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Recombinant DNA Techniques

8 September 2009

Human Factor X

                Coagulation is a process that most people fail to understand or appreciate.  It is necessary for the vitality of animals and humans.  In order to coagulate, Factor X (also known as the Stuart factor) is a necessity.  This protein reacts with prothrombin to make thrombin.   Thrombin, in the coagulation cascade (Figure A) (Raber), reacts with fibrinogen to make fibrin, the cross-linked building blocks that form a blood clot.



Figure : Coagulation Cascade

The primary goal of our project is to create a bacterial system that will produce the Factor X enzyme in order to facilitate the reaction of prothrombin to thrombin in the coagulation cascade.   We may then be able to enter this part in the Biobricks library.

In order to create the Factor X in bacteria, we must complete the following steps:

1. We located the Factor X mRNA sequence in GenBank (Accession number is K01886), but we will only be using the sequence for the portion of the gene that encodes the active X factor (Xa). The sequence for Xa is:

ATCGTGGGAG GCCAGGAATG CAAGGACGGG GAGTGTCCCT GGCAGGCCCT GCTCATCAAT GAGGAAAACG AGGGTTTCTG TGGTGGAACC ATTCTGAGCG AGTTCTACAT CCTAACGGCA GCCCACTGTC TCTACCAAGC CAAGAGATTC GAAGGGGACC GGAACACGGA GCAGGAGGAG GGCGGTGAGG CGGTGCACGA GGTGGAGGTG GTCATCAAGC ACAACCGGTT CACAAAGGAG ACCTATGACT TCGACATCGC CGTGCTCCGG CTCAAGACCC CCATCACCTT CCGCATGAAC GTGGCGCCTG CCTGCCTCCC CGAGCGTGAC TGGGCCGAGT CCACGCTGAT GACGCAGAAG ACGGGGATTG TGAGCGGCTT CGGGCGCACC CACGAGAAGG GCCGGCAGTC CACCAGGCTC AAGATGCTGG AGGTGCCCTA CGTGGACCGC AACAGCTGCA AGCTGTCCAG CAGCTTCATC ATCACCCAGA ACATGTTCTG TGCCGGCTAC GACACCAAGC AGGAGGATGC CTGCCAGGGG GACAGCGGGG GCCCGCACGT CACCCGCTTC AAGGACACCT ACTTCGTGAC AGGCATCGTC AGCTGGGGAG AGGGCTGTGC CCGTAAGGGG AAGTACGGGA TCTACACCAA GGTCACCGCC TTCCTCAAGT GGATCGACAG GTCCATGAAA ACCAGGGGCT TGCCCAAGGC CAAGAGCCAT GCCCCGGAGG TCATAACGTC CTCTCCATTA AAG

1. We will order the following primers (see attachment for information on creating primers):

Forward Primer #1:

 5’ **TCTAG**ATGATCGTGGGAGGCCAGG 3’

Reverse Primer #1:

5’ **TGATCA**CTTTAATGGAGAGGACGTT 3’

Forward Primer #2:

5’ TGGTGGAACCATTCTGAGCGAGTT 3’

Reverse Primer #2:

5’ CTCCTTTGTGAACCGGTTGTGCTT 3’

1. We will isolate and purify Human RNA from a blood sample from Patient A using RNA Isolation from Human Peripheral Blood protocol (Genomic Medicine Biorepository).
2. We will run RT-PCR.
3. We will run an Agarose gel to verify correct size of gene. Should be approximately 752 base pairs.
4. We will purify the PCR product.
5. We will set up the two restriction digests for your PCR product and vector using the XbaI and SpeI restriction enzymes.



1. We will clean up both digests.
2. We will set up and complete the ligation. We will try to be using the pBluescript SK+ vector because it has many restriction sites and also because it allows colonies containing the gene of interest to be easily recognized due to the blue color.
3. We will amplify out product by running a 2nd PCR with 1st PCR product and the following primers that need to be ordered (see attachment for information on creating primers):

Forward Primer #3:

5’ **GAATTCGCGGCCGCTTCTAG**ATGATCGTGGA 3’

Reverse Primer #3:

5’ **TACTAGTAGCGGCCGCTGCAG**CTTTAATAAG 3’

1. We will purify the PCR product.
2. We will set up another two restriction digests for your PCR product and vector using the EcoRI and PstI restriction enzymes.



1. We will clean up both digests.
2. We will set up and complete the ligation. We again are using the pBluescript SK+ vector.
3. We will complete the transformation using the heat shock method.
4. We will streak LB plates with the vectors (blank and ligated).
5. We will observe blue colonies.
6. We will extract proteins from E. coli using the “Analytical Protein Extraction” protocol (Mike).
7. We will run one of our tests (see below) using the extract.

                To determine if the Factor X enzyme has been in fact created and is working properly, we can run multiple tests.

The first test that we could use to prove our validity would be to obtain three equal amounts of blood where one will be exposed to a quantitative amount of the bacterial extract containing Factor X, another containing the untransformed bacterial extract which will be the negative control, and the last which will be regular blood containing no extract at all.  In theory, the blood containing our Factor X should coagulate more readily than both of the controls due to the increase of the Factor X clotting fact, which should then create an increased amount of the other coagulation agents (thrombin and fibrin) and an increased shift in the coagulation cascade. If one of the negative control coagulates at the same rate or a faster rate than our clone, than we can assume that our control has no effect on the rate of coagulation. If the pure blood coagulates at the fastest rate, we can predict that our clone and the untransformed extract had a negative effect on the coagulation rate.

Second, we could add a His-tag to our created protein and run a Western Blot, which would not show us if our protein was working, but it would show us that it had in fact been created. From there we could purify this protein from the blot and use the pure protein extract in conjunction with another test that may have failed, so that we are able to find out that our protein was made correctly, but that it was just not functioning properly.

