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EVALUATING THE OUTCOME OF TRANSFORMING GENETICALLY MODIFIED *E.COLI* CELLS WITH HUMAN COAGULANT FACTOR IX CDNA

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ABSTRACT

Haemophilia B is a rare but severe genetic blood disorder that result in the malfunctioning of the gene that codes for the expression of human factor IX proteins in the coagulation cascade thus leading to an imbalance in haemostatic regulation of blood clot in damage blood vessels. The primary aim of this study was to clone the coding sequence of human factor IX into a bacterial vector (pETBlue 2 plasmid) for transient expression of hFIX protein in *E.coli* cells. RNA extraction from HepG2 cells was carried out from which aliquots obtained were used for cDNA synthesis using M-MLV Reverse transcriptase enzyme. Synthesised cDNA sample was subjected to PCR analysis with different annealing temperatures (57^0C, 55^0C,52^0 and 50^0C) for amplification of hFIX coding sequence using primers designed from the coding sequence. Sucessful transformation of *E.coli* cells was observed through the blue and white screening analysis of colonies. Evidence for transcient expression of hFIX protein in *E.coli* could not be ascertain as at the time of this research.

INTRODUCTION

The human coagulation cascade encompasses series of relatively indispensable proteins required for the formation of blood clots in response to injury or damaged blood vessels.

Within this cascade exist an important vitamin K-dependent hydrophilic protease called coagulation factor IX (Chavali *et al*, 2008). This single chain glycoprotein is synthesised and secreted into the blood stream in an inactive state as a proenzyme classified under the peptidase family S1 (Christophe *et al*, 2001). The coagulation factor IX is circulated in the bloodstream as a zymogen prior to injury or damage of blood vessels (Kulkarni and lusher, 2001). The utterly matured factor IX zymogen structurally comprises of eight exons which constitute; a signalling peptide located at the amino terminal region, a glutamic acid domain, two epidermal growth factor domains (EGF1 and EGF2), activation peptide region which is discarded once the inactive factor IX is activated and a catalytic domain located at the carboxyl terminal which controls protease activity (Schwartz *et al*, 2007).

The ability of the body's haemostatic system to regulate the coagulation cascade can be altered when a mutation occurs in genes coding for the expression of protease found within the coagulation cascade thus resulting in bleeding disorders (Mackman *et al*, 2007). This includes haemophilia A, disorders of fibrinogen and haemophilia B which directly relates with the deficiency of factor IX. The effectiveness of factor IX in the coagulation cascade relies on the state of the FIX gene that codes for its expression.

Haemophilia B is a bleeding disorder and a recessive X-linked genetic disease that is transmitted from parents to offspring and found within the family pedigree (Peters *et al*, 2010). This is caused by heterogeneous mutations that occurs within FIX gene segment located at band Xq27 of the X-chromosome and constitutes 8 exons and 7 introns (Verma *et al*, 2010). The altered FIX protein is ineffective in blood coagulation cascade and cannot initiate blood clot at the site of vascular injury (Kay *et al*, 2000).

Mutations in FIX gene can either cause the synthesis of completely inactive copies of FIX or limit the production of FIX (Collins *et al*, 2010). Haemophilia B can be severe or moderate. Severe haemophilia B occurs when the change in FIX gene structure causes total destruction of its function while, moderate haemophilia B is as a result of incomplete elimination of FIX activity (Mankor-Johnson *et al*, 2007). Treatment of individuals suffering from haemophilia B has evolved over the years. However, FIX deficiency still remains one of the largely neglected bleeding disorders due to its low incidence rate of 15-20% of all cases of haemophilia (Peyvandi *et al*, 2006).

Most frequently used treatment for FIX deficiency involves lifelong intravenous infusion of recombinant FIX concentrates or plasma-derived FIX proteins (Nathwani *et al*, 2014). This requires rigorous screening of human plasma derived from donors to prevent viral contaminants like HIV, hepatitis A, B and C and other blood transmitted viruses (Nathwani *et al*, 2001). This treatment measure has only proven to be effective in the prevention of sudden bleeding episodes but lacks a long lasting curative potentials and its harmful due to prolonged regular injections (Nathwani *et al*, 2014). Intravenous administration of FIX concentrates requires an average cost of $250,000 per year for each patient which is usually unaffordable by most Haemophilia B patients and results in decrease in life expectancy (Gringeri *et al*, 2003).

However *E.coli* cells have proven to be a reliable and cheaper host systems used as a tool for production of useful proteins. Although further protein modifications techniques are required to avoid inclusion bodies.

