DNA INFORMATICS

This protocol is based on the EDVOTEK® protocol "DNA Informatics".

1. EXPERIMENT OBJECTIVE

In this experiment, students will explore the popular bioinformatics tool **BLAST**. First they will read sequences from autoradiographs of automated gel runs. The resulting data will then be analyzed using publicly available databases and **BLAST** to identify genes and gene products.

2. EXPERIMENT COMPONENTS

This experiment contains a total of 3 sets of 4 autoradiographs from gel run sequencing. Students can use any sequence database to perform the activities in this lab. For purposes of simplification we have chosen to illustrate with a database offered by the **NCBI (National Center for Biotechnology Information)**.

COMPONENTS	
Autoradiographs from gel run sequencing	3 sets of 4 Autoradiographs

NOTE: EXPERIMENT CAN BE STORED AT ROOM TEMPERATURE

2.1 Requirements

- Computer with Internet access.
- White Light Box.

NOTE: A white light box is recommended. The EDVOTEK White Light Gel Visualization System is well-suited for this lab.

NOTE: An autoradiograph may also be placed on an overhead projector and shown to the whole class.

3. BACKGROUND INFORMATION

BIOINFORMATICS AS A MODERN CORNERSTONE OF BIOLOGY

DNA sequencing technology allows for the analysis of DNA at the nucleotide level. Nucleotides are the monomer building blocks of DNA (Figure 1). Each deoxynucleotide (dNTP) comprises three basic parts: a phosphate group, a deoxyribose sugar, and a nitrogen-containing base (adenine, cytosine, guanine, or thymine). The 3' hydroxyl group on the sugar of one nucleotide forms a covalent bond with the 5' phosphate group of its neighbor. The nature of this bond results in remarkably stable strands with a distinct polarity, making DNA ideal for genetic information storage.

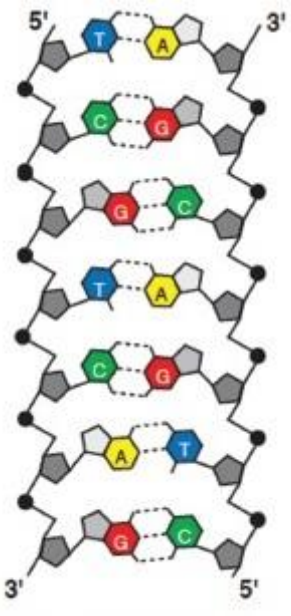


Figure 1: Structure of DNA.

Automated and high-throughput sequencing methods have made generating sequence information vastly more efficient. For example, the human genome, which consists of around three billion nucleotides, initially took over a decade to sequence but can now be completed in less than a day. As a result there is a great emphasis not only on generating sequence data but also on storing and analyzing the data. Bioinformatics represents the intersection of computer science and biology, involving any mathematical or computing approach that advances our understanding of biological processes. One major goal in bioinformatics is to develop computer programs that enable efficient access to and management of large data sets. Other methods aim to develop new algorithms (mathematical formulas) and statistical measures that assess relationships among members of large data sets. These programs often include pattern recognition, data mining, machine learning, and visualization.

THE SANGER METHOD OF DNA SEQUENCING

Chain termination sequencing, often called Sanger sequencing, allows researchers to generate long DNA reads of a target sequence, also known as the template. The DNA template is combined with DNA primer, the DNA polymerase I enzyme (DNA Pol I), and a mixture of two types of free nucleotides, deoxynucleotides (dNTPs) and dideoxy-nucleotides (ddNTPs). Importantly, one of the dNTPs is often labeled with radioactive phosphorus-32 or sulfur-35 to allow for visualization of the DNA fragments at later stages of the experiment. During the sequencing reaction, DNA Pol I copies the DNA template by adding dNTPs to the primer to form a complementary strand of DNA. Occasionally, the DNA Pol I will instead add a ddNTP to the DNA strand.

This ddNTP lacks a 3’ hydroxyl group which makes it impossible for the polymerase to add another nucleotide (Figure 2). As a result the synthesis of that particular strand of DNA is terminated. In Sanger sequencing four separate enzymatic reactions are performed, one for each nucleotide. At the end of the incubation each sample contains a population of molecules each with identical 5’ ends and 3’ ends that terminate with the same ddNTP base. However, since each reaction terminates randomly the sample will contain a mixture of different sized fragments depending on the site at which point the ddNTP was incorporated. Figure 3 gives an example of the fragments produced by a ddGTP reaction.

