UTM
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CELLULAR AND MOLECULAR BIOLOGY FOR BIOINFORMATICS
SEBB4173-01

CLONING CMB PROJECT
Hemagglutinin (HA) protein - Influenza virus

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Introduction

Influenza, commonly called flu, is an infectious and contagious disease caused by influenza viruses. There are 4 types of influenza virus: Influenza A virus (IAV), Influenza B virus (IBV), Influenza C virus (ICV), and Influenza D virus (IDV). In between these viruses, IAV and IBV always cause seasonal epidemics of disease known as flu season while IAV is the only influenza virus known to cause flu pandemic (i.e., global epidemics of flu disease).

IAV is classified into subtypes based on two viral proteins on the surface of the virus: hemagglutinin and neuraminidase. Hemagglutinin (HA) is an integral membrane glycoprotein that is found on the surface of influenza virus and it is integral to the virus infectivity. It has multiple functions that act as an attachment factor and membrane fusion protein. There are 18 subtypes of HA that have been discovered in IAV, named H1 until H18. A well-known example of influenza A virus, A(H1N1) is the subtype combination of hemagglutinin H1 and neuraminidase N1.

For the cloning method of cloning hemagglutinin gene, we will discuss and employ the polymerase chain reaction (PCR) amplification of gene of interest and plasmid vector, purification of DNA, enzyme restriction, gel electrophoresis, DNA ligation, selection and verification of the correct DNA clones.

Sequence of the chosen gene

In this report, we choose hemagglutinin (H1) encoding gene from Influenza A virus (H1N1) segment 4.

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   1 agcaaaagca ggggaaaata aaaacaacca aaatgaaggc aaacctactg gtcctgttat
  61 gtgcacttgc agctgcagat gcagacacaa tatgtatagg ctaccatgcg aacaattcaa
 121 ccgacactgt tgacacagtg ctcgagaaga atgtgacagt gacacactct gttaacctgc
 181 tcgaagacag ccacaacgga aaactatgta gattaaaagg aatagcccca ctacaattgg
 241 ggaaatgtaa catcgccgga tggctcttgg gaaacccaga atgcgaccca ctgcttccag
 301 tgagatcatg gtcctacatt gtagaaacac caaactctga gaatggaata tgttatccag
 361 gagatttcat cgactatgag gagctgaggg agcaattgag ctcagtgtca tcattcgaaa
 421 gattcgaaat atttcccaaa gaaagctcat ggcccaacca caacacaacc aaaggagtaa
 481 cggcagcatg ctcccatgcg gggaaaagca gtttttacag aaatttgcta tggctgacgg
 541 agaaggaggg ctcataccca aagctgaaaa attcttatgt gaacaagaaa gggaaagaag
 601 tccttgtact gtggggtatt catcacccgt ctaacagtaa ggatcaacag aatatctatc
 661 agaatgaaaa tgcttatgtc tctgtagtga cttcaaatta taacaggaga tttaccccgg
 721 aaatagcaga aagacccaaa gtaagagatc aagctgggag gatgaactat tactggacct
 781 tgctaaaacc cggagacaca ataatatttg aggcaaatgg aaatctaata gcaccaaggt
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1021 ggatggttac aggactaagg aacattccgt ccattcaatc cagaggtcta tttggagcca
1081 ttgccggttt tattgaaggg ggatggactg gaatgataga tggatggtac ggttatcatc
1141 atcagaatga acagggatca ggctatgcag cggatcaaaa aagcacacaa aatgccatta
1201 acgggattac aaacaaggtg aactctgtta tcgagaaaat gaacattcaa ttcacagctg
1261 tgggtaaaga attcaacaaa ttagaaaaaa ggatggaaaa tttaaataaa aaagttgatg