In this research, it is expected that the amplified gene of interest (hFIX) will be successfully used to create multiple copies of recombinant hFIX for transient expression in *E. coli* cells (vector system) which will be examined and documented for future research.

MATERIALS AND METHODS

Primers Design: Forward and reverse primers were designed using the nucleotide database of the NCBI website. This was achieved by designing primers from nucleotide sequences for each of the 8 exons. The following primers were used as shown below:

Exon 2 Forward primer: 5’ TCAGTGCTGAATGTACAGTTTTTTCT 3’ (25bp)
Exon 2 Reverse primer: 5’ TCTCCATCAACATACTGCTTCCA 3’ (23bp)
Expected product length from both exon 2 primers is 213 base pairs.
Exon 6 Forward primer: 5’ TGTGAACCAGCAGTGCCATT 3’ (20bp)
Exon 6 Reverse primer: 5’ ACAACCTGCCAAGGGAATTGA 3’ (21bp)
Expected product length from both exon 6 primers is 222 base pairs.
CDS Forward primer: 5’ GTTATGCAGCGCGTGAACAT 3’ (20bp)
CDS Reverse primer: 5’ TCCATCTTTCATTAAGTGAGCTTTG 3’ (25bp)
Expected product length from both coding sequence primers is 1420 base pairs.

Maintenance of HepG2 Cell Line Through Passaging: HepG2 cells were maintained at 37^0C in monolayer culture inoculated in T75 flasks containing DMEM (Invitrogen), 10%

fetal bovine serum and 100 units/ml penicillin plus 100 µg/ml streptomycin for 4 days to obtain a suitable confluence level of 70% - 80% for RNA extraction. Cell passaging was repeated at intervals to keep the cells growing throughout the duration of laboratory sections using the HepG2 cell passaging kit. Spent media from T75 flask containing HepG2 cells was discarded and cells were washed with 10ml Phosphate Buffered Saline to allow for trypsinization. Detached HepG2 cells were transferred into 50ml falcon tube and then 3 ml of fresh media (an equal volume of trypsin) was added to inhibit trypsin activity. Cells were centrifuged at 1000 rpm. Thereafter, the supernatant was discarded and pelleted cells were re-suspended in 6 ml of media from which 1 ml of re-suspended cells was added into sterile T75 flask containing 30 ml of culture medium. HepG2 cells in T75 flasks were visualised under the microscope to determine the presence and volume of cells in the flask. Flasks with adequate volumes of cells were incubated under 37^0C for next harvesting section.

RNA Extraction Analysis: Sub-cultured HepG2 cells with 70% growth confluence were used for RNA extraction, the media was discarded and treated with 7.5ml of TRIzol® reagent. The Homogenized and lysed cells sample was incubated at room temperature for 5 minutes to allow for complete dissociation of the nucleoprotein complex (Chen *et al*, 2014). Phase separation was achieved by centrifugation at 12,000 x g and the aqueous phase was removed for RNA isolation through RNA precipitation, purification and resuspension using the RNA extraction kit.

Spectrophotometric Analysis: Extracted total RNA from HepG2 cell line was used to run spectrophotometric analysis to determine the concentration of RNA being extracted to ascertain volume of Total RNA to be used for cDNA synthesis. Readings were done using 50 µl RNA extracts in cuvette tubes.

Complementary DNA Synthesis: First strand cDNA synthesis was done using mRNAs from coagulant factor IX. 4-6µl of total RNA extract was used alongside 2µl of oligo d(T)20VN primer (50µM), reaction Mix buffer, Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase and nuclease free water. Also, a negative control was prepared by excluding the addition of the Reverse Transcriptase enzyme in the control sample. Gene specific primer was also used to derive gene specificity in cDNA synthesis. The synthesis was done using the First strand cDNA synthesis standard protocol.

Amplification of Factor IX cDNAs by Polymerase Chain Reaction: Synthesised cDNAs of factor IX coding sequence derived from human liver cells with GenBank accession number NM_000133.3 was subjected to PCR amplification with the help of suitable reverse and forward primers as described by Rech *et al* (2008). Three samples were used for PCR analysis. The first sample contained the cDNA, the second sample contained the total RNA extract without reverse transcriptase (negative control 1 (-RT)), and the third sample being PCR positive control was void of template DNA with an equivalent volume (4µl) of sterile water to replace the template DNA. All three samples were run on a PCR machine for 30 cycles at varying annealing temperatures. PCR products were analysed for amplification through gel electrophoresis stained with ethidium bromide.

