Ni-NTA column elution samples.

To prepare the pre- and post-induction samples, thaw the saved pellets at room temperature, and add 24 μL of the 6X sample loading buffer to each pellet. For the elution samples, combine 20 μL of each sample with 4 μL of the 6X gel loading buffer in 1.5-mL Eppendorf tubes. Boil all of the samples for 3 to 5 minutes to fully denature the proteins. The samples can be stored at room temperature until they are used in Session 7.

SESSION 7

Note: You have the option to combine Sessions 7 and 8 into one laboratory day if you would prefer one long session instead of two short sessions.

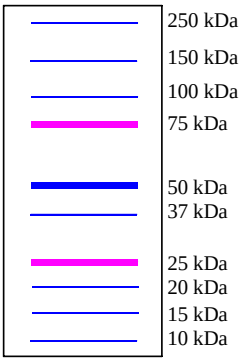
During Sessions 7 and 8 you will analyze the purified H396P Abl(229-511) by SDS-PAGE gel electrophoresis. You will also determine the concentration of your protein domain after purification and dialysis.

1.) Running an SDS-PAGE gel

Remove your cast gel from the storage container, remove the white strip of tape from the bottom of your gel, and place the cassette in the gel running apparatus. Two gels fit in a gel box, so two groups of students should use each box. Fill the apparatus with 1X electrophoresis (TANK) buffer such that the section between the two gels is filled to the top and the outer sections are filled halfway with buffer. Carefully remove the gel comb. Using gel-loading pipette tips on a 20p pipette, load 5 uL of protein ladder (provided by your TA) into a corner well of your gel. The protein ladder consists of proteins with known molecular weights conjugated to visible dyes and does not need to mixed with the sample loading buffer. See the posting on the -20 freezer or the figure below to identify the molecular weight markers in the protein ladder. In the next two wells, load 10 uL of the pre and post-induction samples. In the final 7 wells, load your Ni-NTA elution samples. Run the gel at 200 V until the blue dye reaches the bottom of the gel (approximately 45 to 60 min).

Once the gel running is complete, disassemble the gel apparatus, and carefully pry the cassette apart with a spatula. Transfer the gel into a container for staining. Running a light stream of water over the gel can help prevent gel tearing during cassette separation and gel transfer. Add enough Coomasie blue staining solution to cover the gel (10 to 25 mL), and place the container on the gel rocker. Rock the gel in the stain for 30 minutes. Pour the used staining solution into a designated bottle, and rinse the gel with water. To destain, cover the gel with fast destaining solution. Rock the gel at room temperature for 30 to 50 min, then replace the fast destain with the slow destain solution and rock the gel overnight. For the overnight destain, cover the container with saranwrap to avoid excessive evaporation (especially during the winter months).

Bio-rad precision plus
Molecular weight markers



SESSION 7B

After destaining, pour the destain solution in a designated waste bottle and check your gel for protein bands. You should rinse the gel with water and take a digital picture for your laboratory report. Once you have a decent picture of your gel, you may discard it.

SESSION 8

1.) H396P Abl(229-511) Protein Concentration

Using a syringe, transfer the dialyzed protein solution from the dialysis cassette into a 15-mL conical tube. The cassette cannot be reused and should be discarded after use.

Concentrate the protein solution to a final volume of approximately 500 µL using a Millipore 10-KDa MWCO centrifugal concentrator. For concentration, add up to 12 mL at a time of your dialyzed protein solution to the concentrator tube and replace the cap. Place the concentrator tube with the volume gradations facing up in the centrifuge. Add a counterbalance, either another group's sample or a 50-mL conical tube with the appropriate volume of water to balance your concentrator tube. Spin at 500 x g until you reach the desired volume, approximately 15 to 45 minutes. Use a pipette to recover your concentrated protein from the filter unit in the concentrator tube. For maximum protein recovery, withdraw the protein from the bottom of the filter unit and use a "side-to-side sweeping motion". You should remove the concentrated protein solution as soon as possible following centrifugation.

Store your protein in a clearly labeled eppendorf at 4 °C for use in Sessions 13 and 14.

2.) Protein Quantification

Use the Bio-Rad quantification assay (Session 4) to determine the protein concentration of the H396P Abl kinase domain after purification, dialysis, and concentration. See Appendix A1 for a review on preparing your BSA samples from the 1 mM BSA stock you made in Session 4.

Clearly label the dialyzed protein with your names, your TA's name, and the date. Store the solution at 4 °C.

SESSION 9

During laboratory Sessions 9 and 10 you will perform site-directed mutagenesis to construct the DNA for a mutant Abl kinase domain with a single amino acid substitution. You will transform cells for subsequent isolation of your mutant DNA.

1.) Preparation of mutagenesis primers

For the mutagenesis reactions 10 µM solutions of your forward and reverse primers should be prepared with ddH_20. Before opening the primer containers, which contain your primer DNA as lyophilized power, briefly centrifuge the tubes for 30 to 60 seconds. Calculate and add the necessary amount of water to achieve a 10 µM solution, and store your primer solutions at 4 ºC short term and at -20 °C long term.

2.) Amplification of the mutant plasmid by PCR

You will set up a series of PCR reactions with various concentrations of DNA template (your isolated plasmid DNA) to produce plasmids containing the desired base-pair substitution. By varying the amount of DNA template, you will determine the reaction conditions that ultimately produce colonies with the least amount of template DNA possible, which maximizes the likelihood of isolating a colony with the desired mutation. While there must be enough template DNA to facilitate replication, too much template can result in incomplete digestion after the PCR, resulting in undesired amplification of the wild type DNA. Also essential for successful mutagenesis is the use of PfuTurbo as the DNA polymerase. Unlike standard PCR reactions, which require replication of a 1 to 2 kb insert, mutagenesis requires replication of an entire plasmid, which can be 5 or more kb (the pEt plasmid is approximately 6.1 kb with the Abl insert). PfuTurbo is used for its high fidelity and ability to extend very long templates.

In each of three sterile PCR tubes, add the following components in the order listed, where x = the volume of miniprep elution added (in µL) to give 10 ng of DNA.

	10 ng DNA	**20 ng DNA**	**50 ng DNA**
10X Pfu buffer	5 µL	5 µL	5 µL
template plasmid DNA	x µL	2x µL	5x µL
10 µM forward primer	1.2 µL	1.2 µL	1.2 µL
10 µM reverse primer	1.2 µL	1.2 µL	1.2 µL
10 mM dNTP mix	1.0 µL	1.0 µL	1.0 µL
ddH_20	$40.6 - x$ µL	$40.6 - 2x$ µL	$40.6 - 5x$ µL
Immediately before starting the PCR machine, add:			
PfuTurbo, 2.5 units/ µL	1.0 µL	1.0 µL	1.0 µL
Final volume	50 µL	50 µL	50 µL

Mix the reactions gently by pipetting 4-6 times, and place the tubes in the thermal cycler. The length of the PCR program is critical for successful mutagenesis because the Pfu

polymerase requires ample time to extend around the full plasmid. Your TA will demonstrate how to set the following PCR program: 95 °C for 30 sec; [16-20 cycles of 95 °C for 30 sec: 55 °C for 1 min: 65 °C for 2 min per kb plasmid]; then hold at 10 °C. Since the plasmid you are using is approximately 6 kb, each 65 °C extension will be for 12 min. The class should load the PCR reactions in the thermal cycler together and the TA will start the run. The run will take over 4 hours, and the reaction tubes may be left overnight in the cycler after the run is complete.

SESSION 9B

1.) Remove your reaction from the thermal cycle and store at 4 ºC.

SESSION 10

1.) DpnI digestion of the PCR reaction

As described in the Quikchange mutation overview (in Session 3), the purpose of DpnI treatment is to digest away the template (wild type) DNA, such that the only remaining plasmid is the mutation-containing PCR product. Sufficiently high DpnI concentration and digestion time are essential for complete digestion of the template DNA in order to prevent carrying over the wt plasmid into the transformation, which could result in the isolation of wt, instead of mutated, DNA. For each 50-µL PCR reaction, transfer 10 µL of the crude reaction into a fresh tube. Add 1 µL of DpnI (10 units/ µL) and mix thoroughly by pipetting 4-6 times. Transfer the 10-µL reaction to a new tube to ensure that all the DNA in the tube has been exposed to Dpn1. Incubate the digestion at 37 °C for at least 1 hour. The crude digestion product can be used directly in a transformation reaction. Extra PCR and digestion products should be labeled and stored at -20 °C.

