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5.36 Biochemistry Laboratory  
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## 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- $\beta$ -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 <sub>m</sub>	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.fhrc.org/science/labs/hahn/methods/biochem\\_meth/biorad\\_assay.pdf](http://www.fhrc.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  $\mu$ L of the BSA stock and 30  $\mu$ L of deionized water (to give 0.9 mg/mL), 210  $\mu$ L of BSA and 90  $\mu$ L of water (to give 0.7 mg/mL), 165  $\mu$ L of BSA and 135  $\mu$ L of water (to give 0.55 mg/mL), 120  $\mu$ L of BSA and 180  $\mu$ L of water (to give 0.4 mg/mL), 60  $\mu$ L of BSA and 240  $\mu$ L of water (to give 0.2 mg/mL), and no BSA and 300  $\mu$ L 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  $\mu$ L of each standard into an eppendorf tube. Add 980  $\mu$ L 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  $\mu$ L of water in 980  $\mu$ L 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  $\mu$ L of your sample (or an appropriate dilution) with 980  $\mu$ L 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](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  $\mu$ L of Buffer P1 to the cell pellet and completely resuspend the pellet by vortexing.
- c.) Add 250  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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  $x$   $\mu\text{L}$  (typically, 1-3  $\mu\text{L}$ ) of DNA sample in 100  $\mu\text{L}$  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  $\mu\text{g}/\mu\text{L}$  of your double stranded (ds) DNA, multiply the Abs<sub>260</sub> by  $(0.05)(100/x)$ . Typical concentrations of miniprep DNA are 0.1-2  $\mu\text{g}/\mu\text{L}$ . To calculate the concentration of single stranded (ss) DNA or RNA, multiply the Abs<sub>260</sub> 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  $3.3 \times 10^6$  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](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 gggctctgcac ccgggagccc ccgttctata tcatcactga gttcatgacc  
961 tacgggaacc tcttgacta 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 tgcaccgag agcctggcct acaacaagtt ctccatcaag  
1261 tccgacgtct gggcatttgg agtattgctt tgggaaattg ctacctatgg catgtcccct  
1321 taccgggaa ttgacctgtc ccaggtgtat gagctgctag agaaggacta ccgcatggag  
1381 cgcccagaag gctgcccaga gaaggtctat gaactcatgc gagcatggtg 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<sup>1</sup>

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

```

      R  HF V
    V  V E  RH K  260          270          GA280          A          300
DITMKHKLGG GQYGEVYEGV̄ WKKYSLTVAV̄ KTLKEDTMEV̄ EEFLKEAAVM KEIKHPNLVQ̄
      N
    310 L  I  L320          330          340  T  350  T  G  V
LLGVCTREPP̄ FYIITEFMTȲ GNLLDYLREC̄ NRQEVNAVVL̄ LYMATQISSĀ MEYLEEKNFĪ
      R
    370          I  L  F390          PP400          410          Y420
HRDLAARNCL̄ VGENHLVKVĀ DFGLSRLLMTḠ DTYTAHAGAK̄ FPIKWTAPES̄ LAYNKFSIKS̄

    430          440          450          K          470          480
DVWAFGVLLW̄ EIATYGMSPȲ PGIDLSQVYĒ LLEKDYRMER̄ PEGCPEKVYĒ LMRACWQWNP̄

    S 490          500          510
SDRPSFAEIH̄ QAFETMFQES̄ SISDEVEKEL̄ Ḡ
```

List of mutations:<sup>1</sup>

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 T<sub>m</sub> 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.

$$(396 \times 3) - 2 + 3 = 1189$$

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%.

$$\begin{aligned} \% \text{ GC content} &= ((\# \text{ of G/C bases}) / (\text{total } \# \text{ of bases})) * 100\% = (18/29) \times 100 \\ &= 62 \% \text{ GC} \end{aligned}$$

- ✓ Calculate the % mismatch of your primer.

$$\% \text{ mismatch} = 1/29 * 100\% = 3 \%$$

- ✓ 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.

$$\begin{aligned} T_m &= 81.5 + 0.41(\% \text{GC}) - 675/N - \% \text{ mismatch} = 81.5 - (0.41)(62) - 675/29 - 3 \\ &= 80.6 \text{ }^\circ\text{C} \end{aligned}$$