Analysis of PCR Product by Gel Electrophoresis: PCR products from HepG2 cells cDNA clones were subsequently subjected for analysis using agarose gel electrophoresis. 1x concentrate of Tris-acetate running buffer was prepared from 10x Tris-acetate stock solution and was used to prepare 1%(10mg/ml) pre-stained agarose gel. The gel was placed in the steamer and allowed to completely dissolve. Thereafter, the partially cooled gel was stained with 10mg/ml ethidium bromide and was poured into the gel mould and allowed to solidify. The gel tank was then filled with 1X TAE buffer after which PCR products to be analysed were mixed with loading buffer (0.25 % bromophenol blue) and samples were carefully loaded into the wells. Gels were run at 80v -103V for 50 - 60 minutes and were then visualised using UV light trans-illuminator.

Inoculation of *E. coli* Cells for Plasmid DNA Extraction: *E. coli* bacteria strain having the pETBlue 2 plasmid was plated on L agar containing 10mg/ml ampicillin solution for selective growth of only strains with the pETBlue 2 plasmid having the ampicillin resistance gene for selectivity. *E. coli* cells were cultured under 37^0C in an incubator and left for 16hrs after which a colony was subcultured in L broth medium for 3hrs. Volumes of inoculated E. coli cells in L broth were used to determine the exponential growth phase by measuring the absorbance rate of UV light from spectrophotometer at an interval of 30 minutes until an absorbance of 400nm is achieved. *E. coli* cells in exponential phase were subjected to plasmid DNA extraction and purification protocols as described by the miniprep kit. Extracted plasmid DNA were authenticated using gel electrophoresis to determine if the size of plasmid DNA correlates with the actual size.

Preparation of Competent *E. coli* Cells for Transformation: The ability of the *E. coli* cell strain to take up recombinant plasmid was tested by determining the cells ability to take up non recombinant pETBlue 2 plasmid. Cell were allowed the get to exponential growth phase and determined by optical density of 0.45nm. Thereafter, cells were treated with 5ml 0.1 M of $MgCl_2$ and $CaCl_2$ respectively to make their cell walls permeable for the uptake of plasmid DNA as described by panja *et al*, (2006). Treated cells were cultured in L broth medium containing 10µl of pETBlue 2 plasmid and incubated for 40 minutes under 37^0C. Thereafter, 6µl of the inoculum was spread on culture plates containing L agar with 800µl of ampicillin. This was incubated for 48 hours under 37^0C to allow for maximum uptake of plasmid DNA.

RESULTS

Determination of Size and Purity of Extracted RNAs from HepG2 Cells: For accuracy of results in gene expression analysis and creation of cDNA libraries of desired genes, it is always essential to authenticate the isolation of intact RNA as most techniques involving priming with oligo(dt) demands the utilisation of RNA extracts with high stability. Aliquot of total RNA from cultured HepG2 cell line showed clear 28S and 18S rRNA bands respectively when run on a denaturing agarose gel stained with 10mg/ml ethidium bromide alongside with aliquot of a 100 base pair marker as shown in figure (1) below.

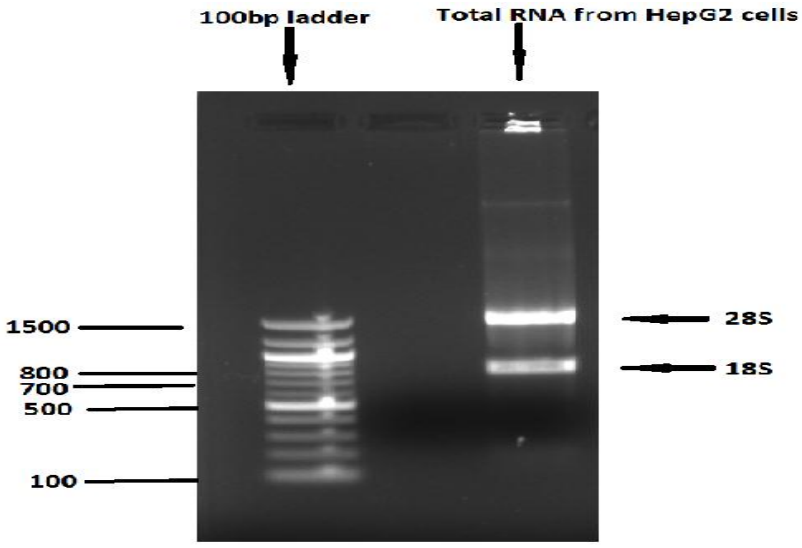

FIGURE 1: Gel electrophoresis showing 28S and 18S bands of rRNA extract from HepG2 cells. Lane(1) represents 100bp marker, lane(3) represents the total RNA from HepG2 cells that was loaded on the gel with an aproximate band size of 1,500bp for the 28S and an aproximate band size of 700bp for the 18S rRNA.