2.) Preparation of LB/kan agar plates

While the DpnI digestion incubates, prepare LB/kan agar plates for your transformation reaction. To prepare 4 plates, weigh out 5 g of LB powder and 3 g of bacto-agar in a 200-mL flask. Add 100-mL of water, and cover the top of the flask with aluminum foil. Add a piece of autoclave-indicator tape to the side of the flask and autoclave the solution for 20 min on the liquid cycle. Several groups should autoclave their media in a single autoclave run to avoid extra waiting time. Once autoclaved, allow the solution to cool to approximately 55 ºC, such that you can hold the flask comfortably. Add 100 µL of 1000X kan to give a final concentration of 50 µg/ mL, and swirl the flask to mix. Immediately transfer 25-mL aliquots of the mixture to cell culture plates using a 100-mL pipette. Allow the agar to solidify for at least 15 minutes before using. Store any unused plates at 4 ºC.

3.) Transformation of DH5α cells

Bacterial transformation involves the uptake of foreign DNA into a cell for subsequent expression. While a small percent of bacterial species (approximately 1%) have the genetic capacity to take in outside DNA through the cell membrane, transformation is commonly achieved using procedures that render bacterial cells passively permeable by heat shock or electroporation. In this procedure, you will use heat-shock competent cells, which work best with plasmid DNA, for your transformation. Heat-shock competent cells are prepared by incubating the chilled cells with divalent metal ions to increase their permeability. After incubation with plasmid DNA, briefly heating the cells results in uptake of the foreign plasmid. Careful timing is essential to maximize DNA incorporation while minimizing cell destruction.

Plates should be placed in the 37 °C incubator with the LB/agar side up.

I. For each transformation reaction, thaw a single 30-uL aliquot of heat-shock competent DH5α cells in an ice bucket. Today you will set up a total of four transformation reactions: three for the mutagenesis products and one as a positive control with your isolated wt plasmid DNA. Competent cells cannot be refrozen for later use, so please do not remove more aliquots then you will use from the -80 ºC freezer.
II. Keep your plasmid DNA from the DpnI digestions and your miniprep of wt Abl DNA chilled in the ice bucket
III. For each transformation reaction, add 25 μL of cells and 1 μL of DNA (either from the crude Dpn1 digestion mixture of from your miniprep) in a 1.5-mL sterile eppendorf tube on ice. Typically, the DNA samples used are 0.5 to 1 μg/ μL. Mix the cells gently by stirring with the pipette tip.
IV. Heat shock the cells by placing the tubes in a 42 °C water bath for exactly 45 seconds. Use a timer to ensure that the cells are not overexposed.
V. Immediately transfer the tubes to an ice bucket. Incubate the cells on ice for 2 minutes, then add 500 μL of SOB to rescue the cells.
VI. Transfer the cell solutions to cell culture tubes.
VII. Incubate the tubes at 37 °C in a shaker (220 rpm) for 30-60 minutes. 30 min. should be adequate for routine transformations, such as this one.
VIII. For each cell solution, plate 200 μL onto a room temperature or 37 °C LB/kan agar plate that has been divided into 4 sections by marker on the bottom of the plate. Streak the plate as shown by your TA across the four quadrants. Label each plate as mutant or wt, specify the point mutation on the mutant plates, and include the amount of DNA template used for the mutant reactions. Write your name and your TA's name on each plate as well.
IX. Incubate the plates overnight in the 37 °C incubator.

SESSION 10B

Pick individual colonies to grow up for DNA isolation (miniprep)

Add 3 mL of autoclaved LB solution (from your 100-mL preparation in week 1) into each of 3 cell culture tubes. Confirm that your LB media is contamination-free by checking that the solution is clear. Add 3 μL of a 1000X (50 mg/ mL) kan stock solution to each tube to give a final concentration of 50 μL/mg kan.

Remove your LB-kan agar plates from the 37 ºC incubator. Confirm that your wt Abl transformation was successful in generating hundreds or thousands of colonies on the designated plate. Estimate and record the number of colonies on the plates transformed with DNA from your mutagesis with 10, 20, or 50 ng of template DNA.

Using a sterile pipette tip, transfer a colony to a cell culture tube from the mutant DNA plate with bacteria transformed with the mutagenesis mixture using the least amount of template DNA. For example, if you have colonies on all of your plates, use the plate from the mutagenesis reaction with 10 ng of DNA. This further decreases the likelihood of selecting a colony that contains wt DNA.

Select 2 more colonies from the same mutant DNA plate, and inoculate the solutions in the other 2 cell culture tubes. Be careful to never mix colonies, since each colony contains a different DNA clone and mixing could result in combining wt DNA with DNA containing the desired mutation.

Place the culture tubes in a 37 °C shaker overnight. During Session 11 you will isolate the plasmid DNA from the 3 selected colonies.

For any groups that experimented with a new point mutation (that hasn't been optimized for this lab), you may or may not have colonies from your mutant DNA transformations. If you are not in one of the lucky groups with colonies, you should pick a colony from another group's plate and record your new target mutation. Quikchange mutatgenesis can require optimization and does not work for every mutation imaginable, and your grade will NOT be affected by whether or not your mutagenesis succeeded, assuming your primer design and Quikchange procedure were competently executed.

SESSION 11

Today you will isolate your mutant DNA from the transformed cells. You will then design primers (or use T7 primers) to sequence your mutant DNA and prepare samples for sequencing.

1.) DNA isolation of mutant plasmid DNA

Isolate the DNA from your three selected colonies. A general miniprep procedure can be found in Appendix A3 and is included below. Note that this is the same procedure that you carried out for isolation of the wt Abl(229-511)-encoding plasmid DNA in session 2. Based on your skillful lab work and a little luck, at least one of these plasmids should contain the desired mutation.

DNA isolation from a bacterial pellet (miniprep)
http://www1.qiagen.com/literature/handbooks/PDF/PlasmidDNAPurification/PLS_QP_Miniprep/1043788_HB_QIAprep_122006.pdf

a.) Harvest the bacterial cells from your 3-mL overnight culture by transferring 1.5 mL into a 1.5 mL eppendorf tube and spinning down the cells in a microcentrifuge for 3 minutes. Discard the supernatant and add the remaining 1.5 mL of cell culture to the tube. Repeat the centrifugation and discard the supernatant. You should have a small bacterial pellet at the bottom of the tube.
b.) Check that RNaseA has been added to Buffer P1. Add 250 µL of Buffer P1 to the cell pellet and completely resuspend the pellet by vortexing.
c.) Add 250 µL of Buffer P2 and mix by inverting the tube 4-6 times. Do not vortex, since that can cause shearing of the DNA. If you have properly lysed the cells, the cell suspension will turn blue after the addition of the P2 buffer. If there are colorless regions or brown clumps in the cells, continue mixing until a homogenous blue solution appears.
d.) Add 350 µL of Buffer N3 and mix immediately by inverting the tube 4-6 times. The solution should become colorless and cloudy.
e.) Centrifuge the tube for 10 min at 13,000 rpm in you bench top microcentrifuge. A compact white pellet should form.
f.) Apply the resulting supernatant (which contains the plasmid DNA) to a QIAprep spin column by decanting or pipetting.
g.) Centrifuge for 30-60 s. Discard the flow through.
h.) Wash the spin column by adding 0.75 mL Buffer PE and centrifuge for 30-60 s. Discard the flow through.
i.) Centrifuge for an additional 1 min to remove residual wash buffer. This is essential for the success of any future enzymatic reactions.
j.) Place the QIAprep spin column into a clean and labeled 1.5-mL microcentrifuge tube. To elute DNA, add 50 µL of Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the spin column, let stand for 1 min, and centrifuge for 1 min.

For long term storage, keep purified DNA at -20 °C.

2.) Quantification of DNA concentration

Calculate the concentration of each of your three miniprep elutions. For each sample, dissolve x µL (typically, 1-3 µL) in 100 µL of water. Measure the absorbance at 260 nm in a quartz cuvette. See Section **Appendix A2** for UV/Vis spectrophotometer instructions. To calculate the concentration in µg/µL of your double stranded (ds) DNA, multiply the Abs260 by (0.05)(100/x). Typical concentrations of miniprepped DNA are 0.1-2 µg/µL.