1321 atggatttct ggacatttgg acatataatg cagaattgtt agttctactg gaaaatgaaa
```

1381 ggactctgga tttccatgac tcaaatgtga agaatctgta tgagaaagta aaaagccaat

1441 taaagaataa tgccaaagaa atcggaaatg gatgttttga gttctaccac aagtgtgaca

1501 atgaatgcat ggaaagtgta agaaatggga cttatgatta tcccaaatat tcagaagagt

1561 caaagttgaa cagggaaaag gtagatggag tgaaattgga atcaatgggg atctatcaga

1621 ttctggcgat ctactcaact gtcgccagtt cactggtgct tttggtctcc ctgggggcaa

1681 tcagtttctg gatgtgttct aatggatctt tgcagtgcag aatatgcatc tgagattaga

1741 atttcagaaa tatgaggaaa aacacccttg tttctact

***Base 33 – 1733 (Bold), Gene = "HA".**

Size of the DNA and protein of interest

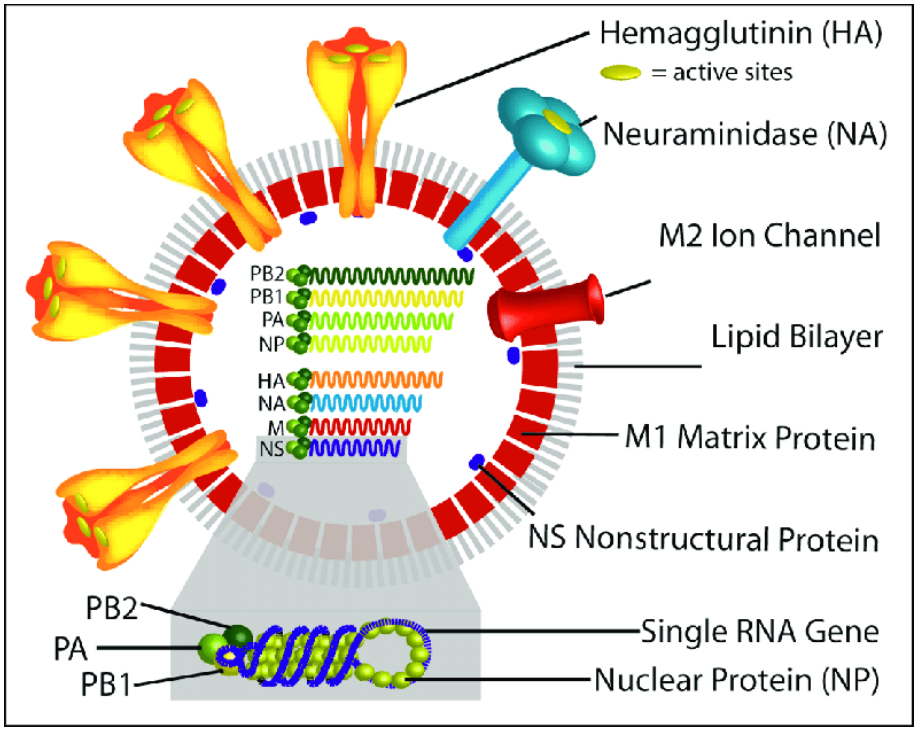

Figure 1

Influenza A virus, Hemagglutinin is a spherical size of about 100 nm in diameter. Hemagglutinin are found on the viral surface as trimeric forms coded from a gene segment. There are eight single-stranded RNA segments that encode 12 viral proteins. Influenza virus HA is coded by RNA segment 4. The HA glycoprotein of influenza virus is an integral membrane protein that has a cylindrical shape with approximate dimensions of 135 A (length) x 35–70 A (radius). The complete sequence of a hemagglutinin gene of influenza is 1768 nucleotides long. HA has a predicted molecular mass of 59 kDa. The location of the gene is at 33 to 1733. So, the size of the gene is 1700.

What disease is related to the gene or protein?

Outbreaks of influenza caused by influenza A viruses have continued to occur in birds and mammals including humans. Each year, seasonal influenza virus infects up to 100 million people worldwide, causing serious illness in 3 to 5 million people and 250,000–500,000 deaths. Diseases that are related to Hemagglutinin (HA) protein such as H1N1. Influenza A(H1N1)pdm09 virus has evolved continually since its emergence in 2009. For influenza virus strains, genetic changes occurring in the HA1 domain of the hemagglutinin cause the emergence of new variants. CDC has determined that this swine influenza A (H1N1) virus is contagious and is spreading from human to human. Swine Influenza is a respiratory disease of pigs (swine) caused by type A influenza virus that regularly causes outbreaks of flu in pigs. Like all influenza viruses, swine flu viruses change constantly

Another disease is influenza H9N2 viruses which are an infection with a rare variant of the virus that causes avian influenza, or bird flu and isolated from poultry flocks in Morocco. Despite the fact that H9N2 is considered as low pathogenic virus, it has been reported that several microbial agents of the respiratory tract and some environmental factors exacerbate IAV H9N2 infections, leading to very severe respiratory disease and causing mortality up to 65 % in broiler chickens and up to 70 % drop in egg production in layers and breeders. It has also been reported that IAV H9N2 affected many species of domesticated and wild birds such as falcon, partridge, quail, houbara, pigeon, sparrow and other species. In Pakistan, sparrows were found to play a very important role in the transmission of the virus between farms.