Clear and visible 28S rRNA band was twice as thick as the 18S rRNA band as shown in figure 1, lane 3 above. The 2:1 intensity ratio of the bands is an indication that the extracted RNA is intact and stable as unstable and degraded RNA will have smeared appearance lacking visible bands across the gel (Becker *et al*, 2010). The visibility of the bands is likely due to the concentration of RNA aliquot that was run on gel, the duration (50min) and voltage (100V) of gel electrophoresis. A concentration of 100µg/ml of total RNA was used for gel electrophoresis.

cDNA Synthesis and Optimization of Human Factor IX Nucleotide Sequence: Synthesis of cDNAs from aliquots of intact RNA yielded varying PCR products due to effects of varying temperature on enzyme activity and elimination of secondary structures. It was observed while using the M-MLV Reverse Transcriptase for cDNA synthesis that the higher the temperature for enzyme activity, the greater the possibility of yielding cDNA clones from which the quality of PCR product through amplification partially depends. This is because the elimination of secondary structures is important and usually achieved at a temperature level of 70^0C, lower temperatures below 70^0C for reverse transcriptase enzyme activity might lead to the formation of hydrogen bonds thus resulting in the formation of secondary structures. However, the possibility of reformation of secondary structures during cDNA synthesis is high when there is significant decrease in temperature from the 70^0C initial temperature for denaturation of RNA thus limiting cDNA synthesis. The production of human factor IX cDNA library using (M-MLV) Reverse Transcriptase at 42^0C resulted in the degradation of RNA and formation of primer dimers as shown in Figure (2).

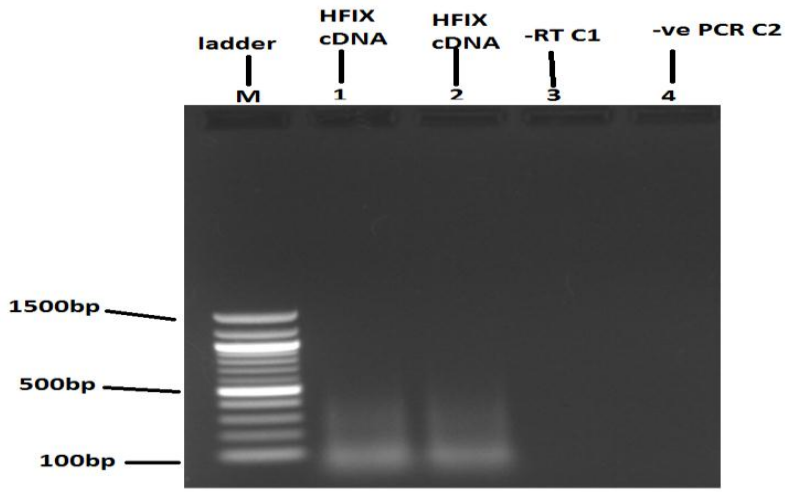

FIGURE 2: Gel electrophoresis showing no visible amplification of hFIX gene except for degraded nucleic acids and the formation of primer dimers in lane 1 (composed of

primers designed from exon 2) and lane 2 (composed of primers designed from exon 6. Bands from DNA marker can be visibly seen in lane (M). Lane 3 represents the negative (-RT) control sample 1 with aliquots of total RNA without reverse transcriptase showing no band formation. Lane 4 represent PCR control 2 with no visible band formation as no template DNA was added to it.

The above PCR product was obtained at an annealing temperature of 57^0C. Further optimization of PCR yielded similar results at varying annealing temperatures of 55^0C, 52^0C and 50^0C as shown in Figure (3), depicting that the quality of cDNA product might be void of synthesised complementary DNA sequence of factor IX gene as no visible bands were seen on gel to ascertain gene amplification.

