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5.36 Biochemistry Laboratory  
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**Laboratory Manual for URIECA Modules 4 and 5  
Course 5.36 U**

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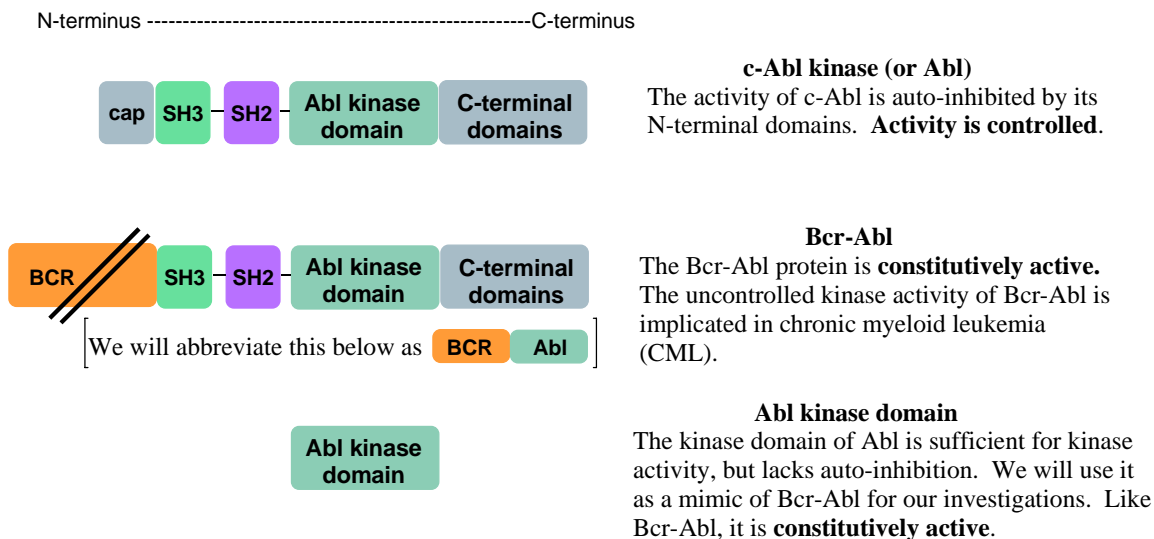
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## An Introduction and Background for URIECA Modules 4 and 5

### Abl and Bcr-Abl Proteins

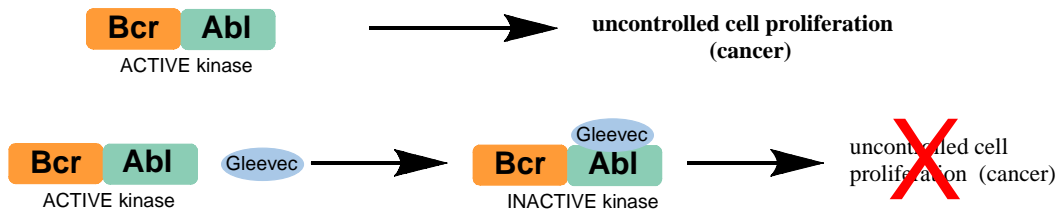
Abelson (c-Abl or Abl) is a protein tyrosine kinase that is involved in a number of highly-regulated cellular processes, including cell division, differentiation and adhesion. In healthy cells, c-Abl is auto-inhibited by domains at its amino (N)-terminus. This means that the kinase activity of c-Abl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

A chromosomal abnormality implicated in chronic myeloid 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 enzyme activity is permanently “on”. This aberrant kinase activity is responsible for uncontrolled cell proliferation, which leads to cancer.



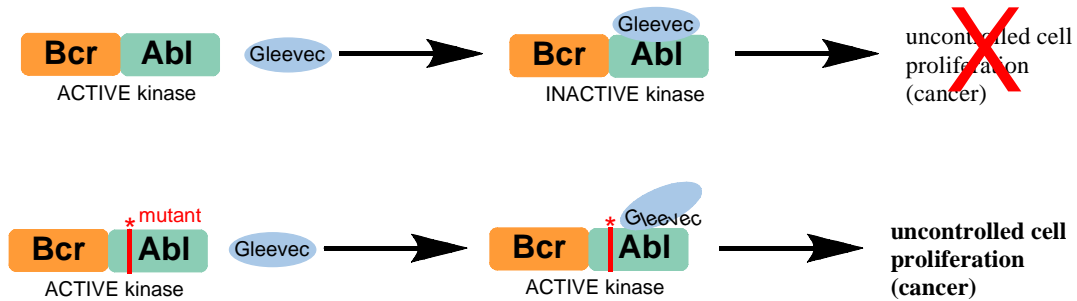
### A Small Molecule Drug for CML Treatment

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. Gleevec inhibits Bcr-Abl tyrosine kinase activity by competitively binding in the ATP binding pocket of the kinase domain and stabilizing the inactive conformation of the protein. 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. 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.

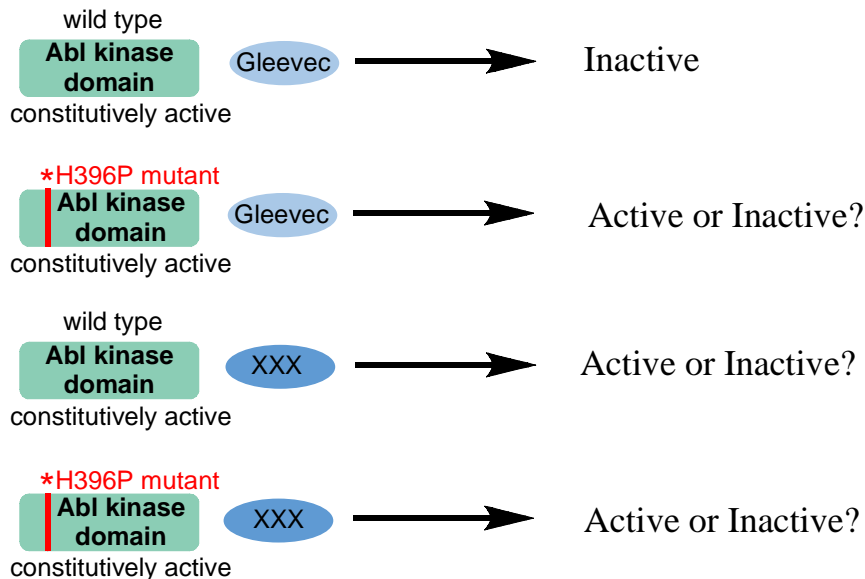


However....A PERCENTAGE OF CML PATIENTS DO NOT RESPOND TO GLEEVEC TREATMENT, AND OTHER PATIENTS THAT INITIALLY RESPOND TO TREATMENT EVENTUALLY DEVELOP GLEEVEC RESISTANCE.

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. Over 30 *different* point mutations have been identified in Gleevec-resistant CML patients (see appendix B3).



The Abl kinase domain is often used 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-resistant 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 another kinase inhibitor, Dasatinib. In addition, you will use site-directed mutagenesis to create a DNA expression vector for future expression of another Gleevec-resistant 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 most 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 an alternative Bcr-Abl inhibitor, Dasatinib, 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 another Gleevec-resistant Abl mutant of your choice. A brief description of the 15 lab sessions is provided below.

	<i>H396P Abl protein expression/ kinase inhibition assays</i>	<i>DNA site-directed mutagenesis</i>
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:

### Module 4 Protein Expression and Isolation of DNA

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**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 °C.

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**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, the 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.

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**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.

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**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**

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**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 °C.

### **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.

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**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.

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**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.**