Calculate the concentration of each of your three miniprep elutions, as was done previously for your wt Abl(229-511)-containing plamid. See Appendix A4 for instructions on measuring and calculating DNA concentration. You will use some of the miniprepped DNA for sequencing and should store the remaining DNA, labeled with the desired mutation and numbered 1-3 at -20 °C.

3.) Preparation of DNA sequencing samples

You will send the plasmid DNA isolated from the three selected colonies for DNA sequencing to check for the desired mutation. For each submission, combine the following in a DNA sequencing tube:

3.2 pmol of your sequencing primer
200-500 ng of the plasmid DNA
ddH20 to a final volume of 12 µL

Label each tube with a 3 letter name that is distinguishable from all other samples in the class, and fill in the information on a numbered sequencing sheet for the class. Make note of the numbers of your samples as well as the names, since the sequencing can come back with numbers only.

SESSION 12

You will be assigned one or several of the following buffers, all of which will be used for the coupled phosphorylation assay in Sessions 13 and 14.

- **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 _____ g Tris, and 0.8 L of room temperature DI water. Adjust the pH to 7.5 with 1.0 M HCl. Add water to a final volume of 1 L.
    2. Next prepare 10 mL of an approximately 3 M solution of $MgCl_2$-$6H_2O$ (FW 203.31) in 0.1 M HCl. Record the actual concentration of your solution. The 0.1 M HCl keeps the solution acidic to prevent the $MgCl_2$ from crashing out of solution during storage.
    3. Prepare 10 mL of the 10 x kinase buffer (to avoid $MgCl_2$ crashing out at neutral pH, do not store it for more than a few weeks). For example, if the $MgCl_2$ solution was 2.66 M, you should add 385 uL of 2.66 M $MgCl_2$ to 9.615 mL of the 1 M Tris buffer, pH 7.5. The Tris concentration will be only negligibly less than 1 M.

- **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 _____ 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). Be careful NOT to overshoot the pH! If the solution becomes too basic, the ATP will hydrolyze and will be useless in the upcoming kinase assay. If you overshoot the pH, you should discard your solution and start over.

    After reaching a pH of 7.5, add water to a final volume of 9 mL. Determine the exact pH of the ATP solution by absorption spectroscopy. The apparent weight of the ATP is likely to include some water, which results in a lower than expected concentration. Record the Abs at 259 for a 1/4000 dilution of the ATP solution and calculate the actual ATP concentration using Beer's Law (Abs = εcl). Aliquot the ATP solution into 100-uL samples in 0.5 mL-eppendorf tubes and store the labeled-aliqots at -20 ºC.

- **35 mM PEP**. FW = 208.04. Add 100 mg of PEP to 13.7 mL of water. Store the solution in 200-µL aliquots in the -20 °C freezer. **(provided by TA)**

- **12 mM NADH**. (FW of the hydrate: 709.40). Since NADH easily takes on water weight, attempt a 14 mM solution by adding "10 mg"/mL of NADH. In a 15-mL conical tube, add "5 mg" of NADH to 0.5 mL of water. Calculate the actual concentration (which will likely be close to 12 mM) by measuring the absorption at 340 (ε_{340} = 6220 $M^{-1}cm^{-1}$). The pH of NADH solution does not need to be adjusted. Store labeled aliquots at -20 °C**. (provided by TA)**

- **20 mM stock of the substrate peptide**, Ac-EAIYAAPFAKKK-NH2. (MW 1377.6584). For a 900 µL solution of peptide (final volume), add 25 mg (18 µmol) of peptide to a 15-mL conical tube. Add 700 µL of 100 mM Tris, pH 7.5 or water to the lyophilized peptide. The peptide solution will initially be very acidic due to TFA contamination from peptide HPLC purification. To neutralize the solution, add 1 M NaOH until the pH is approximately 7.5. For one previous preparation of this peptide, 9 uL of 1 M NaOH was required for neutralization, but this will differ with every batch of peptide, depending on how much residual TFA is with the peptide. After neutralization, add 100 mM Tris buffer or water to give a final volume of 900 µL. The peptide should be labeled, dated, and stored at -20 ºC in 50-µL aliquots. **(provided by TA)**

Once the buffers above are made, all groups should prepare their own 1x assay buffer to use in Sessions 13 and 14:

Preparation of 2 mL* of 1 x assay buffer

In order to save time and allow you to easily and reproducibly repeat the coupled phosphorylation assay, you should prepare a 1x assay buffer that contains all of the reagents in the kinase assay *except* for the kinase, the substrate peptide, and the PK/LDH (coupling enzymes). Once you make up this buffer, you should store it at -20 °C. It is ok to freeze/thaw the buffer and use it as needed.

** 2 mL refers to the final volume after the addition of the kinase, peptide, and PK/LDH. The volume of the assay buffer alone will be 1.44 mL.*

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

- _____ µL of 10x kinase buffer
- _____ µL of 35 mM PEP for a final concentration of 1 mM
- _____ µL of _____ mM ATP for a final concentration of 2 mM
- _____ µL of ____ mM NADH for a final concentration of 0.11 mg/mL or 155 µM. (Make sure the frozen stock that you use is recent.)
- _____ 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 the assay buffer is not confused with the 10x kinase buffer!

SESSION 13 and SESSION 14

During laboratory Sessions 13 and 14 you will

- Use a coupled phosphorylation assay to determine the specific activity of the **wild type Abl** kinase domain 1) in the absence of an inhibitor 2) in the presence of the drug Gleevec and 3) in the presence of one other Abl inhibitor.
- Use a coupled phosphorylation assay to the specific activity of the **H396P Abl** kinase domain 1) in the absence of an inhibitor 2) in the presence of the drug Gleevec and 3) in the presence of one other Abl inhibitor

Researchers use a variety of strategies to monitor the activity of kinases. You will recall that kinases are enzymes that catalyze the phosphorylation of substrate peptides or proteins by transferring a phosphate group from a molecule of ATP to the substrate. Phosphorylation is spectroscopically silent, which means that we can monitor neither the formation of the phosphorylated product, nor the conversion of ATP to ADP directly using spectroscopy. This is because the products would appear identical to the starting materials due to identical absorption features and extinction coefficients. In order to monitor the activity of your wild type and mutant Abl kinase domains, you will therefore use a coupled assay, which "couples" the conversion of ADP to ATP with the conversion of NADH to NAD+, a process that can be detected by a decrease in absorbance at 340 nm. This coupling assay is outlined in **Fig.3**.

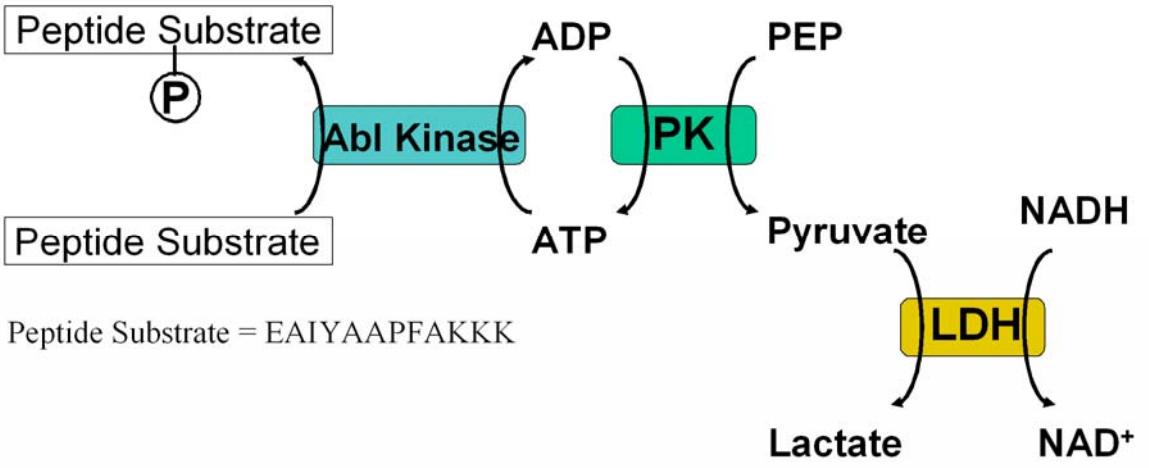


Figure 3. The Kinase Coupled Assay for Abl Activity

It is possible to use the kinase coupled assay to generate quantitative kinetic data on the kinase of interest. You will use this assay to determine the **specific activity** of the wt and mutant Abl kinase domains in the absence and presence of small molecule inhibitors, including Gleevec. Specific activity in biochemistry is defined as the amount of product formed by an enzyme in a given amount of time. Specific activity is often reported in units (U) per mg of enzyme, where 1 unit is equal to 1 µmol of product formed per minute.