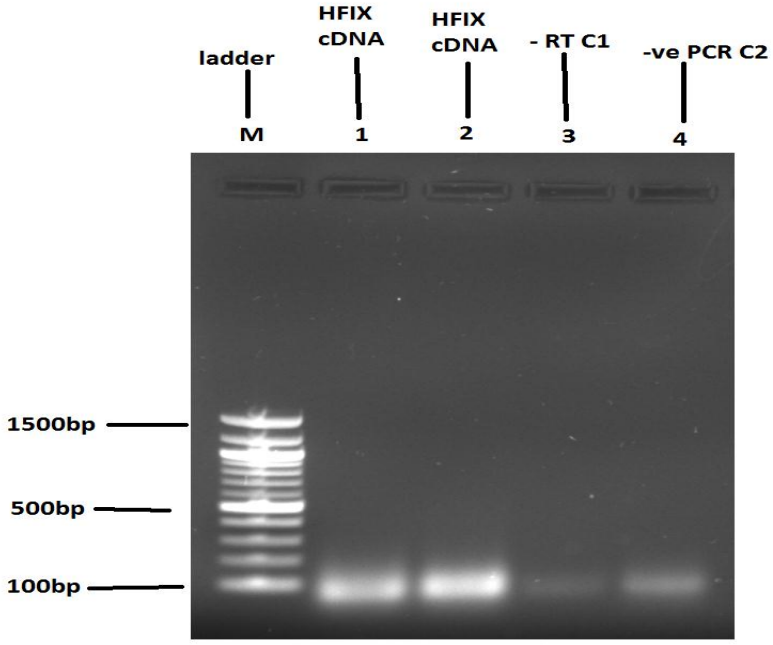

FIGURE 3: Gel electrophoresis showing no visible cDNA amplification of hFIX gene. Lane 1 (hFIX cDNA with primers designed from exon 2), 2 (hFIX cDNA with primers designed from exon 6), 3 (negative control (-RT) 1 without reverse transcriptase) and 4 (PCR control 2 without template DNA) shows degradation of nucleic acids and formation of primer dimers.

cDNA samples used for amplification prior to PCR optimization were obtained at 42^0C of enzyme activity (M-MLV Reverse Transcriptase) and thus hindered PCR optimization because the PCR analysis is highly dependent on the quality of cDNA. However, the optimization of cDNA synthesis by elevation of the temperature of enzyme activity from the

initial 42^{0}C to 70^{0}C at an annealing temperature of 50^{0}C showed positive. Results obtained from gel electrophoresis showed successful amplification of factor IX cDNA sequence (with band size of 1432bp) and plasmid digest when compared with BstEll digest of phage-DNA marker as shown in Figure (4) below.

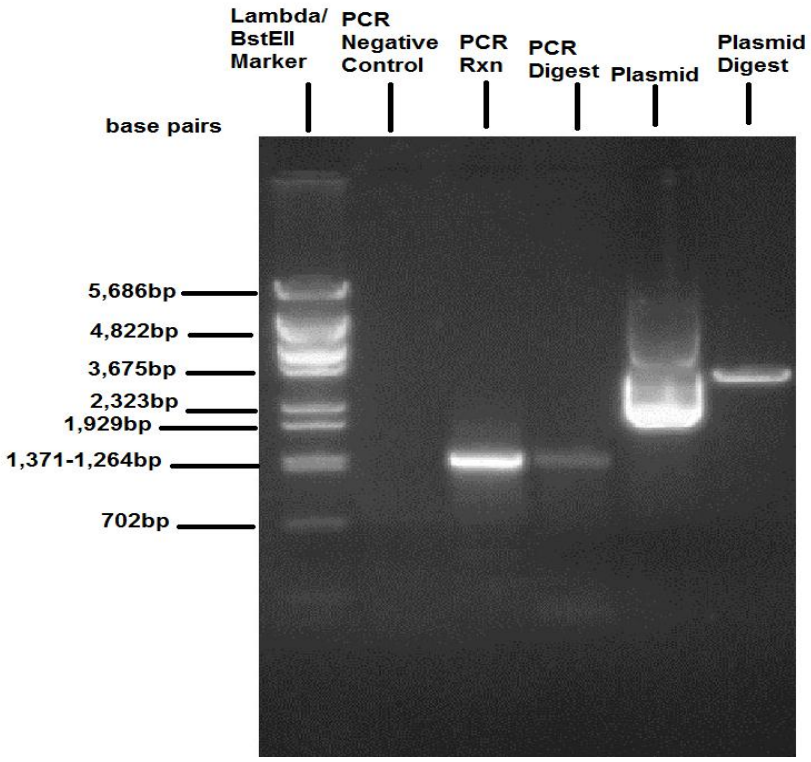

Figure 4: Gel electrophoresis showing band sizes of Lambda/BstEII DNA Marker (line 1), PCR reaction with amplified hFIX cDNA (line 3), PCR Digest with restriction enzymes (line 4), Uncut plasmid (line 5) and plasmid Digest with restriction enzymes (line 6).