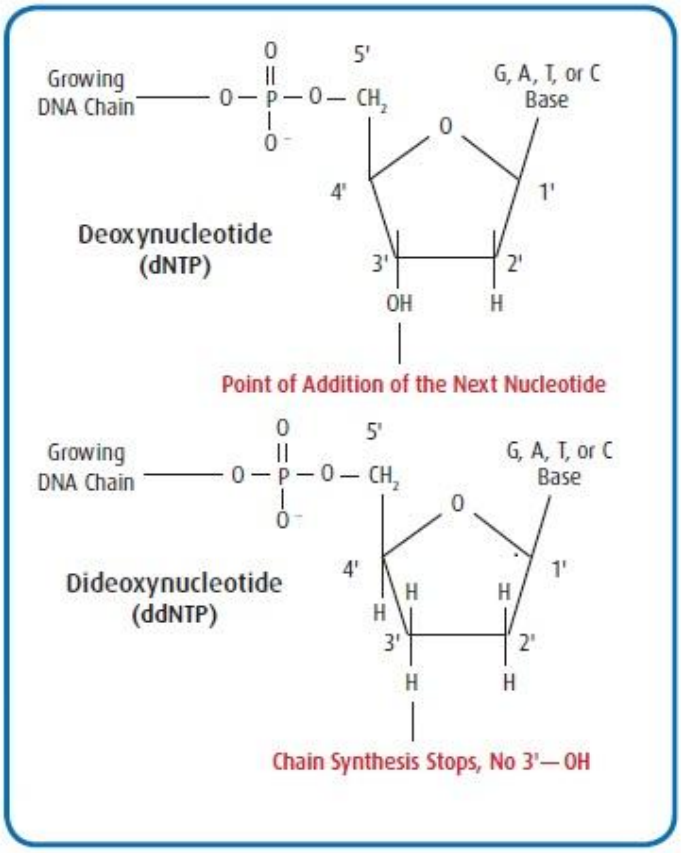


Figure 2: Nucleotide structure.

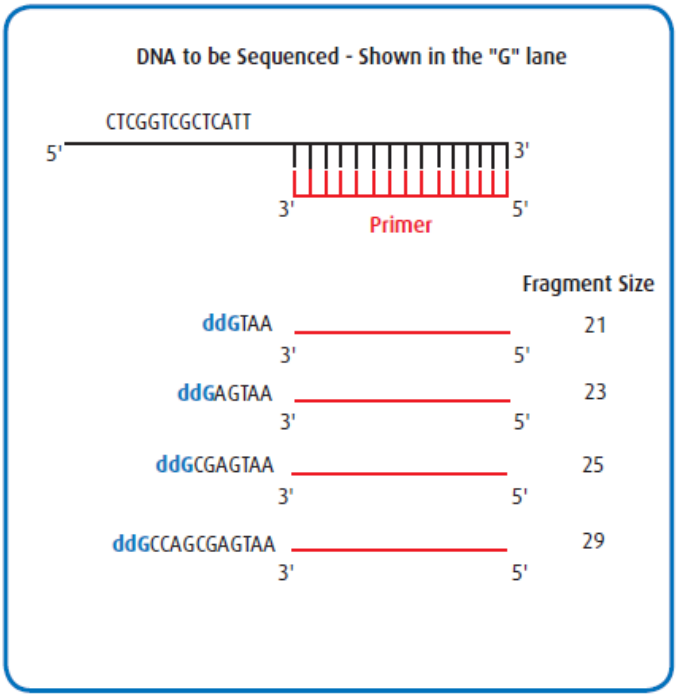


Figure 3: Random Incorporation of ddNTPs.

Once the four Sanger sequencing reactions have been completed the mixtures of fragments can be analyzed by polyacrylamide gel electrophoresis (PAGE). The samples are added into depressions (or "wells") within a polyacrylamide gel and an electrical current is passed through the gel. Because the sugar-phosphate backbone of DNA has a strong negative charge, the current drives the DNA through the gel towards the positive electrode. At first glance, a polyacrylamide gel appears to be a solid at room temperature. However, on the molecular level the gel contains small channels through which the DNA can pass. Small DNA fragments move through these holes easily, but large DNA fragments have a more difficult time squeezing through the tunnels. Because molecules with dissimilar sizes travel at different speeds, they become separated and form discrete "bands" within the gel. This allows PAGE to separate fragments that differ in size by a single nucleotide. Together, the four sequencing reactions contain DNA fragments that cover the entire length of the DNA's sequence, with each fragment corresponding to a different nucleotide.