1.) Determine the specific activity of the wt Abl kinase domain

To run the assay, you will set up a 100-µL reaction in a quartz cuvette and monitor the decrease in absorbance at 340 nm on the UV/Vis spectrometer. You will record the slope of the resulting line (decrease in absorbance over time) and calculate the specific activity of the kinase, as described below.

Combine the following in a 0.65 eppendorf tube:

- 72 µL of the thawed 1x assay buffer
- 8 µL of PK(928 U/mL)/LDH (1300 U/mL) for a final concentration of 74 U/mL // 104 U/mL.
- x µL of Abl kinase domain (typically 2-3 µL for wt Abl- check with your TA)
- 15-x µL of water

Gently mix the solution with tapping and with a pipette, and then transfer the mixed solution to a 100-µL capacity quartz cuvette. Record the initial slope for a background reading. *Note that for a proper reading the meniscus must not be visible in the cuvette window and the cuvette must be pushed down all the way in the holder.* Then add

- 5 uL of 20 mM peptide (for a final concentration of 1 mM) to start the reaction.

Let the reaction run for approximately 5 to 10 minutes. Record the slope and the R^2 value. Subtract your background slope from the slope after substrate addition to determine the slope (change in absorption per minute) from kinase activity.

Now you can calculate the specific activity of the Abl kinase using this slope determined in the coupled phosphorylation assay.

Calculating the Specific Activity (this can be calculated in lab or at home)

$$\text{Specific Activity} = \text{U/mg} = \frac{\dfrac{\mu\text{mol product formed}}{\text{min}}}{\text{total mg of enzyme added}}$$

To find specific activity, you need to determine two values: **A)** the amount of product (phosphorylated peptide) formed per minute (in µmol/min) by the Abl kinase and **B)** the amount of Abl enzyme (in mg) that was used in the assay.

A) Calculate the amount of product (phosphorylated peptide) formed per minute.

Using Beer's Law (Abs = εcl) and the slope determined in the assay, calculate the concentration of product formed per minute:

Beer's Law can be written: $\Delta A/\text{min} = (\Delta c/\text{min})(l\ \varepsilon)$

where, $\Delta A/min$ = change in absorption per minute = |slope|

l = the path length of the cuvette = 1 cm

ε = the extinction coefficient of your chromophore (here NADH)
ε of NADH at 340 nm = 6,220 $cm^{-1}M^{-1}$

$\Delta c/min$ = change in concentration per minute. Solve for this value.

Solving for $\Delta c/min$ gives the concentration of product formed in units of M/min. Multiply this value by 10^6 to get an answer in units of µM/min.

Remember that M = molarity (mol/L). Since the answer should be in terms of µmol/min, NOT µM/min, we need to convert µM to µmol. To do this, multiply your answer by the total volume of the reaction.

Now you have an answer in terms of µM/min, or units (U).

B) Calculate the amount of enzyme used in the assay.

The Calbiochem wild-type Abl has a concentration of 0.01 mg/mL. If you used 3 µL of this kinase stock, the total amount of enzyme in the reaction would be 0.00003 mg. If you used a different stock of enzyme, use the concentration and the volume added to determine the amount of enzyme (in mg).

Now you can divide your µM/min of product formed by the total mg of kinase to determine your specific activity.

2.) Determine the specific activity of the wt Abl kinase domain in the presence of 1 µM Gleevec.

Follow the procedure described in part 1, but include Gleevec in the reaction mixture:

Combine the following in a 0.65 eppendorf tube:

- 72 µL of the thawed 1x assay buffer
- 8 µL of PK(928 U/mL)/LDH (1300 U/mL) for a final concentration of 74 U/mL // 104 U/mL.
- x µL of Abl kinase domain (typically 2-3 µL for wt Abl- check with your TA)
- 1 µL of a 100 µM solution of Gleevec dissolved in DMSO
- 14-x µL of water

Gently mix the solution with tapping and with a pipette, and then transfer the mixed solution to a 100-µL capacity quartz cuvette. Record the initial slope for a background reading. Then add

- 5 uL of 20 mM peptide to start the reaction.

3.) Determine the specific activity of the wt Abl kinase domain in the presence of another kinase inhibitor based on the procedures provided in part 1.) and 2.). Record the name and volume of inhibitor added and the final concentration of inhibitor in the reaction mixture.

4.) Repeat steps 1.) through 3.) with your H396P Abl(229-511). For the x µL of Abl kinase solution, start with x = 10 µL for H396P Abl (double check this value with your TA).

SESSION 15

Session 15 is the final laboratory session in Module 5. Journal Club presentations will take place during Tuesday/Thursday 12-1 lecture times in April.

Today you will

- Analyze your sequencing data from the site-directed mutagenesis.
- Use the *PyMol* structure viewing program to view wild type and mutant Abl crystal structures, and complete the structure viewing worksheet.

1.) Discuss the results of the activity and inhibition assays as a class.

2.) Analyze your sequencing data for the site-directed mutagenesis using DNA strider or another DNA sequencing program. Print out a copy of the DNA analysis for your final report.

3.) Use PyMol to analyze crystal structures of Abl binding to inhibitors and complete the structure viewing worksheet.

STUCTURE VIEWING

For this exercise you will use the structure-viewing program PyMol to analyze crystal structures from the protein data base (PDB) of the Abl kinase domain bound to various small-molecule inhibitors.

You will view the following crystal structures:

- PDB entry **1IEP**: wt Abl kinase domain bound to Gleevec
- PDB entry **2GQG**: wt Abl kinase domain bound to Dasatinib
- *Optional…* PDB entry **2F4J**: the (Gleevec-resitant) H396P mutant of the Abl kinase domain bound to the kinase inhibitor VX-680

You will need to download the PDB file for each structure. Go to www.pdb.org, and in the top search bar type the 4-character ascension code (ie. **1IEP**) for the desired structure.

Once you are on structure page, on the left hand column under "Download Files" choose "PDB text" and save the file somewhere you will be able to find it later. Do this for all three structures.

To access PyMol from an athena terminal, type **add pymol** at the prompt. Then type **pymol &** to start running PyMol.

Please keep the following points in mind as you view these structures

- The wt structures **1IEP** and **2GQG** each include two independent molecules (meaning that two kinase domains are in each crystal, and that the two are not interacting) in addition to the small molecule inhibitors. In viewing these

structures, you will select a single copy of each kinase domain ("chain a" to observe.

- The numbering of the Abl structures is identical to that in your 5.36 lab manual.

Please type or neatly write out answers to the following questions on a separate sheet of paper (complete sentences are not required). This should be turned in with your final lab report. There are a total of 22 questions.

Secondary Structure

First, we will look at the overall secondary structure of the Abl kinase domain. For this part of the exercise you should use the **1IEP** structure.

In the **File** menu, choose **Open** and choose the PBD file you downloaded previously. This will open the molecule in the viewer window.

Here are some instructions to help you work with this structure in PyMol:

- Under the **Display** menu choose **Sequence**, this will display the sequence at the top of the viewer window. You can choose residues in the sequence by clicking on them here, and they will be highlighted in the viewer window.
- In the command line, type **select chain a, Chain a**. A tab called "Chain a" will show up in the right hand column of the viewer window. Selecting an individual chain will allow you to view a single copy of the kinase domain, rather than view both copies that are present in the 1IEP structure.

Each selection in the right hand column has five menus: Actions, Show, Hide, Label, and color (A,S,H,L,C)

- Next to 1IEP click on **H** (for hide), choose **everything**. This should make the whole structure disappear.
- Next to "Chain A" click on the **S** (for show), and choose **cartoon**. Now the cartoon ribbon diagram of just chain A should appear on your screen.
- To make manipulating this chain easier, in the "Chain A" menu click on **A** (for actions), then **center**. To help orient yourself, you may want to turn the molecule around until it is in the orientation of the kinase domains in the Lecture 4 notes.

Other cool things you can do:

- Color by secondary structure: under **C** choose **ss**, choose either color scheme. This can help you locate secondary structure elements.
- Color as a rainbow: under **C** choose **spectrum**, then **rainbow**. This will color the structure from blue at the N-terminus to red at the C-terminus, the sequence at the top will also change color accordingly.