H3N8 equine influenza virus from the Swedish epizootic of 1991 differ from those representative of the Swedish vaccine strain in hemagglutination inhibition tests. The amino acid sequence of the hemagglutinin (HA) of an isolate from the 1991 outbreak was deduced from the nucleotide sequence and comparison was made to the strain. Twenty-three amino acid substitutions were found, 10 mapping onto areas of the HA known to bind antibodies in human H3 influenza viruses. The amino acid changes together with the serological data suggest that a major antigenic drift has taken place in equine H3N8 viruses in Sweden and we conclude that recent strains of the virus must be incorporated into vaccines on a regular basis if epizootics of equine influenza are to be controlled in the future. The clinical symptoms in unvaccinated horses

were typical of classical H3N8 equine influenza with coughing, pyrexia, rhinitis and general loss of condition.

Next, low pathogenic avian influenza subtype H9N8 was diagnosed on a Korean native chicken farm in Gyeonggi province, South Korea, in late April 2004. Clinical signs included H9N2 isolated from Korean poultry.Poultry are not the natural hosts of this virus, but infections moderate respiratory distress, depression, mild diarrhoea, loss of appetite and a slightly elevated mortality (1.4% in 5 days). Pathologically, mucopurulent tracheitis and air sacculitis were prominently found with urate renal deposition. The isolated H9N8 had an Ala-Ser-Gly-Arg (A/S/G/R) motif at the cleavage site of haemagglutinin, which has been commonly found in with AIVs produce a variety of syndromes ranging from asymptomatic, to respiratory disease with low mortality, to highly pathogenic forms with high mortality.

In conclusion, Influenza viruses have developed ways to evade the body's immune response using an antigenic variation known as antigenic shift (replacement of HA and NA antigens with novel subtypes from non influenza viruses) and drift (mutations within antibody-binding sites in HA and or NA). Because of new influenza viruses constantly emerging from antigenic shift and drift, new influenza vaccines are required each year. Human-to-human transmission of influenza occurs each winter and early spring through small-particle aerosols or droplets. The influenza virus attacks epithelial cells of the upper and lower respiratory tract, with the potential for secondary bacterial infection and acute respiratory distress syndrome (ARDS). The symptoms of influenza infection include fever, headache, cough, sore throat, myalgia, and nasal congestion. Lower respiratory tract manifestations such as pneumonia and bronchiolitis are virtually indistinguishable from other viral infections. Children with certain comorbidities, such as chronic lung disease and severe neurologic impairment, are at higher risk of influenza-related complications. The most reliable test for influenza is reverse transcription polymerase chain reaction (RT-PCR). Rapid antigen tests have lower sensitivity and specificity and are not reliable during periods of low influenza activity. Antiviral treatment with NA inhibitors can shorten the duration of fever, symptoms, and hospitalization, especially when started within 48 hours of influenza illness onset. Prevention of influenza through annual influenza vaccination is recommended for all children 6 months of age and older. The vaccines contain three or four influenza subtypes, chosen depending on the circulating strains. The two formulations approved

for children are the inactivated influenza vaccine (IIV) and live-attenuated influenza vaccine (LAIV).

What is the role of the gene/protein of interest in the pathogenesis?

The genome of influenza viruses is segmented, with negative sense RNA encapsulated by a virally defined nucleoprotein. There are three varieties of influenza viruses that cause sickness in humans, type A, type B, and type C, which are distinguished by the serological reactivity of internal proteins. Influenza virus can be subtyped further based on the serological reactivities of its surface antigens, HA and NA, which are encoded on different genomic segments.

The Hemagglutinin (HA) protein is a type 1 membrane glycoprotein that binds to sialic acid-containing cell surface receptors. The HA protein facilitates the fusing of the viral and cellular membranes after endocytosis by the host cell. When an influenza virus infects a host cell, the nascent HA protein is synthesised as a precursor polypeptide (HA0). Then, the host proteases break this precursor at a conserved arginine residue to form two subunits, HA1 and HA2, which are connected by a single disulphide bond. The cleavage of precursor polypeptide is required to activate HA protein's membrane fusion potential. Similar cleavage activation of viral polypeptides is required for a range of viruses, including HIV and parainfluenza virus, to enter the host cells. Tissue tropism of influenza viruses and their ability to spread systemically are thus dependent on the availability of functional HA receptors on the host cell, the distribution of host proteases capable of cleaving HA0, the cleavage properties of the HA0 precursor, and the cell's permittivity for viral replication.

