

Using BLAST and ExPASy for Genetic and Protein analysis of H1N1 variability, including mutations that confer resistance to antiviral medications.

Objectives:

- Students will become familiar with the online databases available to researchers including GenBank, BLAST and ExPASy utilities.
- Students will analyze normal and mutant strains of H1N1 viruses to look for nucleotide mutations that confer resistance to two antiviral therapies: zanamivir and oseltamivir.
- Students will observe the effects of nucleotide changes on viral protein structure
- Students will hypothesize if a previously unknown, but sequenced resistant strain has a similar resistance mutation or a new type
- Students will research the glycoprotein upon which the anti-viral therapies act, neuraminidase, to investigate the reason for antiviral resistance due to the known mutations

Previous Knowledge:

1. In all cells and viruses, DNA is made of nucleotides and triplets of As, Ts, Cs and Gs code for specific amino acids
2. Proteins are chains of amino acids
3. The amino acid composition of the protein determines the shape, and therefore function of the protein, and changing the amino acids can change the shape and function of the protein
4. Proteins are essential components of all cells and viruses
5. Viruses are intracellular parasites and have molecules on their surface, that among other things, allow the viruses to infect cells, replicate themselves and leave cells thereby destroying them

Introduction: Influenza is an infection of the upper respiratory tract that causes sickness and death and widespread outbreaks also cause a significant economic impact as well. The H1N1 influenza A pandemic of 2009 caused millions of people to become sick, hundreds of thousands to be hospitalized and thousands of deaths in the United States alone¹. The new flu strain contained genes from influenza viruses from avian (bird), swine (pig) and human. New strains often cause more infection because our immune systems have been conditioned to respond to previously encountered strains. When strains mutate it allows them to more easily slip by our body's defenses. In addition, mutation can make previous vaccines and antiviral medications ineffective against them.

¹ http://www.cdc.gov/h1n1flu/estimates_2009_h1n1.htm Numbers are for United States only; worldwide projections are not given.

Background: Most of us are familiar now with the nomenclature “H1N1” but few of us know the meaning of these letters and numbers. Scientists label flu viruses based on the presence of two antigens on their surface. The first letter, “H” refers to the type of Hemagglutinin present on the virus’ surface. This molecule is primarily responsible for the virus’ ability to infect cells. The second letter, “N”, refers to the type of Neuraminidase present. This molecule helps the replicated virus leave from the cell in which it has replicated, allowing the many copies to infect more cells. These two proteins together determine much about the virus, including, in general, which species the virus can infect and the virulence of the particular strain. Even though there are many different strains of flu virus, they infect mammals and birds and there remains a lot of similarity between them. These similarities can be exploited to develop vaccines and/or antiviral drug therapies that could be used against a wide array of similar viruses. Between 1999 and 2002 two new antiviral drugs were introduced into the population: zanamivir and oseltamivir. These same drugs were used to treat patients with H1N1. In just about 10 years it is possible for flu viruses to evolve resistance to these drugs. In order to monitor the potential development of resistant strains the Neuraminidase Inhibitor Susceptibility Network was established.

Directions: You are a member of the Neuraminidase Inhibitor Susceptibility Network and the World Health Organization has contacted you with information about a new viral strain that might have resistance to the antiviral drugs zanamivir and oseltamivir (Tamiflu). In this case study you will:

- Use BLAST to compare H1N1 sequences to observe conserved and variable regions
- Align nucleotide sequences of normal strains of H1N1
- Align multiple nucleotide and protein sequences to look for mutations.
- Compare the newly identified strain and hypothesize its resistance based on its mutations

Part A: Introduction to blastn. Listed below are the identifying numbers for several “normal” (non-resistant) strains of H1N1 that were sequenced during the 2009 pandemic. In part A of this activity you will use online database tools to look at virus variation that does not confer antiviral resistance.

Step 1: Go to the National Center for Biotechnology Information’s website at: <http://www.ncbi.nlm.nih.gov>. From the menu at the top right, click on BLAST. BLAST stands for “Basic Local Alignment Search Tool” and it is one of the many web-based applications available to investigate, analyze, research and identify genes and proteins of interest. The applications of BLAST and other tools are almost limitless! All of these resources are free and open to the public. This particular site is maintained by the National Institutes of Health (NIH).