Questions:

1) What type of secondary structure is most prominent in Abl?
2) What type of secondary structure is most prominent in the N-lobe (residues 225-350) of Abl?
3) What type of secondary structure is most prominent in the C-lobe (residues 354-498)

of Abl?

4) The protein contains a five-stranded β-sheet. Is this sheet located at the C or N terminus of the kinase domain?
5) What residues comprise this beta-sheet? For this answer do not include the helix that is between the strands, so you should list two ranges. (For example "S229-G250 and L340-A350" would in the correct form- although this is *not* the correct answer). It may be helpful to zoom in on your structure to answer this question.

One can also evaluate the type of residue present in different parts of a protein. In this case it may be easier to look at the structure as a stick model and not a ribbon model.
In the "Chain A" menu show (**S) sticks** and hide (**H) cartoon**.

To color the structure by residue type in the command line type
color white, resn val+trp+..... (type in the three letter codes for each residue of the type you are coloring)

Using the command above, color all of the hydrophobic residues white (leave out glycine). Remember to include the aromatic residues for a total of 9 hydrophobic amino acids.

Questions:

6) Where are most of the hydrophobic residues located, on the inside or the outside of the protein? (You may want to switch back and forth from viewing the sticks to the cartoon form to get a better view of this.)
7) Where are most of the polar residues located?
8) Does this make sense in terms of protein folding? Explain.

Molecular Details

Now we will look more closely at specific regions and residues in the 1IEP structure to understand the molecular basis for inhibition of Abl by Gleevec and how mutations can confer Gleevec resistance.

- If your structure is currently in the stick form, switch back to the cartoon form. In the "Chain A" menu hide (**H) sticks** and show (**S) cartoon**.
- To reset the color of your structure in the Chain A tab choose **color, by element** and choose the first coloring scheme, then choose **color, by chain** and choose the first coloring scheme.

Binding of Gleevec

Now let's look more closely at the binding of Gleevec to the Abl kinase domain.

- In the command line type: **select Gleevec, organic**. Press enter. A new tab will appear on the right hand menu. This is the menu that controls the Gleevec molecule.
- For Gleevec, change the color to a different color from Abl. For example choose

color and select **orange**. Next go to **color**, **by element** and choose the first coloring scheme.

Although Bcr-Abl is constitutively active, it may adopt an activated state or one of many inactivated states. Consider Gleevec binding in relation to the activation loop (A loop) of the Abl kinase domain. The A loop is comprised of Abl residues 381-402.

- In the command line type: **select Aloop, resi 381-402**. Press enter. A new tab will appear on the right hand menu to control the A loop.
- Highlight the A loop with a new color. Under the "Aloop" tab, select **color** and then **magenta**.
- Select the conserved DFG (Asp-Phe-Gly) motif within the A loop by typing: **select DFG, resi 381-383**. Press enter.
- In the "DFG" menu on the right of the screen, select show (**S)** as **sticks**.

Questions (It may be helpful to refer to pages 3 and 4 of lecture #4 notes):

9) When bound to Gleevec, is the A loop of the Abl kinase domain in the open (extended) or closed conformation? Very briefly explain your answer.
10) When bound to Gleevec, is Abl kinase domain in the active or inactive form. Very briefly explain your answer.

<u>Hydrogen bonds in the active site</u>

Gleevec binds in the ATP-binding pocket, the active site, of Abl. One important thing to note is that the resolution of an X-ray crystal structure is not high enough to see hydrogen atoms, so they are not included in the model. To assign hydrogen bonding interactions, which are important in substrate binding, you must read beyond what the structure can tell you and use your chemical knowledge.

It may be easier to see molecular interactions if you restrict the amount of atoms you see on the screen.

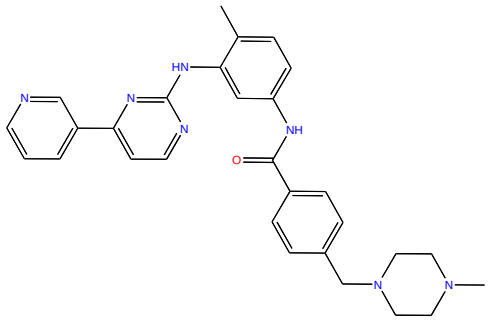
- In the command line type: **select bindingpocket, (Gleevec expand 8)**. This will select all atoms within 8 angstroms of the Gleevec molecule and make a menu for it in the right hand menu column called "bindingpocket". *Note: do not use spaces in your selection names. Also, PyMol is case sensitive so if you named the selection "Gleevec" it will not understand "gleevec".*
- In the Chain A menu choose **H**, **everything**.
- In the bindingpocket menu choose **S, sticks** or **lines** (sticks are thicker than lines).

To measure distances between bonds, click on the **Wizard** menu (at the top of the command line window) and choose **measurement.** In the window it will prompt you to click on the first atom. Follow the prompts to measure distances as needed to answer the following questions.

In looking for hydrogen bonds, make sure the atoms you are measuring are atoms that could be hydrogen bonding partners, and remember that reasonable H-bonding distances

are 2.4 - 3.0 Å.

If your screen is getting cluttered with distance values, click "**Delete all Measurements**" in the right hand menu. When you are done measuring distances, click **Done**.

Gleevec 

Questions:

11) What groups on the structure of Gleevec above could participate in hydrogen bonds? (Draw Gleevec and indicate the groups by circling).

12) Looking at the Abl structure, name the residues that appear to hydrogen bond to Gleevec and give the distances between the atoms. Draw these on your Gleevec structure. *Hint: there are 5 possible hydrogen bonds within 3.0 Å .*

Note that only *new* hydrogen bonds make inhibitor binding energetically more favorable. H-bonds with an inhibitor that result from breaking H-bonds among residues within the binding pocket or with coordinated water do not significantly stabilize inhibitor binding.

13) Name two other interactions that may be stabilizing the inhibitor in the active site.

Let's now consider the most common mutation site found in cases of Gleevec-resistant CML, site 315.

14) What amino acid is at this site, and what sort of interaction does this particular residue have with the inhibitor?

15) What might you predict would happen if this residue were mutated to Asn or Ile?

16) What if this residue were mutated to Ala?

Now consider residue 315 in the structure of Abl complexed with Dasatinib, the other inhibitor you tested in the Session 13/14 kinase activity assays. For this we will use the structure with PDB **ID: 2GQG**. In this structure there are again two molecules in the file, so you should select a single chain to look at.

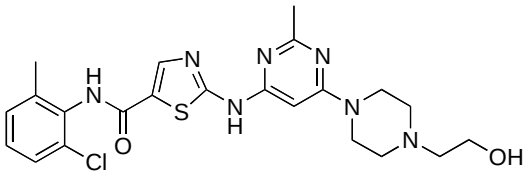
Close the **1IEP** structure and open **2GQG** in the viewer window.

- Under the **Display** menu choose **Sequence**.
- Type **select chain a, Chain a**. Next to "2GQG" click on **H**, and choose **everything**. Next to "Chain A" click on the **S**, and choose **cartoon**.
- As with the Gleevec-bound structure, to make manipulating this chain easier, click on **A**, then **center** in the "Chain A" menu.

Once you have opened the structure, consider the inhibitor-binding site. This is the same site Gleevec binds to, but Dasatinib binds in a different way.

Select the inhibitor (Dasatinib) by typing **select Dasatinib, organic** and pressing enter. Change its color so that you can distinguish it from the protein. (See the directions above for Gleevec if you do not remember how to do this.)

As a reference, here is a structure of the Dasatinib:



Dasatinib (Sprycel, BMS-354825)

Look at residue 315 of Abl.

Highlight this residue on the protein by clicking on the corresponding "T" in the sequence display at the top of the screen. The chosen residue (315) will be highlighted, and in the right hand menu there will be a tab labeled "sele". This tab controls whatever you have selected in the viewer window. Use this tab to display residue 315 in stick form and change the color. Refer to detailed instructions from above to manipulate the residue as needed to answer the following questions.

17) Is residue 315 interacting with Dasatinib? If so, describe the interaction.

18) Would you expect Dasatinib to effectively inhibit an Abl mutant with residue 315 mutated to an Ile? Very briefly explain why or why not.

<u>Conformational considerations</u>

Now consider Dansatinib binding in relation to the activation loop of Abl kinase.

- As you did with the previous (Gleevec-bound) structure, select the activation loop by typing **select Aloop, resi 381-402** and pressing enter.
- Next to the "Aloop" tab, select **color** and then **magenta**.
- Select the conserved DFG (Asp-Phe-Gly) motif within the A loop by typing: **select DFG, resi 381-383**. Press enter.
- In the "DFG" menu on the right of the screen, select show (**S)** as **sticks**.