- ✓ 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 cccaactacg acaagtggga gatggaacgc
721  acggacatca ccatgaagca caagctgggc gggggccagt acggggaggt gtacgagggc
781  gtgtggaaga aatacagcct gacgggtggcc gtgaagacct tgaaggagga caccatggag
841  gtggaagagt tcttgaaaga agctgcagtc atgaaagaga tcaaacaccc taacctgggtg
901  cagctccttg gggctctgcac cggggagccc ccgttctata tcatcactga gttcatgacc
961  tacgggaacc tcctggacta cctgagggag tgcaaccggc aggaggtgaa cgccgtgggtg
1021 ctgctgtaca tggccactca gatctcgtca gccatggagt acctggagaa gaaaaacttc
1081 atccacagag atcttgctgc ccgaaactgc ctggtagggg agaaccactt ggtgaaggta
1141 gctgattttg gcctgagcag gttgatgaca ggggacacct acacagccca tgctggagcc
1201 aagttcccca tcaaattggac tgcacccgag agcctggcct acaacaagtt ctccatcaag
1261 tccgacgtct gggcatttgg agtattgctt tgggaaattg ctacctatgg catgtcccct
1321 taccgggaa ttgacctgtc ccaggtgtat gagctgctag agaaggacta ccgcatggag
1381 cgcccagaag gctgcccaga gaaggtctat gaactcatgc gagcatggtg gcagtggaa
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')

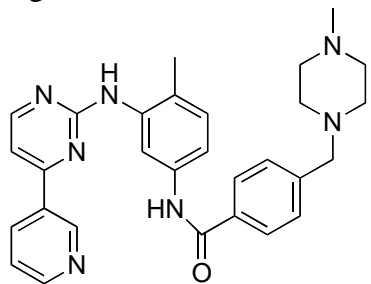
\_\_\_\_\_

Percent GC \_\_\_\_\_

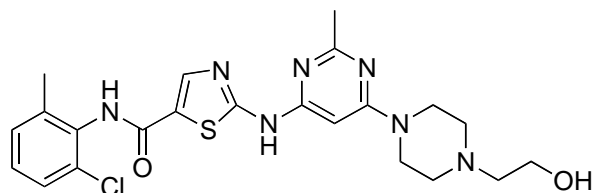
$T_m$  \_\_\_\_\_

## APPENDIX D: Abl inhibitors:

A great recent review<sup>2</sup>



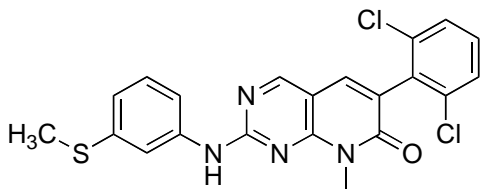
Imatinib (Gleevec, STI571)



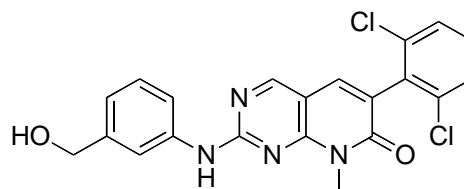
Dasatinib (Sprycel, BMS-354825)

Imatinib (Gleevec) is an inhibitor of Bcr-Abl that was FDA approved in 2001 as a first-line treatment for CML.<sup>3</sup> 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.<sup>4,5</sup>

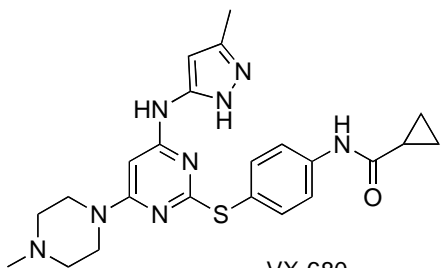


PD173955  
originally identified as a Src inhibitor

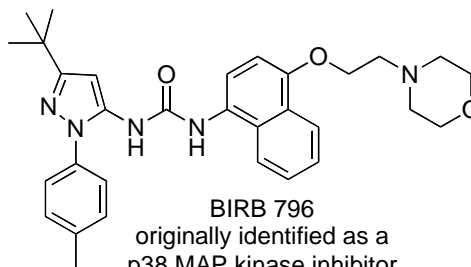


PD166326

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.<sup>6</sup> A similar compound (PD180970) was found to inhibit several relevant Bcr-Abl isoforms, but NOT the T315I mutation.<sup>7</sup>



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.<sup>8</sup> VX-680 is currently in clinical trials.<sup>9</sup> In session 15, you will evaluate a crystal structure of VX-680 bound to the H396P mutant of the Bcr-Abl kinase domain.<sup>10</sup>

Abl autophosphorylation reference<sup>11</sup>

Abl kinase activity assay (coupled to NADH oxidation) references<sup>12, 13</sup>

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

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12. Barker, S. et al. Characterization of pp60c-src Tyrosine Kinase Activities Using a Continuous Assay: Autoactivation of the Enzyme Is an Intermolecular Autophosphorylation Process. *Biochemistry* **34**, 14843-51 (1995).
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