Step 2: Choose nucleotide blast from the menu of choices under the heading “Basic BLAST”. To become familiar with BLAST output we will compare the nucleotide sequences of two strains of H1N1. Enter this accession number in the box, CY056295. Give your job the title, “align two non-resistant H1N1 strains”.

Step 3: Click on the box in front of “Align two or more sequences”. This will open a new box. Enter accession number CY062530 in this box.

Step 4: At the bottom section called “Program Selection” select the choice “Highly similar sequences (megablast)”. Then click “show results in a new window” so we can refer back to our search criteria if needed.

Step 5: Click the “BLAST” button. Your search will show up in a new window or tab and if you scroll down you can see the aligned nucleotides for the two neuraminidase genes in our two strains.

Answer the questions for Part A once the BLASTN search is finished.

Part A Questions:

1. What are the lengths of the two sequences used in this comparison?

Query: _____ nucleotides Subject: _____ nucleotides

2. Now that the sequences are aligned for best fit, how many differences can be found between the two aligned regions? (count them!)

_____ nucleotides

3. Since every 3 bases codes for 1 amino acid, what is the maximum number of amino acids in the protein that would be made from the shorter of the two sequences?

_____ amino acids

4. Will every nucleotide difference result in an amino acid difference? Explain your answer.

5. Since neither of these strains is resistant to anti-viral therapies, what can be concluded about the variation in the gene for neuraminidase between these two strains?

Part B: Collecting Info on your sequences. There are a lot of details recorded about the sequences that are uploaded to the GenBank database. In Part B of this activity we will collect information about our strains, including a reference number to their translated amino acid (protein) sequence. We will use these sequences in Part C of this activity.

Step 1: From the top of the BLAST results page, click on the link after “Query ID”. This will bring up all of the information about this sequenced gene. Look over the information and answer the questions:

Part B Questions:

1. When was this sample collected?
2. Who was the host of this virus? Be as specific as possible.
3. From which country was this host?
4. If you scroll down to the bottom you can find the entire sequence and translated amino acid sequence. See appendix A or <http://www.bio.davidson.edu/courses/genomics/jmol/aatable.html> for a list of amino acid abbreviations. Write down the protein_id number: _____

Step 2: Use the back button of the web browser to return to your BLAST results. Scroll down and click on the accession number of our subject sequence, CY062530.1. Answer the same 4 questions about this viral strain.

1. When was this sample collected?
2. Who was the host of this virus? Be as specific as possible.
3. From which country was this host?
4. If you scroll down to the bottom you can find the entire sequence and translated amino acid sequence. See appendix A or <http://www.bio.davidson.edu/courses/genomics/jmol/aatable.html> for a list of amino acid abbreviations. Write down the protein_id number: _____

Part C: Using blastp. In Part C of this activity we will use blastp to align two protein sequences.

Step 1: Navigate back to the BLAST input page. From the tabs at the top click on “blastp”. If you have closed this window, go back to <http://www.ncbi.nlm.nih.gov>. From here choose “blast” and then “protein blast”.

Step 2: Input the first of the two protein_id numbers from Part B into the box. Click on “align two or more sequences” and enter the second protein reference number in the second box.

Step 3: Choose “show results in a new window” and click the BLAST button. The blastp output will show a large number of identifiers listed below the query sequence. The reason for the large number of entries is because we are searching against the non-redundant protein database (nr). As the database name suggests, NCBI tries to collapse entries in GenBank with the same sequence into a single record and then simply append the redundant sequences in the description.

Step 4: Scroll down to the bottom to see the two sequences aligned. It is difficult to look for amino acid differences in this view. To change the view to make it easier, scroll to the very top. Click “Formatting options”. In the “alignment view” pull down menu, choose “query-anchored with dots for identities”. Click the blue “reformat” button to see the changed view.

Step 5: Scroll back all the way to the bottom. Now you can see the identical amino acids marked with a dot, and the differences are marked with their respective amino acid letters.

Answer the questions for Part C once the blastp search is complete.