After electrophoretic separation is complete, auto- radiography is performed. The polyacrylamide gel is placed into direct contact with a sheet of x-ray film. Since the DNA fragments are radiolabeled their position can be detected as dark bands on the x-ray film (Figure 4). Remember, since the smallest DNA fragments migrate further through the gel the sequence must be read from the bottom up. This provides a complementary sequence to the original DNA template for further analysis.

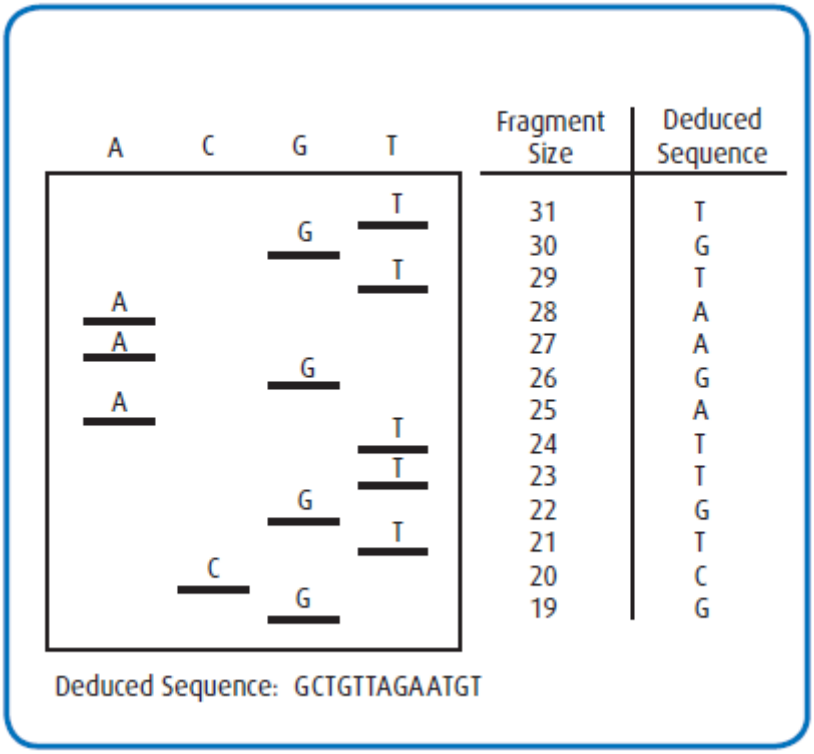


Figure 4: Simulated Sequencing Gel.

SEQUENCE COMPARISON USING BLAST

Data from DNA sequencing is of limited value unless it is converted to biologically useful information, making bioinformatics a critical part of DNA sequencing. After collecting DNA sequence data, molecular biologists will often search public databases for similar sequences. Comparing sequences can lead to the identification of genes and other conserved sequence patterns. In addition, sequence comparison can be used to establish functional, structural, and evolutionary relationships among genes. Another potential benefit is that sequence comparison provides a reliable method for inferring the biological functions of newly sequenced genes.

One of the largest and most influential databases is known as GenBank. This free, open source database contains over a trillion nucleotide bases of publically available sequence data. Each entry in GenBank contains a sequence and a unique accession number, as well as supporting bibliographic and biological annotations such as author references and taxonomic data. The NCBI (National Center for Biotechnology Information) oversees and maintains the entire database, but each entry is submitted directly by individual laboratories. Direct submission has allowed the database to keep pace with the rapid growth in sequence data production. However, it also means that heterogeneity in entry quality exists, especially in the certainty of each nucleotide's identity and in the extent of attached annotation. The impact of this uncertainty can vary depending on the goals of the study, the physical properties of the DNA region(s), and the chosen sequencing method. To address this, GenBank classifies the sequence information based on the sequencing strategy used to obtain the data.

Associated with this database is the Basic Local Alignment Search Tool, or BLAST. The BLAST program finds regions of local similarity between a user's DNA sequence and sequences in the GenBank database. In BLAST terminology, the user's input sequence is known as the query sequence, sequences in the database are known as target sequences, and sequences with similarities to the input sequence are hits. The user can draw inference about the putative molecular function of the query sequence by looking at the hits. Similarities between hits suggest homology, the existence of shared ancestry between the genes. In addition, BLAST can be used to identify unknown species, locate known protein domains, and find potential chromosome locations.

BLAST takes a heuristic approach to the problem of searching through such a mammoth database of target sequences. This means the program takes shortcuts in order to find sequence matches in a reasonable time frame. These shortcuts are based on the assumption that biologically similar sequences will contain short identical stretches of sequences. The entire database is scanned for the presence of these short stretches, and sequences containing two stretches within a preset distance are set aside. These sequences are then locally aligned with the query sequence to see if the nucleotides order beyond the two stretches matches. By searching the GenBank database this way BLAST can return results very quickly, although it sacrifices some accuracy and precision.