product/PCR digest (digested with FspI and EcoRI restriction enzymes) of the hFIX cDNA showed visible thick and light band sizes of approximately 1432bp respectively thus indicating amplification of target sequence and the activity of enzymes used as shown in line 3 and 4 of the gel photograh in Figure 1 above. The pETBlue-1 plasmids used for cloning in *E. coli* cells were also digested with endonuclease enzymes (EcoRV and EcoRI) to allow for integration of insert (PCR digest of hFIX cDNA). Result obtained from gel electrophoresis of plasmid digest revealed varying band sizes of approximately 2,323bp for uncut plasmid and 3,476bp for cut plasmid as expected thus differentiating between supercoiled and linear forms of the plasmid analysed as shown in figure 4 above.

Spectrophotometric Analysis of Plasmid DNA for Quality and Quantity

Result obtained from spectrophotometric analysis of pETBlue-1 plasmid DNA samples extracted from genetically modified strain of *E. coli* showed UV absorbance of 1.69 at 260nm and 0.9 at 280nm respectively. Plasmid DNA purity was determined by calculating the ratio of absorbance at 260nm (1.69) divided by absorbance at 280nm (0.9) thus having an optical density (OD260:OD280 ratio) of 1.87. The concentration of plasmid DNA was calculated by multiplying its absorbance rate at 260nm (1.69) × the dilution factor (10) × Extinction coefficient (50µg/ml for 1.0 absorbance at 260nm) = 845µg/ml.

Estimation of *E. coli* Bacterial Growth by Measurement of Optical Density

The growth estimation of cultured *E. coli* cells was determined by measuring the optical density of cultured samples at 600nm.Spectrophotometric analysis showed an optical density of 0.481. The estimated cell number at 0.481 optical density was 0.380×10^9 cells/ml as shown in the calibration curve in Figure (5) below. *E. coli* cell number was calculated as 1 OD= -log10.

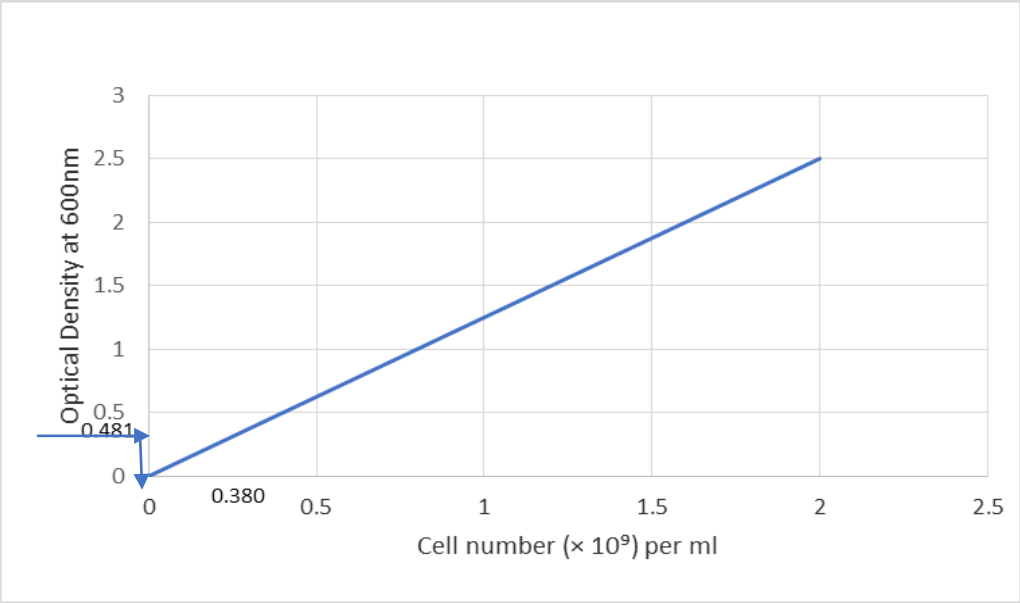

FIGURE 5: Shows the calibration curve for estimated *E. coli* cell number (0.38×10^9) per ml of culture media with an optical density of 0.481 at 600nm.