Part C Questions:

1. How many amino acid differences are there between the two sequences? _____
2. Scientists use the single letter amino acid abbreviations and their position number to label and refer to specific amino acids. Record the amino acid differences below:

Position 274: Query	_____	Position 453: Query	_____
Subject	_____	Subject	_____

3. Use Appendix A or <http://www.bio.davidson.edu/courses/genomics/jmol/aatable.html> to record what change in the properties of amino acids has occurred at each variation site.

Site 274: _____ --> _____ Site 453: _____ --> _____

4. Even though we know these strains do not cause anti-viral resistance, which amino acid variation is more likely to confer resistance? Why?

5. What can be concluded about this variation?

Part D: Aligning multiple protein sequences. In Part D of this activity we will align multiple neuraminidase protein sequences to find the nature of the mutation that confers resistance to the two antiviral drugs, zanamivir and oseltamivir.

Step 1: Navigate back to the blastp tool (see part C). Keep the query sequence the same as in Part C (ADD52538.1). Now click align two or more sequences and enter **all** of the following numbers, each followed by the enter/return key. Be sure to keep them in order so we will know which ones are resistant.

4 normal strains:

ADG27998

ADB98137

ACT67242

BAJ05804

and 4 mutant strains:

ADB98138

ADF97837

ACT10319

ADG28013

Step 2: Choose “Show results in a new window” and click the BLAST button.

Step 3: The default view for the results will show EACH sequence compared to the query sequence. We want to see them all together. At the top, change the formatting options to “query anchored with dots for identities”. Don’t forget to click the “Reformat” button for your changes to take effect. Then scroll down to view all of your amino acid sequences aligned. Imagine doing this analysis without a computer!

Part D Questions:

1. What conclusions can you draw from the information? Be as specific and complete in your explanation as possible.

Part E: Analyzing a new mutant strain. You have been asked to analyze a new resistant strain of H1N1. You have been provided with the nucleotide sequence. In Part D of this activity you will translate the nucleotide sequence into an amino acid sequence to draw conclusions about the nature of its mutation.

Step 1: Navigate to <http://expasy.org/tools/>

There are many tools available to researchers at this site maintained by the SIB (Swiss Institute of Bioinformatics.) Scroll to the bottom and choose translate. It can be found in section labeled DNA-->protein.

Step 2: Copy and paste the sequence from appendix B into the box.

Step 3: Click on translate sequence. This will translate the nucleotide sequence in all of the possible “frames”, 3 from the top strand and 3 from the bottom strand.

Step 4: Examine the translated sequences. Your protein will not have many stop codons in the middle of the sequence and it should start with a “Met” for Methionine.

Once you have located the most likely reading frame, click on this frame number.

Step 5: You will see a new window with your sequence. Copy this protein sequence starting with the earliest M and ending with the last letter before the STOP codon.

Step 6: Return to the BLASTp search page from Part D. Paste this new protein sequence into the second box. Be sure it is separate from the last entry by a enter/return.

Step 7: Choose results in a new window and click BLAST.

Step 8: Reformat the results like you did in Part D to view all of the sequences aligned together. Don't forget to click the reformat button.

Part E Questions:

1. What conclusions can you draw from the aligned sequences of the wild-type strain, original mutant strain and the new mutant strain? Be as specific as possible in your analysis. Be sure to include where researchers should focus their study in the future to further analyze the nature of this new mutant strain.

EXTENSION--Part F: The anti-viral drugs zanamivir and oseltamivir target the active site of the catalytic component of neuraminidase. This active site has several key amino acids that function in the chemical reaction. In Part F of this activity you will research the new mutations amino acid substitutions to draw conclusions about which mutation is the likely cause of the antiviral resistance.

Step 1: Use Google to search for “PMCID: PMC1563878”. Click on the first result. This will bring up the Journal of Virology article, “Importance of Neuraminidase Active-Site Residues to the Neuraminidase Inhibitor Resistance of Influenza Viruses”.

Step 2: Scroll down about halfway looking for figure 1. Click to enlarge this figure. This image shows the active site of neuraminidase and its interactions with its substrate, sialic acid (yellow). Antiviral drugs, such as Tamiflu act by mimicking the structure of sialic acid and blocking this active site.