This exercise introduces students to bioinformatics through the analysis of sequencing data. In order to gain experience in database searching, students will use the free service offered by the NCBI. At present, GenBank comprises several databases including the GenBank and EMBL (European Molecular Biology Laboratory) nucleotide sequences, the non-redundant GenBank complementary DNA translations (protein sequences), and the EST (expressed sequence tags) database. These exercises will involve using BLASTN to compare nucleotide sequences.

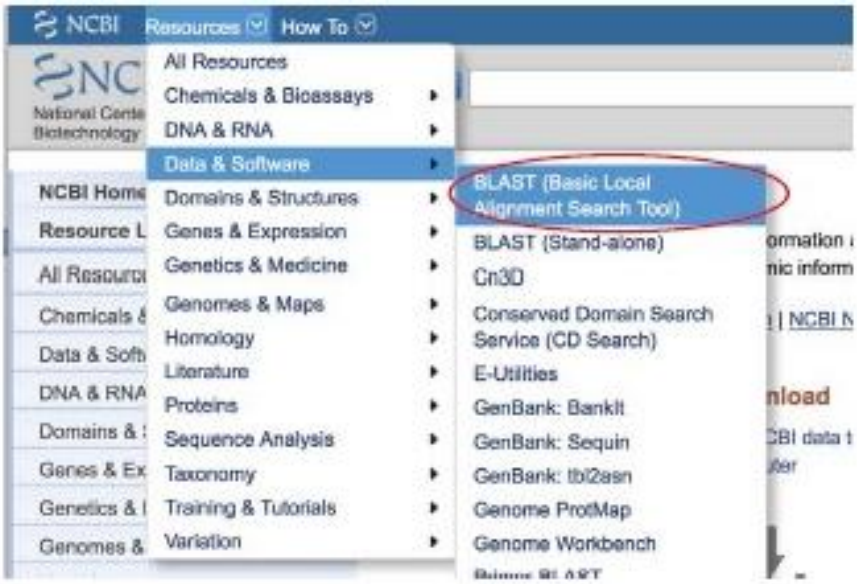
4. EXPERIMENTAL PROCEDURES

In this experiment, students will explore the popular bioinformatics tool **BLAST**. First they will read sequences from autoradiographs of automated gel runs. The resulting data will then be analyzed using publicly available databases and **BLAST** to identify genes and gene products.

5. STUDENT EXPERIMENTAL PROCEDURES

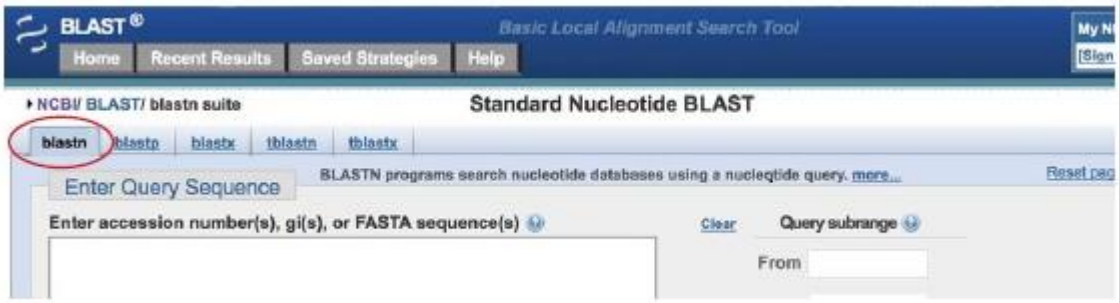
A. Guide to Using BLASTN

1. Type: www.ncbi.nlm.nih.gov to log on to the NCBI web page.
2. On the top left of the screen click on the drop down menu "Resources".
3. Click on "Data and Software", and then click on "BLAST" (Basic Local Alignment Search Tool).



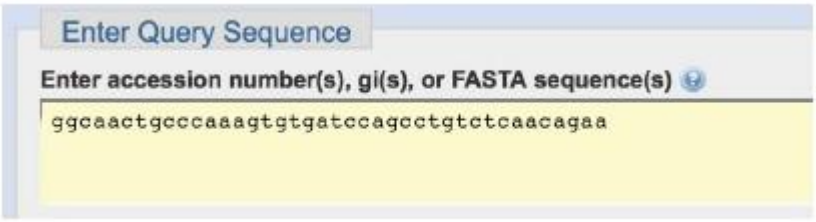
4. On the new BLAST Home screen select "nucleotide blast" which is the first opinion under the Basic BLAST list.