Besides that, the antigenic glycoprotein hemagglutinin (HA) or haemagglutinin (BE) is present on the surface of influenza viruses and it is in charge of binding the virus to the infected cell. The process goes like this, firstly, Hemagglutinin (HA) binds to the monosaccharide sialic acid found on the surface of its target host cells. The virus is subsequently engulfed by the cell membrane via endocytosis, resulting in the formation of an endosome. The cell next tries to break down the endosome's contents by acidifying its interior and changing it into a lysosome. When the pH of the endosome falls below 6.0, the HA molecule partially unfolds, revealing a particularly hydrophobic section of its peptide chain that had previously been contained within the protein. This so-called "fusion peptide" behaves like a molecular grappling hook which enters itself into the endosomal membrane and fastens on. The rest of the HA molecule then refolds into a new form and drags the endosomal membrane right up close to the virus particle's

own membrane, allowing the two to merge. Once this occurs, the viral RNA genome enters the cell's cytoplasm.

Moreover, due to the obvious presence of particular receptors on host cells, the HA protein is essential in the host range and tissue tropism of distinct influenza subtypes. The host range of a given influenza subtype, however, is also limited by inhibitors of receptor binding that are present in the host. These inhibitors, which are found in serum and other bodily fluids, prevent the virus from binding through steric hindrance or by competitively inhibiting the HA's receptor-binding site. Thus, the host specificity of influenza viruses is controlled by a number of viral genes, as well as their interactions and roles in various cellular conditions.

Cloning methods employed

PCR Amplification of the Gene of Interest and Plasmid Vector

Polymerase Chain Reaction (PCR) is a simple laboratory technique that amplifies a DNA template to produce specific DNA fragments in vitro. This method only requires around two hours compared to traditional methods of cloning a DNA which requires days to weeks to amplify the DNA sequences.

PCR set up requires:

- DNA template (concentration within 30 ng to 50 ng)
- Primers (15-30 bp long for both reverse and forward primer)
- DNA precursors (generally 0.2 mM of deoxynucleoside triphosphates, dNTPs)
- Magnesium ion (using $MgCl_2$ with concentration 1 to 4 mM)
- Taq polymerase enzyme (obtained from Thermus aquaticus)
- Buffer Solution (8.0 to 9.5 pH of KCl buffer stabilized by Tris-HCl)

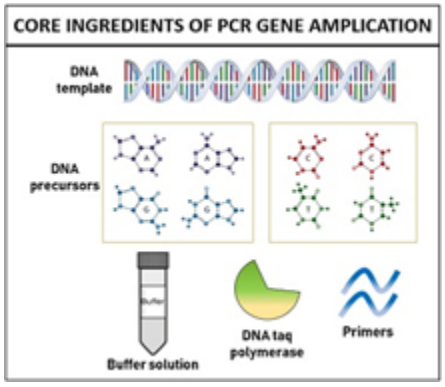

Figure 2

PCR method consists of 3 basic steps which are:

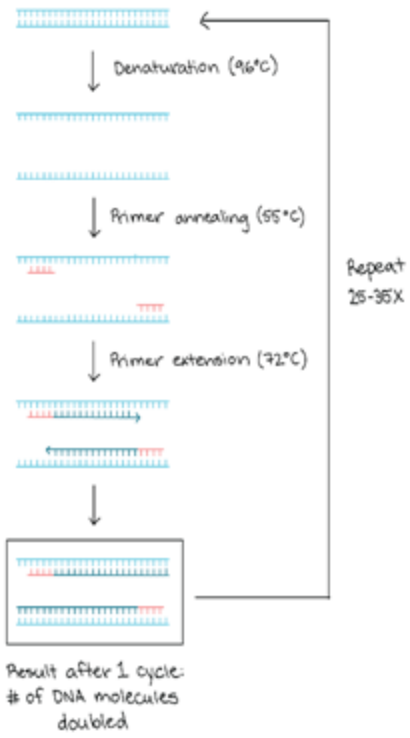

Figure 3

1. Denaturation (96ºC) – DNA strands are separated or denatured by strongly heating the reaction. This step will produce a single-stranded template to be used in the next step.

2. Annealing (55-65ºC) – The reaction is cooled to let the primers bind to their complementary sequences on the single-stranded template DNA.

3. Extension (72ºC) – The temperature of the reaction is raised so that Taq Polymerase extends the primers and synthesizes new strands of DNA.

This cycle repeats 25 to 35 times and it can take 2 to 4 hours depending on the length of the DNA region that needs to be copied.