If time allows, we also have other options we can complete. First, We could clone the human factor V (also known as labile factor). Factor V is necessary in the coagulation cascade by working with Factor X to turn prothrombin to thrombin. We could clone the Factor V using relatively the same process and compare the effects of adding this factor to blood alone and in conjunction with Factor X.

Second, as a secondary option to extracting the proteins from *E. coli*, we could add a specific peptide signal, such as the ompA signal peptide (Takahara, Hibler and Barr), to our Factor X, which would allow the protein to be secreted outside of the membrane of the *E. coli*. This would not require us to extract any proteins ourselves and would allow us to place the transformed *E. coli* into our blood samples, therefore simplifying all testing methods.

The great importance of Factor X cannot be under minded.  Its significance can be depicted in people who have a Factor X, or Stuart-Prower, deficiency (National Hemophila Foundation).  In the rare, even when someone inherits this Autosomal recessive deficiency, hemorrhagic symptoms are prominent.  The individual may have frequent nosebleeds, easy bruising, soft tissue hemorrhages, and bleeding of the joints.  It is even thought by researchers that the entire absence of Factor X is incompatible to life.

Today, virtually the only way to help patients with this condition is to give them a transfusion of blood plasma.  The major dilemma is this is only a quick fix as the body quickly uses up the Factor X.  If successful, this experiment may be the beginnings of a solution to this problem.  By directly giving a patient Factor X, most likely intravenously or possibly via the capillaries, there would no longer be a need for blood transfusions and cauterizations.  The patient would no longer have to live their entire life in defense.  In order to complete such a task, much study and research would have to go on after this project is finished.  Researchers would have to perfect the procedure and ultimately mass-produce a product that can help Factor X deficient people all over the world.

ATTACHMENT:

How we found our primers:

1. We looked at our specific Xa gene sequence and wrote out the first and last 20 (approx) base pairs of the sequence:

ATCGTGGGAG GCCAGGAATG CAAGGACGGG GAGTGTCCCT GGCAGGCCCT GCTCATCAAT GAGGAAAACG AGGGTTTCTG TGGTGGAACC ATTCTGAGCG AGTTCTACAT CCTAACGGCA GCCCACTGTC TCTACCAAGC CAAGAGATTC GAAGGGGACC GGAACACGGA GCAGGAGGAG GGCGGTGAGG CGGTGCACGA GGTGGAGGTG GTCATCAAGC ACAACCGGTT CACAAAGGAG ACCTATGACT TCGACATCGC CGTGCTCCGG CTCAAGACCC CCATCACCTT CCGCATGAAC GTGGCGCCTG CCTGCCTCCC CGAGCGTGAC TGGGCCGAGT CCACGCTGAT GACGCAGAAG ACGGGGATTG TGAGCGGCTT CGGGCGCACC CACGAGAAGG GCCGGCAGTC CACCAGGCTC AAGATGCTGG AGGTGCCCTA CGTGGACCGC AACAGCTGCA AGCTGTCCAG CAGCTTCATC ATCACCCAGA ACATGTTCTG TGCCGGCTAC GACACCAAGC AGGAGGATGC CTGCCAGGGG GACAGCGGGG GCCCGCACGT CACCCGCTTC AAGGACACCT ACTTCGTGAC AGGCATCGTC AGCTGGGGAG AGGGCTGTGC CCGTAAGGGG AAGTACGGGA TCTACACCAA GGTCACCGCC TTCCTCAAGT GGATCGACAG GTCCATGAAA ACCAGGGGCT TGCCCAAGGC CAAGAGCCAT GCCCCGGAGG TCATAACGTC CTCTCCATTA AAG

First: ATCGTGGGAGGCCAGG

Last: TAACGTCCTCTCCATTAAAG

1. We then needed to add XbaI (**TCTAGA**) as a restriction site and a start codon (ATG) because our mRNA did not include a start codon to begin transcription. We added these two sequences to the first base pairs in the gene sequence to create forward primer #1:

 5’ **TCTAG**ATGATCGTGGGAGGCCAGG 3’

1. We found the reverse complement to the last base pairs so that it was arranged 5’ to 3’:

5’ CTTTAATGGAGAGGACGTT 3’

1. We needed to add SpeI (**ACTAGT**) to the reverse complement in reverse order to create reverse primer #1:

5’ **TGATCA**CTTTAATGGAGAGGACGTT

1. Since we are only able to work with mRNA since the gene sequence for Factor X is 26,731 base pairs long, which is too long to run a PCR, we needed two primers found within the mRNA gene sequence to help us verify our primers and our PCR product. To find the best primers that would fit our sequence, we plugged the gene sequence into the IDT PrimerQuest tool. It came up with the following primers:

Forward Primer #2 (found in middle of gene at start position 81):

5’ TGGTGGAACCATTCTGAGCGAGTT 3’

Reverse Primer #2 (also found in middle of gene, but downstream from forward primer #2 at start position 120):

5’ CTCCTTTGTGAACCGGTTGTGCTT 3’

1. We will then add the rest of the biobrick prefix sequence (see bolded portion below), which includes a EcoRI restriction site, to the forward primer to create forward primer #3 and to make it biobrick compatible:

5’ **GAATTCGCGGCCGCT**TCTAGATGATCGTGGA 3’

1. We will add the rest of the biobrick suffix sequence (see bolded portion below), which includes a PstI restriction site, to the reverse primer to create the reverse primer #3 and to make it biobrick compatible:

5’ **TACTAGTAGCGGCCG**CTGCAGCTTTAATAAG 3’

# Bibliography

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