19) When bound to Dasatinib, is the A loop of the Abl kinase domain in the open (extended) or closed conformation? Very briefly explain your answer.

As you may recall from Lecture #4, Tyr 393 within the activation loop mimics the target tyrosine (to be phosphorylated) on the peptide or protein substrate, and it binds in the substrate-binding site of Abl. When Tyr 393 is phosphorylated, it can no longer bind in this site. Tyr393 is often phosphorylated in the active form of a kinase, but is never phosphorylated in the inactive form.

Find residue Tyr393 in the Dasatinib-bound Abl structure. Note the location of this residue in this structure. (Residue 393 is listed as "PTR" instead of "Y". "PTR" stands

for “phosphoryl tyrosine”, although the phosphoryl group cannot be seen in the structure.)

We can get a better look at conformational differences, such as with the Abl A loop, by making an overlay of the two structures.

With the **2GQG** structure already loaded, open the 1IEP structure in the same viewer window. Display both molecules as cartoons. In the command line type **align 1IEP, 2GQG.**

Locate residue 393 in both structures. It may help to view the structures in cartoon mode (like above) then display atoms only for the desired residues by selecting residue 393 in both molecules and to the right of **(sele)** click, **S**, **sticks**. You can also highlight the A loop of each structure in a different color.
20) How does the location of residue 393 differ in the two structures?

Find the inhibitor in each structure (for example by typing **show sticks, organic**) in the command line.
21) Very briefly compare the binding orientation of Gleevec and Dasatinib.

22) The H396P mutation in Abl destabilizes the inactive conformation of the kinase. Based on the Gleevec-bound and Dasatinib-bound structures of wt Abl, explain how your laboratory results from the kinase activity assays with wt and H396P Abl are consistent (or not consistent) with these structures. (If you have not yet completed Session 14, predict what you would expect to see in the H396P assays based structural evidence. Compare this to your actual results after completing your assays.)

*Optional: If you are interested in exploring the structure of the H396P mutant, you can bring up the PDB file **2F4J**, which is H396P Abl bound to another inhibitor, VX-680. This is NOT required to answer any of the questions above.*

List of Abbreviations:

Abl (or c-Abl)	Abelson kinase
Abl(229-511)	the Abelson kinase domain, which is constitutively active for kinase activity when expressed as an isolated domain
APS	ammonium persulfate
bp	base pairs
EtBr	ethidium bromide
IPTG	Isopropyl-*ß*-D-thiogalactopyranosid
kan	kanamycin, an antibiotic
kb	kilobase pairs
MW	molecular weight (reported in g/mol)
OD600	optical density (absorbance) at 600 nm
RE	restriction enzyme
strep	streptomycin, an antibiotic
TAE	tris acetate electrophoresis
TBS	tris buffered saline
T_m	melting temperature
TEMED	etramethylethylenediamine
wt	wild type
w/v	weight/volume

APPENDIX A: Common biochemistry laboratory procedures used multiple times in URIECA modules 4 and 5.

A1: Bio-Rad assay for quantifying protein concentrations
http://www.fhcrc.org/science/labs/hahn/methods/biochem_meth/biorad_assay.pdf

First prepare bovine serum albumin (BSA) solutions to use as standards. To prepare 5 mL of a 1 mg/mL aqueous solution of BSA, weigh out 5 mg of BSA into a 15-mL conical tube, add 5 mL of water, and invert or vortex the tube to completely dissolve the BSA. BSA aliquots may be stored long term at -20 °C. Prior to each assay, prepare five dilutions of the BSA stock solution to create a standard curve. The linear range of the Bio-Rad protein assay is 0.2 to 0.9 mg/mL. Therefore in six separate 0.65-mL eppendorf tubes, label and add the following: 270 µL of the BSA stock and 30 µL of deionized water (to give 0.9 mg/mL), 210 µL of BSA and 90 µL of water (to give 0.7 mg/mL), 165 µL of BSA and 135 µL of water (to give 0.55 mg/mL), 120 µL of BSA and 180 µL of water (to give 0.4 mg.mL), 60 µL of BSA and 240 µL of water (to give 0.2 mg/mL), and no BSA and 300 uL of water (to use as your "blank").

Prepare 20 mL of the assay dye reagent by diluting one part Bio-Rad dye concentrate with four parts DI water. Filter the resulting solution and store any unused reagent for up to two weeks at room temperature. To create a standard curve using the BSA standards, pipette 20 µL of each standard into an eppendorf tube. Add 980 µL of the prepared dye reagent to each tube and vortex briefly. Incubate the tubes at room temperature for 5 to 50 minutes. Measure the absorbance of each solution at 595 nm by UV/Vis spectrometry using disposable polystyrene cuvettes. The sample with 20 µL of water in 980 µL of assay reagent should be used as your "blank" in the spectrophotometer. Plot the absorbance vs. concentration for the BSA standards using Microsoft Excel or another graphing program and determine the R-squared value and slope of the best-fit line to establish that you can obtain a strait line with your standards. If your R-squared value is less than 0.9, repeat the exercise until you achieve an acceptable line. Good laboratory practice calls for the creation of a new standard curve each time you run a protein assay.

To determine the concentration of unknown samples, combine 20 uL of your sample (or an appropriate dilution) with 980 µL of assay reagent and measure the absorbance at 595. Use the slope of the standard curve to calculate the sample concentration in mg/mL.

A2: Varian Cary 100 Spectrophotometer Instructions

1. Turn on the spectrophotometer.
2. Open the "simple reads" program, found as a desktop icon or under the Cary WinUV menu under the "Start" button.
3. The lamps are on if under the "Commands" menu "Lamps Off" is displayed. If "Lamps On" is displayed, click on it to turn on the lamps. For accurate measurements, allow the lamp to warm up for at least 15 minutes before use.
4. To set the instrument, click the "Setup…" command button on the left side of the screen. In the "Read the wavelength" scroll box, type in the desired wavelength.

The "Abs" button in "Y mode" should be selected. When the setup is complete, click the "OK" button to exit the window.

5. Prior to measuring the absorbance of your samples, you must zero the instrument. To do this, open the lid and determine which cell is in the light path. Insert a cuvette with your blanking solution in the instrument. Close the lid completely, and press the "Zero" command button on the left side of the screen to zero the instrument at the chosen wavelength. The blank should now have a absorbance read of zero give or take the small amount of "noise" present at all times.
6. You are now ready to measure the absorbance of your samples. Insert a cuvette containing a sample into the instrument, close the lid completely, and press the "Read" button at the top of the screen. Record the absorbance measurement.
7. Under the "File" menu, select "Print" to print your data.
8. When finished, close the program and return to the desktop.
9. Turn off the lamps, then turn off the instrument.

A3: DNA isolation from a bacterial pellet (miniprep)
http://www1.qiagen.com/literature/handbooks/PDF/PlasmidDNAPurification/PLS_QP_Miniprep/1043788_HB_QIAprep_122006.pdf

Miniprep Procedure:
a.) Harvest the bacterial cells from your 3-mL overnight culture by transferring 1.5 mL into a 1.5 mL eppendorf tube and spinning down the cells in a microcentrifuge for 3 minutes. Discard the supernatant and add the remaining 1.5 mL of cell culture to the tube. Repeat the centrifugation and discard the supernatant. You should have a small bacterial pellet at the bottom of the tube.
b.) Check that RNaseA has been added to Buffer P1. Add 250 µL of Buffer P1 to the cell pellet and completely resuspend the pellet by vortexing.
c.) Add 250 µL of Buffer P2 and mix by inverting the tube 4-6 times. Do not vortex, since that can cause shearing of the DNA. If you have properly lysed the cells, the cell suspension will turn blue after the addition of the P2 buffer. If there are colorless regions or brown clumps in the cells, continue mixing until a homogenous blue solution appears.
d.) Add 350 µL of Buffer N3 and mix immediately by inverting the tube 4-6 times. The solution should become colorless and cloudy.
e.) Centrifuge the tube for 10 min at 13,000 rpm in you bench top microcentrifuge. A compact white pellet should form.
f.) Apply the resulting supernatant (which contains the plasmid DNA) to a QIAprep spin column by decanting or pipetting.
g.) Centrifuge for 30-60 s. Discard the flow through.
h.) Wash the spin column by adding 0.75 mL Buffer PE and centrifuge for 30-60 s. Discard the flow through.
i.) Centrifuge for an additional 1 min to remove residual wash buffer. This is essential for the success of any future enzymatic reactions.
j.) Place the QIAprep spin column into a clean and labeled 1.5-mL microcentrifuge tube. To elute DNA, add 50 µL of Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the spin column, let stand for 1 min, and centrifuge for 1 min.