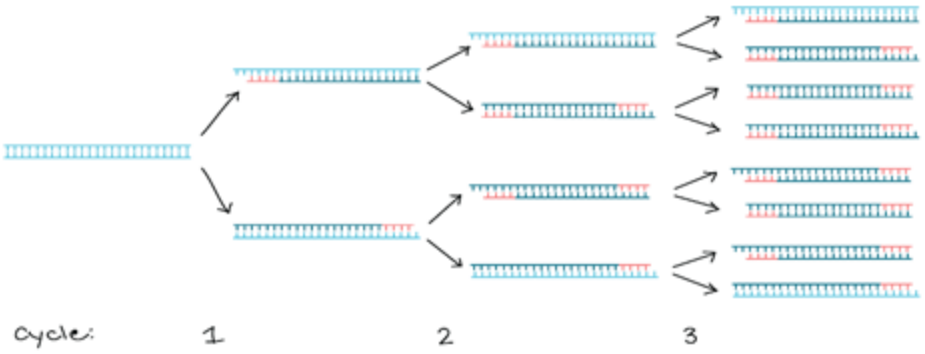

Figure 4

Purification of DNA

1. The sample obtained is spun at high speed and the larger or heavier components ground at the bottom of the tube due to centrifugal force.
2. A solution containing the fragments of the cell wall (small molecule) and the DNA (gigantic molecule) will be obtained after the cell wall of bacteria is destroyed by lysozyme and detergents.
3. To centrifuge, DNA and some other large components are hurled to the bottom of the tube and the garbage from the destruction of the cell wall will remain in solution then will be discarded.
4. The sedimented DNA is then redissolved. But, lots of protein and RNA are mixed in it.
5. Phenol (a very corrosive and extremely dangerous acid) is used to dissolve and denature the proteins in the DNA sample. By adding water to phenol, a separate layer below the water will be formed by the denser phenol.
6. The mixture is shaken to mix the two layers temporarily. Once the shaking is stopped, the layers are separated again. Almost all of the proteins are dissolved by the phenol, but the water layer still contains nucleic acids, DNA and RNA.
7. Centrifuge it briefly to ensure a good separation of the layers.
8. DNA and RNA in the water layer will be sucked off and stored.

9. An enzyme named ribonuclease (RNase) is used to get rid of the RNA. It will convert RNA into tiny fragments but DNA remains unchanged as giant macromolecules.

10. An equal volume of alcohol is added and it is dissolved in the water so enthusiastically that all of the water is occupied and the larger and less soluble DNA are pushed out of solution. Only the small RNA fragments remain dissolved.

11. Centrifuge it again and DNA is sedimented to the bottom of the tube and the solution containing the RNA fragments can be poured off.

12. Tiny pellets of DNA left at the bottom of the tube are often scarcely visible but billions of DNA molecules are contained and sufficient for most experiments. The DNA is redissolved and ready to be used.

Restriction enzyme treatment on DNA

Restriction enzymes found in bacteria that have been used to defend them from phages. Restriction enzymes act like scissors and cut foreign DNA like phages to unactivated it. However, the restriction enzyme does not cut the foreign DNA randomly. They will cut at a specific area called **restriction sites**. Restriction enzymes will make a double-stranded cut in DNA molecules. EcoRI is one of the examples for restriction enzymes. EcoRI recognizes DNA palindromes where both strands have the same nucleotide sequence but in antiparallel orientation.

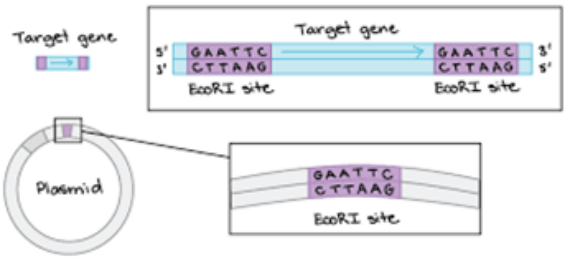

Figure 5

Enzyme EcoRI will cut within the sequence but in pair of staggered cuts between the G and A nucleotides as follows:

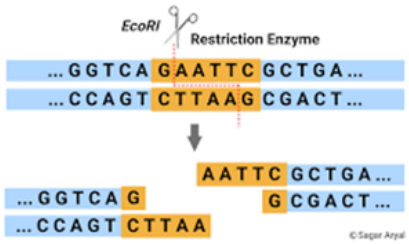

Figure 6

The EcoRI restriction enzyme will produce a pair of identical single-stranded sticky ends. Production of the sticky ends makes them suitable for recombinant DNA. It is called sticky ends because they can stick to a complementary sequence by **hydrogen bonding**. If two different pieces of DNA were cut with the same restriction enzyme, both will produce fragments with the same complementary sticky ends and make it possible for the two different pieces of DNA to be bound together by matching the sticky ends. Even though the hydrogen bond between the pieces of DNA is only temporary, this gives time for the permanent bonding of the sugar-phosphate backbone (ligation).