5. On the new screen make sure the tab selected is "blastn".

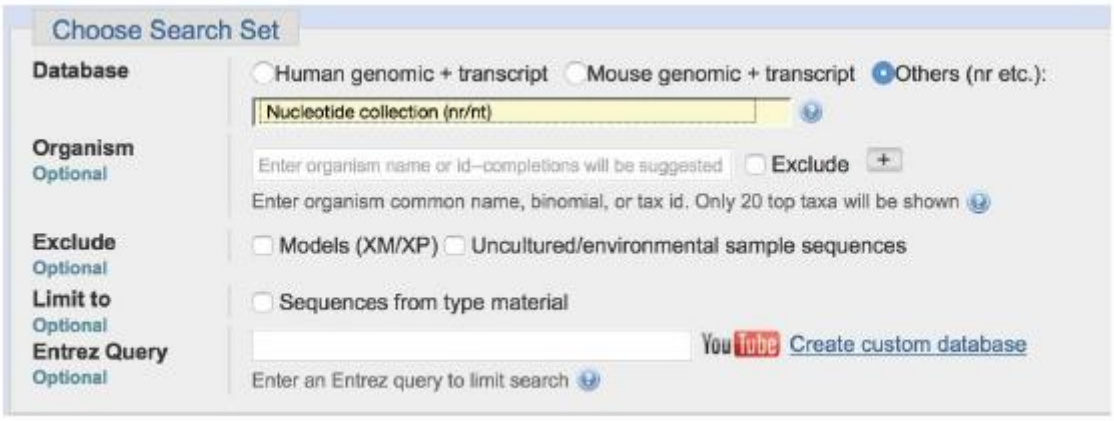


6. Enter the nucleotide sequence into the large box in the "Enter Query Sequence" section; be careful to type the following sequence exactly:

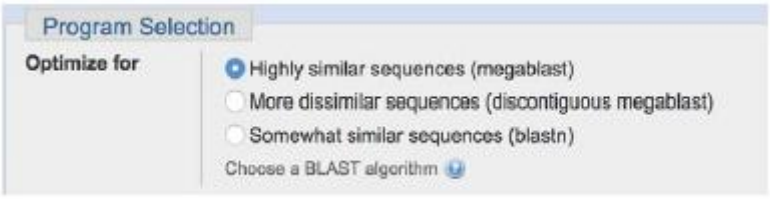
ggcaactgcccaaagtgtgatccagcctgtctcaacagaa



7. Under "Choose Search Set" make sure that "Others (nr etc.)" is selected and that "Nucleotide collection (nr/ nt)" is highlighted in the dropdown menu. The remaining entries should be left blank.



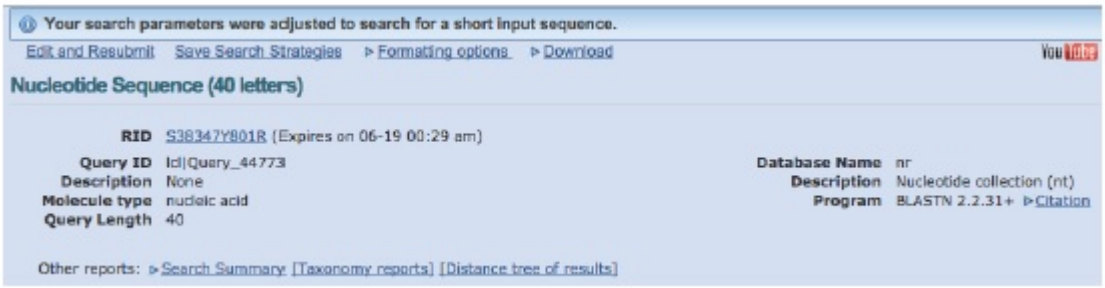
8. Under "Program Selection" select "High similar sequence (megablast)".



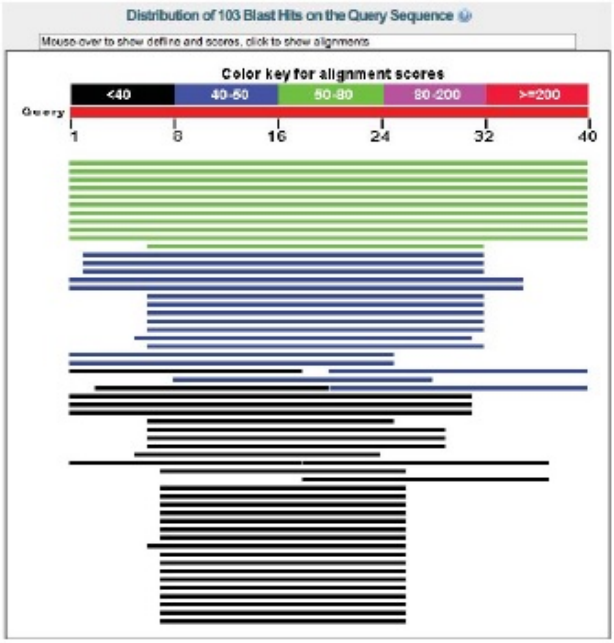
9. Click on the blue "BLAST" query box.