***E. coli* Cells Transformation and Screening for Plasmid Uptake**

Transformed *E. coli* cells with non-recombinant and recombinant plasmids containing the ligated target sequence for Human factor IX protein expression showed integration of both plasmids observed through the blue/white screening of colonies of cultured competent cells

on solidified L-agar plates as shown in the table below. Ampicillin and petBlue2 plasmid with Amp resistant gene that allow recombinant cells to grow on ampicillin medium were all contained in the variousplates. Also, X-gal was spread in all the plates as an anologue of lactose. The two P (positive control) contained the non-recombinant plasmid, the two N (negative control) contained competent cells and sterile water, and the two L (ligation) plates contained the competent cell with recombinant plasmid and an uncultured plate as control for possible contamination. 20µl and` 100µl aliquots of each content prepared for the 6 plates was spread on each plate and incubated at 37ºC for 48hours. No visible colony was observed on the 20ul ligation plate, but at 100µl of P & L they were visible colony. The N(negative control) is needed to check the contamination within the competent cell and therefore should not have colonies as indicated in table(1). The positive control (P) at 100µl has 186 blue colonies and 41 blue colonies at 20ul.The ligation plate (L) has Four (4) white colonies at 100µl but lack at 20ul.However, both P & L plates were meant to have colonies at 20µl and 100µl. But blue colonies should not be in Ligation (L) but white colonies. And positive control (P) should have only blue colonies as shown below.

Table1: An obtained data from blue/white screening analysis for cell transformation (6): The values 0 symbolize no visibility or absent of colonies. 4,41 and 186 values show visibility of colony.

	Negative control(N)	Negative control(N)	positive control(P)	positive control(P)	Ligation(L)	Ligation(L)
	White	Blue	White	Blue	White	Blue
20µl	Nil(0)	Nil(0)	Nil(0)	41	Nil (0)	Nil(0)
100µl	Nil (0)	Nil (0)	Nil(0)	186	4	Nil(0)

DISCUSSION

Low cost production of recombinant proteins is an important factor considered when choosing expression systems for production of valuable human proteins (Lau and Sun, 2009). However, these recombinant proteins need be extracted from cells that actively express the gene of interest through synthesis of desired protein from which complementary DNA clones to be amplified are generated. It is from this point that successfully amplified coding gene sequence is used for transformation through suitable vectors and expression systems. This was applicable in this study as the primary goal of this research was to evaluate the competency of GM *E.coli* strain in the uptake of recombinant plasmid DNA incorporated with human factor IX.

In this study, complementary DNA sequence from mRNA was synthesised using total RNA extracts from HepG2 cells. First strand cDNA protocol used for cDNA synthesis revealed the importance of choice of reverse transcriptase and effect of different incubation temperature for enzyme activity during synthesis of cDNA (Bustin, 2000). M-MLV Reverse Transcriptase used could only transcribe hFIX gene at high incubation temperature of 70^0C depicting that the extracted RNA contained high amounts of secondary structures and required a thermostable reverse transcriptase like the one used in this study, mostly in cases where transcription is only achieved at high incubation temperature. Apart from being capable to withstand high thermal conditions, M-MLV-RT is also known to have low RNase H activity with the ability to transcribe mRNA with secondary structures and also enhances priming specificity (Gerard *et al*, 2002). It has a half-life of 120 minutes at 65^0C. Increased yield in first strand cDNA is achievable due to reduced RNase H activity (Herschhorn *et al*,2010). In other to enhance gene specificity during cDNA synthesis, the reverse primer designed from coding sequence was used in place of oligo(dt) primer. Since transcription was rarely achievable with oligo(dt) priming, it was necessary to determine the possibility of achieving transcription using a gene specific primer as it allows the transcript to start much further in from the 3' end thus avoiding some of the potential secondary structures during transcription (Stahlberg *et al*, 2004). Generated cDNA clones containing the gene of interest had to be amplified through polymerase chain reaction with series of optimization to obtain better PCR product.

PCR optimization was done in several ways. There are basically four factors to look out for while optimising PCR product. This includes, annealing temperature, concentration of Mg^{++}, buffer pH and the condition of cycling. The most important is annealing temperature. This is because of its importance in the activity of primers used (Korbie *et al*,2008), in this case, the primer pairs designed should closely match in annealing temperature to avoid the effectiveness of one primer against the other. Concentrations of Mg^{++} can easily be manipulated as it can be used separately in form of $MgCl_2$ from the rest of the standard reaction buffer making adjustment in concentration easier. Although, adjustment should be made with care as Mg^{++} and dNTPs are interdependent. An increase in concentration of dNTPs decrease the concentration of free Mg^{++} ions (Roux, 2009). Adjustment can be made between 0.5 – 5.0 Mm of the solution (Kramer and Coen, 2002). Optimization of annealing temperature should be done within the range of 10^0C above or below estimated temperature of both primers (Frey *et al*, 2008). It has been noted that some primers are refractory to

optimization, in this regard, it is usually advisable to design new set of primers that cleave to relative DNA sequence (Chou *et al*, 1992). The use of certain substances during preparation of template DNA like sodium dodecyl sulphate, phenol, heparin, xylene, bromophenol blue and cyanol exhibit inhibitory effects on the performance of PCR when used in excess or if purification of extracts is done inaccurately. When this is suspected as a limiting factor for PCR analysis then a 100-fold dilution of template DNA is recommended to eliminate inhibitory effects or a re-extraction can be done with more accuracy (Weyant *et al*, 1990).