Separation of the DNA (electrophoresis)

Agarose gel electrophoresis is a technique used to separate DNA (and RNA and proteins) fragments based on their size and charge. Electrophoresis involves running a current through a gel containing the molecules of interest. The molecules will travel through the gel in different directions or at different speeds based on their size and charge and allowing them to be separated from one another. However, since DNA has the same amount of charge per mass, gel electrophoresis of DNA fragments separates them based on size only.

The gels are made of Jelly-like material made out of a polysaccharide called agarose. When a hot solution of agarose cools, it congeals to form a meshwork like gelatin. At the molecular level, the gel is a matrix of agarose molecules that are held together by hydrogen bonds and form tiny pores.

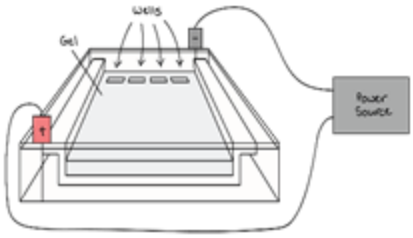

Figure 7

At one end, the gel has pocket-like indentations called wells, which are where the DNA samples will be placed. At the main body of the box is where the gel is placed and filled with a salt-containing buffer solution that can conduct current. One well is reserved for **DNA ladder**, a standard reference that contains DNA fragments of known lengths.

The power to the gel box is turned on and current is passing through the gel, the gel is said to be running. The DNA molecules have a negative charge because of the phosphate groups in their sugar-phosphate backbone, so they start moving through the matrix of the gel towards the positive pole. The shortest pieces of DNA will be close to the positive end of the gel while the longest pieces of DNA will remain near the wells.

Since both DNA and agarose are colourless, a type of stains is used to locate DNA fragments for example SYBR green. SYBR green stained DNA green if viewed under UV light.

Procedure to prepare gel:

1. Weigh out an appropriate mass of agarose into a conical flask. Agarose gels are prepared using w/v percentage solution. The concentration of agarose in a gel will depend on the sizes of the DNA fragments to be separated, with most gel ranging between 0.5% to 2.0%.

2. Add a running buffer, TBE (45mM Tris-borate, 1 mM EDTA) to the agarose- containing flask. The volume of the buffer should not be greater than 1/3 of the capacity of the flask. Swirl to mix.

3. Melt the agarose and buffer mixture by heating with the Bunsen burner for 30 s. Then remove the flask and swirl the contents to mix well. Repeat until the agarose has completely dissolved.

4. Add SYBR green to a concentration of 0.5 μg/ml. Alternatively, the gel may also be stained after electrophoresis in a running buffer containing 0.5 μg/ml SYBR green for 15 to 30 minutes, followed by destaining in the running buffer for an equal length of time.

5. Allow the agarose to cool either on the benchtop or by incubation in a 65 °C water bath. Failure to do so will warp the gel tray.

6. Place the gel tray into the casting apparatus and tape the open edges of a gel tray to create a mold. Place an appropriate comb into the gel mold to create the wells.

7. Pour the molten agarose into the gel mold. Allow the agarose to set at room temperature.

Procedure to setting up gel apparatus and separation of DNA fragments:

1. Add loading dye (help track DNA and allow DNA to sink into the gel) to DNA samples.

2. The chamber connected electrodes with a power supply of 5V.

3. Add enough running buffer to cover the surface of the gel.

4. Attach the leads of the gel box to the power supply and turn it on.

5. Remove the lid and carefully load the DNA sample into the gel.

6. Replace the lid to the gel box.

7. Turn on the power and run the gel until the dye has migrated to an appropriate distance.

Observing separated DNA fragments:

1. Turn off the power supply after the electrophoresis has completed and remove the lid of the gel box.

2. Remove gel from the gel box and drain off excess buffer from the surface of the gel. Place the gel tray on paper towels to absorb any extra running buffer.

3. Remove the gel from the gel tray and expose the gel to UV light. DNA bands should show up as green fluorescent bands. Take a picture of the gel.

4. The DNA bands are visualized in each lane corresponding to a chamber wall. The DNA ladder that was loaded is also visualized and the length of the DNA bands can be estimated.

5. The size of DNA fragments is measured and the preferred size and gene sequence of Hemagglutinin HA oncogene are cut out from the gel.