Part F Questions:

1. List below the neuraminidase amino acids that seem to play important roles in binding of sialic acid:

_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

2. Which, if any, of these amino acids are mutated in our new strain?

3. How should researchers use this new information?

Author's note:

This work written in 2010 by Julie Ertmann and updated and edited by Wilson Leung in 2012. It was supported by a NSF Mathematics and Science Partnership grant #06344780, to B Schaal, Washington University in St Louis. All of the information contained in this activity is based on actual viruses, science and research with the exception of the new mutant strain. This new mutant strain sequence is fictional and was created only for this activity. This work is designed as a stand alone activity using BLAST for AP or second year high school biology students. If you have questions about this activity, please email the author at: jertmann@ucityschools.org.

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Appendix A

Amino Acids: Their Properties and Structures

Nonpolar (hydrophobic)

amino acid	three letter code	single letter code
glycine	Gly	G
alanine	Ala	A
valine	Val	V
leucine	Leu	L
isoleucine	Ile	I
methionine	Met	M
phenylalanine	Phe	F
tryptophan	Trp	W
proline	Pro	P

Polar (hydrophilic)

serine	Ser	S
threonine	Thr	T
cysteine	Cys	C
tyrosine	Tyr	Y
asparagine	Asn	N
glutamine	Gln	Q

Electrically Charged (negative and hydrophilic)

aspartic acid	Asp	D
glutamic acid	Glu	E

Electrically Charged (positive and hydrophilic)

lysine	Lys	K
arginine	Arg	R
histidine	His	H

Appendix B

>gi|NewResistantH1N1|2010|

agtttaaaat gaatccaaac caaaagataa taaccattgg ttcggtctgt atgacaattg
gaatggctaa cttaatatta caaattggaa acataatctc aatatggatt agccactcaa
ttcaacttgg gaatcaaaat cagattgaaa catgcaatca aagcgtcatt acttatgaaa
acaacacttg ggtaaactcag acatatgta acatcagcaa caccaacttt gctgctggac
agtcaagtgt tccctgaaa ttagcgggca attcctctct ctgccctgtt agtggatggg
ctatafacag taaagacaac agtataagaa tcggttccaa gggggatgtg tttgcataa
ggggaccatt catatcatgt tcccccttgg aatgcagaac cttctctctg actcaagggg
ccttgctaaa tgacaaacat tccaatggaa ccattaaagt taggagccca tctgaacce
taatgagctg tctatttgg gaagttccct ctccatacaa ctcaagattt gagtcagtcg
cttggtcagc aagtgcctgt catgatggca tcaattggct aacaattgga atttctggcc
cagacaatgg ggcagtggct gtgttaaagt acaacggcat aataacagac actatcaaga
gttgagagaaa caatatattg agaacacaag agtctgaatg tgcattgtga aatggttctt
gctttactgt aatgaccgat ggaccaagtg atggacaggc ctatacaag atcttcagaa
tagaaaaggg aaagatagtc aatcagtcg aatgaatgc ccctaattat cactatgagg
aatgctcctg ttatctgat tctagtgaaa tcacatgtgt gtgcagggat aactggcatg
gctcgaatcg accgtgggtg tcttcaatc agaatctgga atatcagata ggatacatat
gcagtgggat ttccggagac aatccacgcc ctaatgataa gacaggcagt tgtggtccag
tactgtctaa tggagcaaat ggagtaaaag gattttcatt caaatacggc aatggtgttt
ggatagggag aactaaaagc attagttaa gaaacggttt tgagatgatt tgggatccga
acggatggac tgggacagac aataacttct caataaagca agatatcgta ggaataaatg
agtggtcagg atatagcggg agtttaagc agcatccaga actaacaggg ctggattgta
taagacctg cttctgggtt gaactaatca gagggcgacc caaagagaac acaatctgga
ctagcgggag cagcatatcc ttttgcgggtg taaacagtga cactgtgggt tggcttggc
cagacgggtg tgagttgcca tttaccattg acaagtaatt tgttcaaaaa act