For long term storage, keep purified DNA at -20 °C.

A4: Quantification of DNA concentration

Dissolve x μL (typically, 1-3 μL) of DNA sample in 100 μL of water. Measure the absorbance at 260 nm in a quartz cuvette. See Section **Appendix A2** for UV/Vis spectrophotometer instructions. To calculate the concentration in μg/μL of your double stranded (ds) DNA, multiply the Abs260 by (0.05)(100/x). Typical concentrations of miniprepped DNA are 0.1-2 μg/μL. To calculate the concentration of single stranded (ss) DNA or RNA, multiply the Abs260 by (0.04)(100/x) instead.

To convert the concentration to mol/L, use the fact that 1 base pair has a molecular weight of ~650 g/mol, so a 5 kb plasmid has a typical molecular weight of 3.3×10^6 g/mol. For ssDNA or RNA, use a molecular weight of 325 g/mol per nucleotide.

APPENDIX B: Protein and Nucleotide Sequences

B1: Protein Sequence of c-Abl(229-511) (283 AA; 32,730 MW) **from ABL1_HUMAN**
Swissprot accession number: P00519 (http://www.expasy.org/uniprot/P00519).
Numbering is for isoform 1A. For isoform 1B numbering, add 19 (Abl(248-530)). We will use 1A numbering throughout this course.

```
                                                (229)SP NYDKWEMERT

       250        260        270        280        290        300
DITMKHKLGG GQYGEVYEGV WKKYSLTVAV KTLKEDTMEV EEFLKEAAVM KEIKHPNLVQ

       310        320        330        340        350        360
LLGVCTREPP FYIITEFMTY GNLLDYLREC NRQEVNAVVL LYMATQISSA MEYLEKKNFI

       370        380        390        400        410        420
HRDLAARNCL VGENHLVKVA DFGLSRLMTG DTYTAHAGAK FPIKWTAPES LAYNKFSIKS

       430        440        450        460        470        480
DVWAFGVLLW EIATYGMSPY PGIDLSQVYE LLEKDYRMER PEGCPEKVYE LMRACWQWNP

       490        500        510
SDRPSFAEIH QAFETMFQES SISDEVEKEL G
```

B2: Nucleotide sequence encoding Abl(229-511)
The nucleotide sequence of the Abl(229-511) construct that we are working with is identical to the kinase domain of the Bcr-Abl protein. The DNA sequence for the kinase domain is shown below.
Genbank accession number for the full human Bcr-Abl protein: NM_005157
(http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=NM_005157)

Note: amino acid 229 corresponds to nucleotides 688-689 (not 685-687 as might be expected) because there are 3 bases prior to the start of the open reading frame (ORF). Before working with any nucleotide sequence, you should confirm that the DNA is in-frame and encodes the expected peptide or protein sequence using a DNA to protein translation tool (ie. http://www.expasy.ch/tools/dna.html).

```
                              (688)tcc cccaactacg acaagtggga gatggaacgc
721  acggacatca ccatgaagca caagctgggc gggggccagt acggggaggt gtacgagggc
781  gtgtggaaga aatacagcct gacggtggcc gtgaagacct tgaaggagga caccatggag
841  gtggaagagt tcttgaaaga agctgcagtc atgaaagaga tcaaacaccc taacctggtg
901  cagctccttg gggtctgcac ccgggagccc ccgttctata tcatcactga gttcatgacc
961  tacgggaacc tcctggacta cctgagggag tgcaaccggc aggaggtgaa cgccgtggtg
1021 ctgctgtaca tggccactca gatctcgtca gccatggagt acctggagaa gaaaaacttc
1081 atccacagag atcttgctgc ccgaaactgc ctggtagggg agaaccactt ggtgaaggta
1141 gctgattttg gcctgagcag gttgatgaca ggggacacct acacagccca tgctggagcc
1201 aagttcccca tcaaatggac tgcacccgag agcctggcct acaacaagtt ctccatcaag
1261 tccgacgtct gggcatttgg agtattgctt tgggaaattg ctacctatgg catgtcccct
1321 tacccgggaa ttgacctgtc ccaggtgtat gagctgctag agaaggacta ccgcatggag
1381 cgcccagaag gctgcccaga gaaggtctat gaactcatgc gagcatgttg gcagtggaat
1441 ccctctgacc ggccctcctt tgctgaaatc caccaagcct ttgaaacaat gttccaggaa
1501 tccagtatct cagacgaagt ggaaaaggag ctgggg
```

B3: Point mutations in the kinase domain of Abl detected in leukemia patients[1]

Amino acid substitution locations in mutant Bcr-Abl are indicated in red with the amino acid substitution(s) in bold directly above the wild type residue:

```
                                                    (229)SP NYDKWEMERT
             HF V
   V   V E   RH K  260         270       GA280          A          300
DITMKHKLGG GQYGEVYEGV WKKYSLTVAV KTLKEDTMEV EEFLKEAAVM KEIKHPNLVQ
             N                                                    A
       310 L   I L320         330         340   T    350 T   G   V
LLGVCTREPP FYIITEFMTY GNLLDYLREC NRQEVNAVVL LYMATQISSA MEYLEKKNFI
                                       R
       370          I   L    F390       PP400         410        Y420
HRDLAARNCL VGENHLVKVA DFGLSRLMTG DTYTAHAGAK FPIKWTAPES LAYNKFSIKS

       430         440         450          K          470         480
DVWAFGVLLW EIATYGMSPY PGIDLSQVYE LLEKDYRMER PEGCPEKVYE LMRACWQWNP

     S 490         500         510
SDRPSFAEIH QAFETMFQES SISDEVEKEL G
```

List of mutations:[1]

The amino acid substitutions are indicated in bold, followed by the corresponding nucleotide sequence* in parenthesis, and a fraction (Y/Z), where X = the number of patient cases in which the given base pair mutation was detected and Z = the number of cases tested for the given mutation.

* This nucleotide numbering has been converted from GenBank entry M1472 numbering to the nucleotide numbering found in the GenBank entry in **Appendix B2** (entry NM_005157) and used throughout Modules 4 and 5.

M244V (A733G) in 3/125, **L248V** in 2/29, **G250E** (G752A) in 6/87, **G250R** (G751A) in 1/117, **Q252R** in 1/32, **Q252H** (G759C/T) in 12/125, **Y253H** (T760C) in 9/154, **Y253F** (A761T) in 6/125, **E255K** (G766A) in 28/182, **E255V** (A767T) in 3/101, **D276G** (A830G) in 1/33, **T277A** in 1/117, V289A, **F311L** in 1/24, **T315I** (C947T) in 27/194, **T315N** (C947A) in 1/33, **F317L** (C954G) in 4/60, **M343T** (T1031C) in 1/32, **M351T** (T1055C) in 24/204, **E355G** in 4/25, **F359V** (T1078G) in 4/59, **V379I** (G1138A) in 1/32, **F382L** (T1147C) in 1/32, **L387M** (T1162A) in 2/149, **L387F** in 3/117, **H396P** (A1190C), **H396R** (A1190G) in 5/12, **A397P** in 1/117, **S417Y** (C1253A) in 1/27, **E459K** (G1378A) in 1/27, **F486S** (T1460C) in 1/27

APPENDIX C: Primer design for site-directed mutagenesis. Primers may also be designed using a primer design program (http://www.stratagene.com/qcprimerdesign), or checked by that method following design. The Tm must be calculated using the formula below.

C1: Example of step-by-step design of primers for the Abl H396P mutation

Design of the forward (5' to 3') primer:

- ✓ Determine the DNA code that corresponds to His396. To do this, multiply the residue number by 3, and then subtract 2 to get the first bp in the 3 bp codon. Add 3 bases to this number (see Appendix B2 for an explanation of why) to convert to the numbering to correspond to Genbank entry NM_005157.