One major challenge in the production of recombinant proteins is usually the difficulty in creating cDNA library with efficient amplification of the gene of interest. This is usually because the effectiveness of transcribing and amplifying a particular gene sequence is based on a complex variation of factors ranging from the cell line to be used and its ability to express the gene of interest, the type of primers to be used, the choice of reverse transcriptase and annealing temperature (Tang *et al*, 2009) suitable for both forward and reverse primers to bind to 3' and 5' ends of the coding sequence and initiate amplification through polymerase chain reaction. It is usually advisable to examine the product of cDNA clones before amplification to be more specific when optimising PCR analysis. Where possible, this could be achieved by the application of southern blot techniques with the help of gene specific probe to determine if the desired cDNA is present and to ascertain the complexity of the cDNA clones (Rasool *et al*,2016). With this, it will be much easier to determine if the HepG2 cell line used for RNA extraction have the potential for expressing the desired gene or probably regulate the annealing temperature or optimise other PCR factors that enhance the quality of PCR product.

To assess the integrity of amplified PCR product (hFIX), it was adequate to analyse using gel electrophoresis as an effective technique used in identifying the size of amplified DNA sequence for correlation with the expected band size with the help of a molecular marker. Gel electrophoresis involves running DNA/RNA samples on a denaturing agarose gel to allow adequate downstream separation of DNA/RNA molecules into bands of different sizes (Westermeier, 2016). It requires minimal electric voltage needed to cause migration of negatively charged DNA molecules from the cathode to the anode region of the apparatus (Xia *et al*, 2016). Analysed DNA molecules on gel electrophoresis as shown on the result section proved accurate amplification of the target sequence. The reason for lack of visible differences between band sizes of cut (PCR digest) and uncut PCR products is because the

length of nucleotides incised by EcoRI and FspI endonucleases are so little and not enough to make visible differences that could be noted on the gel. Also, the differences in band sizes of cut plasmid and uncut plasmid digest is because of differences in the compartment of DNA molecules. The uncut plasmid is in a supercoiled form with compressed DNA molecules thus preventing accurate analysis of the DNA size. However, the plasmid digest incised with endonucleases (EcoRV and EcoRI) caused the unwinding of DNA double helix thus revealing the actual size of the plasmid DNA on the gel.

Transformation of *E. coli* cells with non-recombinant and recombinant (with hFIX DNA insert) plasmid vector revealed an unseen metabolic activity between the inoculated cells and its environment (growth media) that is vital in the blue/white screening for transformed cells. Cell colonies with blue colouration only indicated the cells' ability in taking-up non-recombinant plasmid because of unaltered LacZ gene responsible for expressing β-galactosidase which breaks down lactose (X-gal was used and added in the media as a substitute) to give glucose + galactose thus producing bromophenol blue in the process which is responsible for the blue colouration. On the contrary, white cell colonies indicated the cells' competence in taking-up recombinant plasmid. The reason for the white or colourless appearance of colonies is because the *LacZ* gene has been altered by the insertion of hFIX nucleotide sequence and so the production of functional β-galactosidase needed for the breakdown of X-gal present in the media is hindered. Although cell transformation showed positive result yet there are possibilities of having recombinant plasmid in transformed *E. coli* cells with untranscribed hFIX sequence to produce mRNA needed for expression of the human factor IX protein. To achieve this, certain laboratory techniques are applied such as the use of real-time PCR and enzyme-linked immunosorbent assay (ELISA).

The application of real-time PCR techniques is useful in analysing for transcription of target DNA sequence that was inserted into plasmids taken-up by host cells. This technique involves labelling of target DNA with sequence specific DNA probes that has a fluorescent reporter molecule which allows detection of labelled DNA sequence on hybridization of the probe with complementary sequence (Arya *et al*, 2014). Once this is achieved, further application of enzyme-linked immunosorbent assay is required to make sure the mRNA has been successfully translated into target protein (hFIX protein).

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