10. Once the "BLAST" query box has been clicked you will be assigned an ID#. Record this number so you can check your results at a later time.

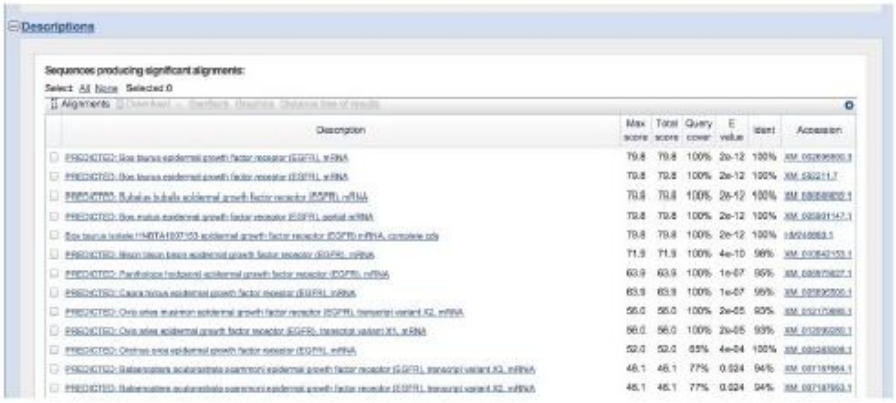
11. Examine the BLASTN search report. The report includes:
    a. Search Summary Report that shows an overview of the BLASTN search parameters.



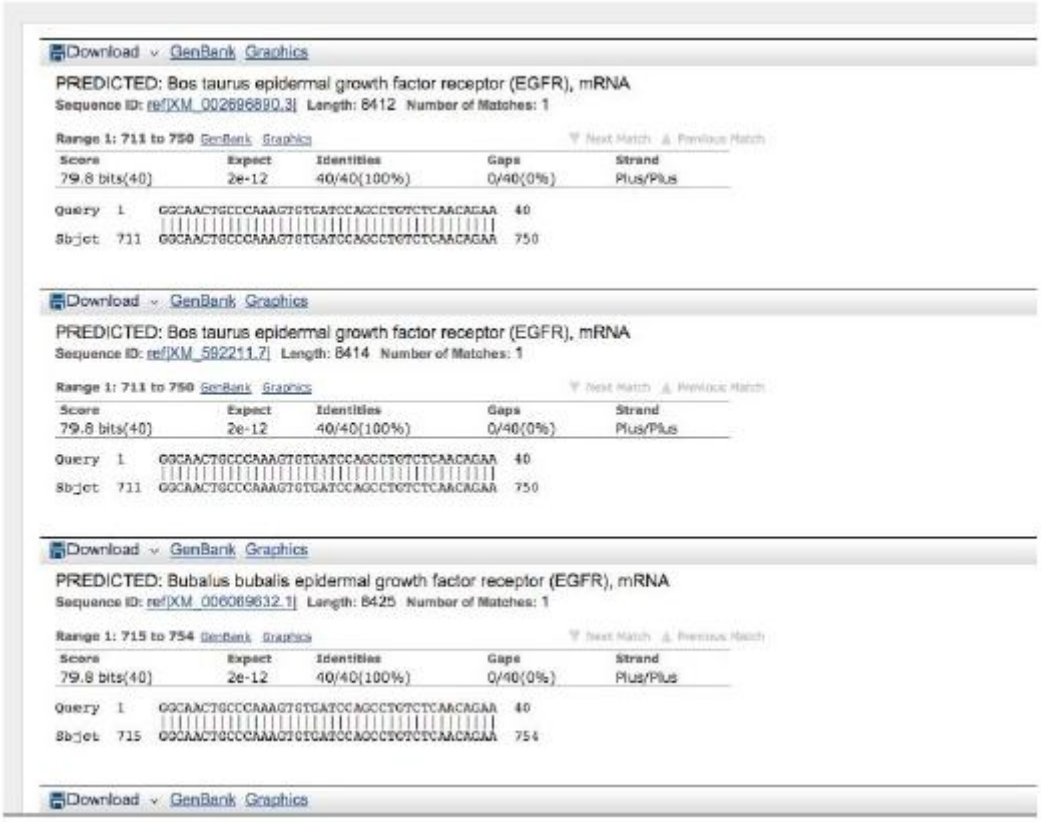
b. Graphic Summary section that shows the alignment of database matches to the query sequence. The color of the boxes corresponds to the score of the alignment with red representing the highest alignment scores.