  (396*3) – 2 + 3 = 1189

  His396 is encoded by base pairs 1189-1191, which are CAT.
- ✓ Check that the codon corresponds to the correct amino acid using a DNA to protein translatation tool (http://www.expasy.ch/tools/dna.html). If provided in Appendix B3, confirm that the numbering of the nucleotide substitution falls within the codon determined above (here 1189-1191), and use a translator tool to ensure that the bp change results in the expected amino acid substitution. If the DNA change is not listed, determine which nucleotide substitution gives the desired amino acid change. For H396P, the A1190C mutation results in a CCT (Pro) codon, as expected.
- ✓ Write out the desired mutation (shown in bold italics below) with 12 flanking bases on each side.

  CC TAC ACA GCC C***C***T GCT GGA GCC AA
- ✓ The first and last residues of your primer should be a G or a C. Add bases as needed to each end of your primer so that each end terminates with a G or C.

  CC TAC ACA GCC C***C***T GCT GGA GCC AAG TTC
- ✓ Check that your primer has at least 40% GC content. If needed, added more bases to one or both ends to achieve a higher GC%.

  % GC content = ((# of G/C bases)/(total # of bases)) * 100% = (18/29) X 100 = 62 % GC
- ✓ Calculate the % mismatch of your primer.

  % mismatch = 1/29 * 100% = 3 %
- ✓ Calculate the melting temperature (T_m) of your primer. In the equation below, N is the primer length in bases, and %GC and % mismatch should be written in whole numbers.

  T_m = 81.5 + 0.41(%GC) – 675/N - % mismatch = 81.5 – (0.41)(62) – 675/29 -3 = 80 .6 ºC
- ✓ The T_m of your primer should be greater than or equal to 78 ºC. If the T_m is less than 78 ºC, increase the length of your primer or increase the % G/C content to increase the T_m. However, make sure your primer does not exceed 45 bases.

Forward primer: 5' **CC TAC ACA GCC C*C*T GCT GGA GCC AAG TTC** 3'

Design of the antisense primer, which is the reverse complement of the forward primer:

- ✓ Write the complement of the forward primer.

  Forward: 5' CC TAC ACA GCC C***C***T GCT GGA GCC AAG TTC 3'

  Complement: 3' GG ATG TGT CGG G***G***A CGA CCT CGG TTC AAG 5'
- ✓ Rewrite the complement primer from 5' to 3'.

  5' GAA CTT GGC TCC AGC A***G***G GGC TGT GTA GG 3'
- ✓ Double check everything. Then triple check everything.
- ✓ Reverse primer: 5' **GAA CTT GGC TCC AGC A*G*G GGC TGT GTA GG** 3'

C2: Primer design worksheet: Spring 2009

Name:_____

Group members: _____

Section/TA name_____

Point mutation (use numbering from Appendix B2)_____
Please also circle your desired mutation below:

```
                          (688)tcc cccaactacg acaagtggga gatggaacgc
721  acggacatca ccatgaagca caagctgggc gggggccagt acggggaggt gtacgagggc
781  gtgtggaaga aatacagcct gacggtggcc gtgaagacct tgaaggagga caccatggag
841  gtggaagagt tcttgaaaga agctgcagtc atgaaagaga tcaaacaccc taacctggtg
901  cagctccttg gggtctgcac ccgggagccc ccgttctata tcatcactga gttcatgacc
961  tacgggaacc tcctggacta cctgagggag tgcaaccggc aggaggtgaa cgccgtggtg
1021 ctgctgtaca tggccactca gatctcgtca gccatggagt acctggagaa gaaaaacttc
1081 atccacagag atcttgctgc ccgaaactgc ctggtagggg agaaccactt ggtgaaggta
1141 gctgattttg gcctgagcag gttgatgaca ggggacacct acacagccca tgctggagcc
1201 aagttcccca tcaaatggac tgcacccgag agcctggcct acaacaagtt ctccatcaag
1261 tccgacgtct gggcatttgg agtattgctt tgggaaattg ctacctatgg catgtcccct
1321 tacccgggaa ttgacctgtc ccaggtgtat gagctgctag agaaggacta ccgcatggag
1381 cgcccagaag gctgcccaga gaaggtctat gaactcatgc gagcatgttg gcagtggaat
1441 ccctctgacc ggccctcctt tgctgaaatc caccaagcct ttgaaacaat gttccaggaa
1501 tccagtatct cagacgaagt ggaaaaggag ctgggg
```

Corresponding amino acid substitution (use numbering from Appendix B1)_____

Forward Primer (5’ to 3’)

_____

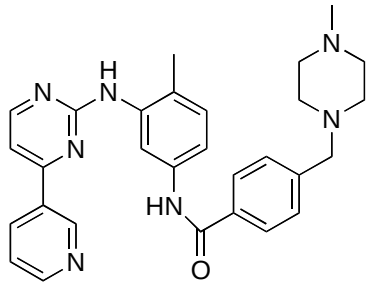
Reverse Primer (5’ to 3’)

_____

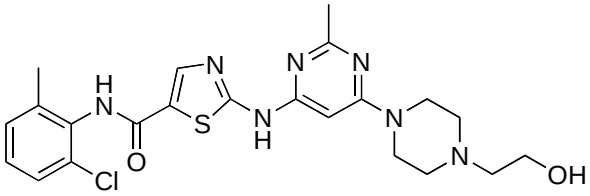
Percent GC _____ T_m_____

APPENDIX D: Abl inhibitors:

A great recent review[2]



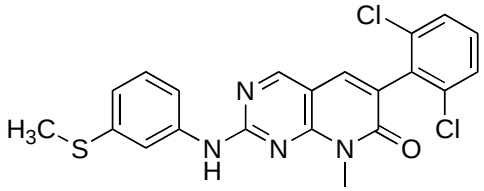
Imatinib (Gleevec, STI571)



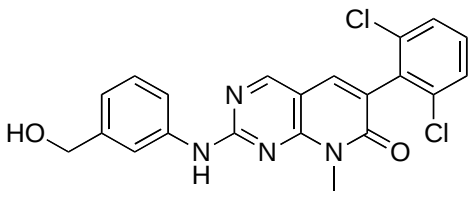
Dasatinib (Sprycel, BMS-354825)

Imatinib (Gleevec) is an inhibitor of Bcr-Abl that was FDA approved in 2001 as a first-line treatment for CML.[3] Gleevec resistance in CML patients has been linked to a number of point mutations resulting in amino acid substitutions in the Bcr-Abl protein.

Dasatinib was granted accelerated approval by the FDA in 2006 for treating patients with Gleevec-resistant forms of CML. Dasatinib has been shown to inhibit 14 or 15 Gleevec-resistant mutants of Bcr-Abl. It does not inhibit the T315I mutant.[4,5]

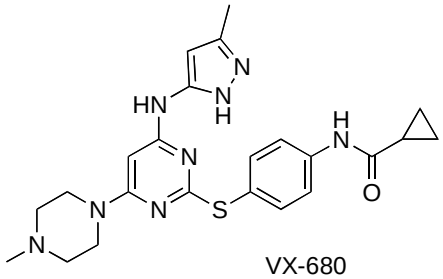


PD173955
originally identified as a Src inhibitor

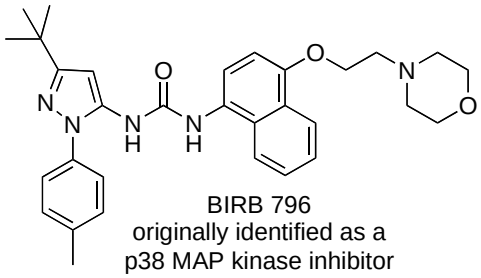


PD166326

These compounds were studied as potential CML drugs thought to target Src kinase. Instead they appear to inhibit Bcr-Abl activity. PD173955 and five other related compounds were studied for inhibition of Bcr-Abl-dependent cell growth.[6] A similar compound (PD180970) was found to inhibit several relevant Bcr-Abl isoforms, but NOT the T315I mutation.[7]



VX-680
originally identified as an Aurora inhibitor



BIRB 796
originally identified as a
p38 MAP kinase inhibitor

These compounds have been identified as inhibitors of Bcr-Abl mutants (namely the T315I mutant) that are resistant to Gleevec, dasatinib, and PD180970.[8] VX-680 is currently in clinical trials.[9] In session 15, you will evaluate a crystal structure of VX-680 bound to the H396P mutant of the Bcr-Abl kinase domain.[10]

Abl autophosphorylation reference[11]
Abl kinase activity assay (coupled to NADH oxidation) references[12, 13]

Appendix References (see the list of lecture topics for a more complete list)

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