5.36 Biochemistry Laboratory
Spring 2009

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**List of Abbreviations:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Abl (or c-Abl)</td>
<td>Abelson kinase</td>
</tr>
<tr>
<td>Abl(229-511)</td>
<td>the Abelson kinase domain, which is constitutively active for kinase activity when expressed as an isolated domain</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranosid</td>
</tr>
<tr>
<td>kan</td>
<td>kanamycin, an antibiotic</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight (reported in g/mol)</td>
</tr>
<tr>
<td>OD600</td>
<td>optical density (absorbance) at 600 nm</td>
</tr>
<tr>
<td>RE</td>
<td>restriction enzyme</td>
</tr>
<tr>
<td>strep</td>
<td>streptomycin, an antibiotic</td>
</tr>
<tr>
<td>TAE</td>
<td>tris acetate electrophoresis</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TEMED</td>
<td>etramethylethlenediamine</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
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</tbody>
</table>
APPENDIX A: Common biochemistry laboratory procedures used multiple times in URIECA modules 4 and 5.

A1: Bio-Rad assay for quantifying protein concentrations

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 μL 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 straight 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)


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 TrisCl, 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 3.3x10^6 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
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 GQYGEYEGV WKKYSVTAV KTLKDTEMEV EEFLKEAVM KEIKHPMLVQ

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.


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 caaagctggc gggtctgcac ccgttctata tcatcactga gttcatgacc
901  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 tgctgagcgc
1201 ctgctgtaca tggccactca gatctcgtca gccatggagt acctggagaa gaaaaacttc
1261 atccacagag atcttgctgc ccgaaactgc ctggtagggg agaaccactt ggtgaaggta
1321 tacgggaacc tcctggacta cctgagggag tgcaaccggc aggaggtgaa cgccgtggtg
1381 ccctctgacc ggccctcctt tgctgaaatc caccaagcct ttgaaacaat gttccaggaa
1501 tccagtatct cagacgaagt ggaaagagc ctgggg
B3: Point mutations in the kinase domain of Abl detected in leukemia patients

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

List of mutations:

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.

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 translation 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 CCT 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 CCT 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 °C

- The T_m of your primer should be greater than or equal to 78 °C. If the T_m is less than 78 °C, 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 CCT 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 CCT GCT GGA GCC AAG TTC 3’

Complement: 3’ GG ATG TGT CGG GGA CGA CCT CGG TTC AAG 5’

- Rewrite the complement primer from 5’ to 3’.

5’ GAA CTT GGC TCC AGC AGG GGC TGT GTA GG 3’

- Double check everything. Then triple check everything.

Reverse primer: 5’ GAA CTT GGC TCC AGC AGG 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 cccactacg acaagtggga gatggaacgc

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

Forward Primer (5’ to 3’)
________________________________________________________________________

Reverse Primer (5’ to 3’)
________________________________________________________________________

Percent GC _______________                       Tm_______________________________

55
APPENDIX D: Abl inhibitors:
A great recent review²

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