c. Description section that shows all the sequences in the database with significant sequence homology to our sequence. By default the results are sorted according to the E-value but you can click on the column header to sort the results by different categories. Notice that there can be several different entries with identical high scores.



d. Alignment section that shows alignment blocks for each BLAST hit. Each alignment block begins with a summary that includes the Max score and expected value, sequence identity, the number of gaps in the alignment, and the orientation of the query sequence relative to the subject sequence.



12. Select a sequence to focus on in-depth. You can do this by clicking on a colored bar in the graphic section, clicking on the sequence name in the description section, or scrolling down to the alignment section. Then click on the sequence ID. This brings up additional information about the subject sequence, including the gene name, the genus and species of origin, and articles written about the gene. After performing this search, one of the top hits should be Bos taurus epidermal growth factor receptor (EGFR), mRNA. Sequence ID: ref|XM_002696890.3|. If top hit does not match, try re-entering the sequence. Be sure to double check the search parameters before the BLASTN searching.

B. EXERCISES

EXERCISE 1

Familiarize yourself with the autoradiograph by reading the DNA sequence for sample #1.

1. Start at the arrow and read up the gel for 20 nucleotides. Record the DNA sequence. Submit it to NCBI using the BLASTN program.

2. Start at the arrow and read up the gel for 30 nucleotides. Record the DNA sequence. Submit it to NCBI using the BLASTN program.

A few notes on reading a sequencing gel:

- Either enter the sequence directly into the query box or write the sequence down on a piece of paper and then enter it.
- It is critical that you do not confuse lanes when reading the sequence. The gel contains the A, C, G, T lanes from left to right.
- Reading a sequence gel requires that you read the nucleotides in the 5'—>3' direction. This can be accomplished by reading "up" the gel (from the bottom of the gel to the top).
- Notice that for the most part that the spacing and intensity of most of the bands is fairly constant. Ignore lightly colored bands and choose only the darker ones. Occasionally the sequence will be dark and all four lanes will be of relatively similar intensity. This is called a DNA sequencing compression and is common when there are stretches of G and C's. This type of pattern should be treated as an ambiguous position (see next note).
- If an exact band at a position is ambiguous, you can enter an N which denotes that it could be either A, C, G or T.

Results to be obtained for autoradiograph 1:

a. Do the BLASTN results for the first and second search resemble each other?

b. What is the name of this gene?

c. What organism's DNA was likely sequenced in this exercise?

EXERCISE 2

Now that you have familiarity with the entry and submission process, read the DNA sequence analysis from the autoradiograph corresponding to number 2. Notice that it is sometimes difficult to judge the spacing and strongest intensity of the band in each lane and therefore you need to use your best judgment.

1. Begin the exercise by reading the DNA sequence for sample number 2 approximately 6 cm from the bottom of the strip. The first 12 nucleotides should be: 5'...GGACGACGGTAT...3'.

2. Submit the sequence to NCBI using the BLASTN program.

3. After receiving the BLASTN results, scroll down to the alignment section and look at the entries that have nucleotide matches with your query sequence.

Some more notes on reading a sequencing gel and interpreting BLAST results:

- Remember that DNA sequence is always entered in the 5'—>3' direction.

- DNA is double stranded and contains a top (5'—>3') and bottom (3'—>5') strand (sometimes this corresponds to the coding and noncoding strands). When a query sequence is searched against the database both strands of the query are examined. The entered sequence is known as the plus strand and reverse complement of this sequence is known as the minus strand.

- As a general rule, identical nucleotide sequence spanning greater than 21 bp between two samples usually indicates that the sequences are related or identical.

Results to be obtained for autoradiograph 2:

a. What is the name of this gene?

b. What strand does the matched query sequence represent? What strand does the hit sequence represent?

EXERCISE 3

1. Read the DNA sequence from sample number 3. Start at the bottom of the strip and record the DNA sequence.

2. Submit the sequence to NCBI using the BLASTN program.

3. Click on the GenBank accession number of a sequence hit to access further information about the DNA sequence and/or gene.

Results to be obtained for autoradiograph 3:

a. What is the name of this gene?

b. Approximately how many base pairs does this gene have?