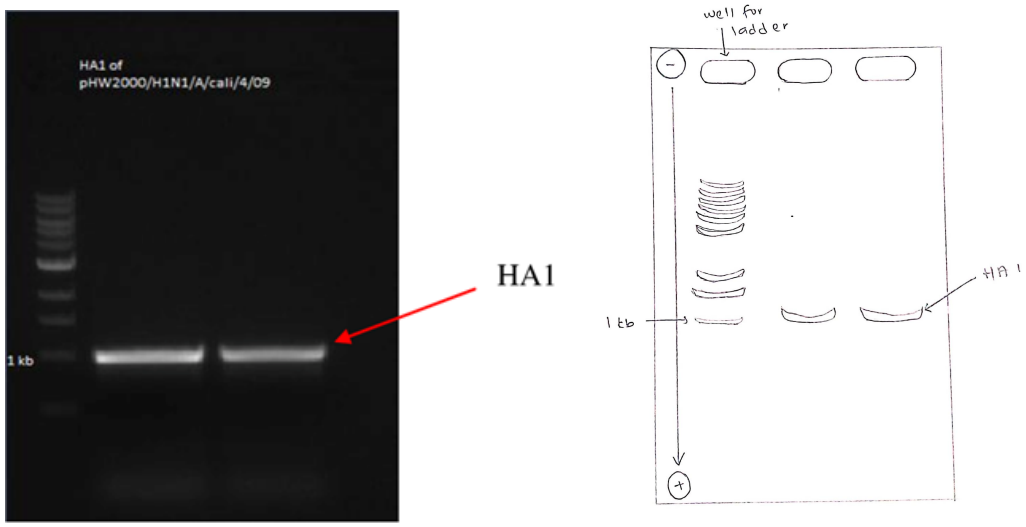

Figure 8

DNA Ligation

This step is carried out to join together two DNA fragments with matching ends by using the enzyme, DNA ligase. DNA ligase can be grouped into two families – ATP-dependent ligases and NAD^+-dependent ligases (Shuman, 2009). Other than using DNA ligase, DNA also can be ligated by using topoisomerase and site-specific recombination. The most common method used is the T4 DNA ligase, as it allows the ligation of sticky end or blunt end.

In this procedure, we use the pGEX-4T-1 plasmid which has multiple cloning site (MCS) that give a wide choice of restriction enzymes to be used due to many recognition sites available in the plasmid.

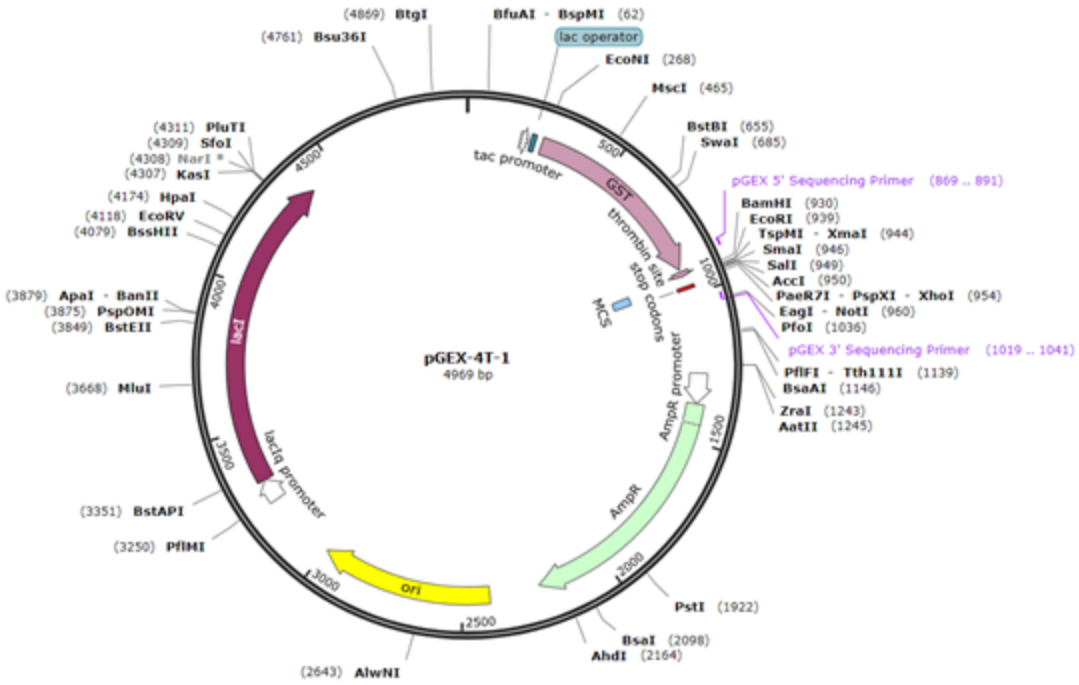

Figure 9, plasmid pGEX-4T-1 (SnapGene, n.d)

1. Plasmid was cut using the restriction enzyme, EcoRI to ensure it has the same ends as the gene.
2. In a microcentrifuge tube on ice, 5µL pGEX-4T-1, 10µL HA PCR product, 2µL H_2O, 2µL 10X T4 DNA ligase buffer and 1µL T4 DNA ligase.