EXERCISE 4

This section demonstrates the interaction of two proteins encoded by two genes. Protein-protein interactions play a fundamental role in virtually every process in a living cell.

For example, signals from the exterior of a cell are mediated to the inside of that cell by the protein-protein interactions of the signaling molecules. This process, called signal transduction, is of central importance in many biological processes such as cell division and cytoskeleton formation. In this exercise, we will use DNA sequences to characterize two human genes.

1. Read the DNA sequence obtained from sample number 4. Start at the bottom of the strip and record the DNA sequence for around 30 base pairs.

2. Next, move approximately a third of the way up the strip (~14cm) and read a portion of this section of the DNA sequence.

3. For this exercise limit the database search to human genes. To do this go to "Choose Search Set" and select "Human genomic + transcript". See Guide to Using BLASTN step 7.

4. Submit each sequence section individually to the BLASTN program.

5. Once you have identified the name of these two genes, perform a general Internet search to collect more information about the two proteins.

Results to be obtained for autoradiograph 4:

a. This sample contains two DNA sequences (from bottom section and starting from the middle section). What are the corresponding names of these genes?

b. What are the functions of the two proteins encoded by these genes?

c. How do these two proteins interact in a living cell?

6. EXPERIMENTAL RESULTS

Below are the answers obtained by performing a BLASTN search with the sequences provided with this kit as of its publication (2015). Because the GenBank database is constantly being revised, we recommend that you run the below sequences through GenBbank prior to performing this lab with your students. If you observe difference in the answers, please contact Edvotek® Technical Support at 1-800-EDVOTEK or info@edvotek.com

#1 sequence (20 nucleotides) 5'....ACAAATAGTTACCTTGGAAC.....3'

#1 sequence (30 nucleotides) 5'.... ACAAATAGTTACCTTGGAACATCAAACGTG....3'

#2 sequence 5'...GGACGACGGTATGGAATAGAGAGGAAGTTCCTC...3'

#3 sequence 5'...GATTTGTAATGTAAGTGAATAAGGAATACCATCTA...3'

#4 sequence (bottom) 5'... CAGCTTGGGTGGTCATATGGCCATGGAG...3'

#4 sequence (middle)
5'...GAGACGGAGCTGTAGGTAAAACTTGCCTACTGATCAGTT...3'

6.1 Experimental Results

EXERCISE 1

A. The sequence matches should be close to identical for searches one and two but the values associated with each hit (max score, total score, e-value etc.) will be higher for the second search.

B. Replication factor C

C. Mus musculus (house mouse)

EXERCISE 2

A. UEV and lactate/malate dehyrogenase domains

B. The matched query sequence represents a plus strand (the entered sequence). The hit sequence represents a minus strand (the reverse complement sequence).

EXERCISE 3

A. Rho GTPase activating protein 5

B. 7933 bp

EXERCISE 4

A. The first DNA sequence is that of Bai1. The second sequence is that of Rac1.

B. Bai1 codes for BAI1, a brain specific angiogenesis inhibitor. Angiogenesis involves the growth of new blood vessels from pre-existing vessels, and is a normal process in growth, development, and wound healing. However, angiogenesis has been shown to be essential for growth and metastasis of solid tumors. In order to obtain blood supply for their growth, tumor cells are potently angiogenic. BAI1 is believed to inhibit new growth from blood vessel cells, thus suppressing growth of glioblastomas (malignant brain tumors). BAI1is also believed to function in cell adhesion and signal transduction in the brain. Rac1 codes for a small GTPase called RAC1. RAC1 acts as a molecular switch in signaling pathways that can switch signal transduction in a cell on and off. RAC1 is active or 'ON' when bound to GTP and inactive or 'OFF' when bound to GDP. The inactive form of RAC1 (GDP-form) is activated by the exchange of GDP for GTP by Guanosine Nucleotide Exchange Factors (GEFs). Inactivation of RAC1 is achieved by GTPase activating proteins (GAPs), which revert the conformation back to the inactive GDP-bound form through hydrolysis of the GTP.

C. In a living cell, after RAC1 is activated by GTP binding, it interacts with BAI1. This interaction at the cytoplasmic membrane is crucial to the function of BAI1, which is thought to be involved in neuronal growth. BAI1 also associates with other down-stream effectors of Rho small G proteins, which is associated with the formation of stress fibers and cytokinesis.

6.2 Study Questions

Answer the following questions in the lab notebook:

1. **What is DNA sequencing?**
2. **What does each band in the autoradiograph represent?**
3. **What is BLAST? Why is it considered a bioinformatics tool?**