3. The reaction was mixed gently by pipetting up and down and microfuge briefly.
4. Incubated at 16℃ overnight or at room temperature for 10 minutes.
5. Heated inactivate at 65℃ for 10 minutes.
6. Chilled on ice, 1-5µL of the reaction was transformed into 50µL competent cells, which in this procedure is E.coli Rosetta 2(DE3) cells.

Selection for the correct clones and verification

To check whether the right HA protein DNA has been inserted in the vector, we use the Brute force (physical) and ignorance approach to detect it.

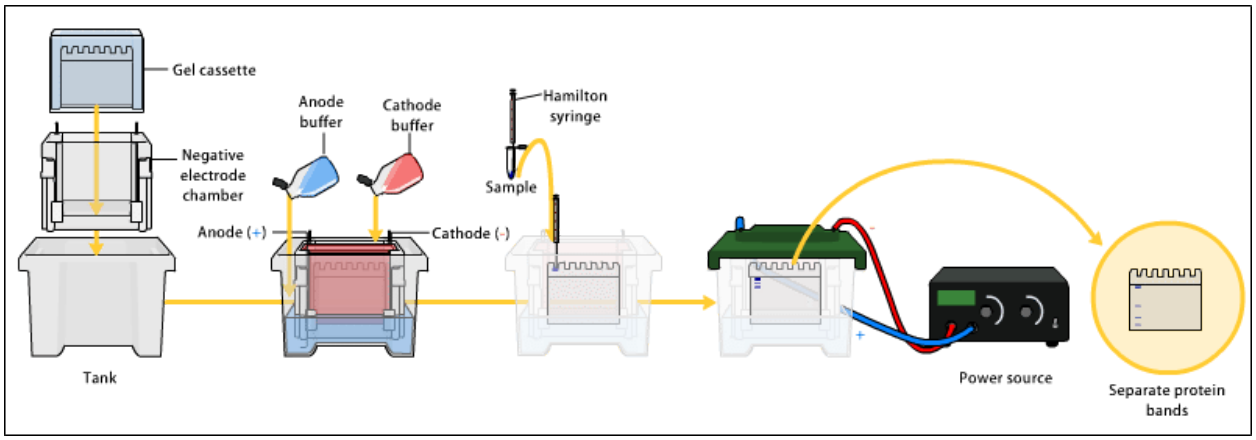

Figure 10

1. 5 µg of purified H1-HA protein was separated and transferred to a polyvinylidene fluoride (PDVF) membrane.
2. 5% bovine serum albumin (BSA) in phosphate buffered saline (PBST) was used to block the membranes for 1 hour.
3. 500 ng 5'-biotinylated selected aptamers in PBST was added and incubated for 1 hour.
4. The membranes were washed with PBST for 5 minutes, for four times.
5. The membranes were incubated with streptavidin-HRP for 1 hour.
6. Washed, then the enhanced chemiluminescence (ECL) was prepared. The membrane was incubated for 1-2 minutes.
7. The membranes was exposed to LAS 4000.

Conclusion

Influenza virus is a virus that is transmitted among living things either mankind or animal, adults or child. From the disease history of influenza virus, there are various sorts and varieties of influenza virus with different subtypes of essential proteins and their encoding gene. During research, we can observe that the name and information of influenza virus are labelled with virus type, country, strain number, year and patient age or animal type. This means that information above are the important factors that should be taken into consideration to observe gene revolution. Influenza virus is able to mutate and modify its encoding gene itself to adapt to its environment, maintain its infectivity, increase its immunity and other properties, so there are also different types of vaccine, antibody and agent like inhibitor invented to handle the influenza virus from time to time.

Infectivity of influenza virus is significantly dependent on two major viral proteins, hemagglutinin and neuraminidase. IAV and IBV are well known to cause seasonal flu disease because of the presence of these two proteins. In the molecular biology field, it is important for us to know the properties and characteristics of targeted proteins or genes before solving a problem. For example, the scientist took into consideration the infectivity characteristic of HA protein, aiming HA protein to invent its agent, antibody or vaccine to treat the disease.

Living things keep on experiencing evolution from a long time ago until now, genetic genes play an important role in it. We should appreciate scientists' contribution to genetic engineering and the genetic field that protects life from many fatal disease viruses. Looking forward to the future, we should learn and pass on the dedicative spirit of scientists to future generations to improve human life and living things' genetic quality.

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