The quantification of human factor IX gene expression in transfected mammalian liver cells, employing the real time qPCR and the ELISA techniques.

Zaid Al-Obaidi
Kerbala University, College of Pharmacy, Dep. of Pharmaceutics,
Mobile: +964(0)7702751265, Email: zaid.alobaidi@uokerbala.edu.iq

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Abstract:
Real-time qPCR and ELISA are very sensitive powerful techniques that were commonly and universally employed in the quantification of gene expression. The aim of this study has two parts; the first part is to quantify the level of the hFIX gene expression employing the real time qPCR for quantifying the newly transcribed mRNA. The second part is to detect the translated rFIX protein using the ELISA technique. In this study the mRNA levels present in mammalian liver cells (HepG2) that were transfected with hFIX gene has been successfully quantified by qPCR using hACTB as a housekeeping gene. Also the level of the translated rFIX protein has been observed utilizing the ELISA instrument. ΔCt and ΔΔCt for the transfected cells were -1 and -21 respectively, while the concentration of the rFIX in these cells was 5,495 ng/ml, which is more than 800 times increase when compared with the control cells. The validation of such methods, which are used for quantification of both the gene and the translated protein, is of significant importance. Furthermore the examination of the functionality of the target protein is even more important due to the challenges associated with these techniques.

القياس الكمي للتعبير الجيني لعامل التخثر التاسع في الانسان في خلايا كبد الثديات بتوظيف الوقت الحقيقي لتفاعلات الكوثرة الكمية و كذلك فحص الانزيم المرتبط مناعياً.

زيد مهدي جابر العبيدي
جامعة كربلاء\ كلية الصيدلة\ فرع الصيدلانيات

مفتاح البحث: عامل التخثر التاسع في الانسان، الوقت الحقيقي لتفاعلات الكوثرة الكمية، و فحص الانزيم المرتبط مناعياً.

الخلاصة:
الوقت الحقيقي لتفاعلات الكوثرة الكمية (rt-qPCR) و كذلك فحص الانزيم المرتبط مناعياً (ELISA) هي تقنيات قوية و حساسة للغاية و التي تم استخدامها عالميا في القياس الكمي للتعبير الجيني. الهدف من هذا العمل يمتلك جزئين: الجزء الاول هو لقياس مستوى التعبير الجيني لعامل التخثر التاسع للانسان (hFIX) بوساطة توظيف (qPCR) لقياس كمية الحمض النووي الرايبوزي الناقل (mRNA) المنسوخ حديثاً. الجزء الثاني هو لكشف بروتين عامل التخثر التاسع المركب (rFIX) المترجم باستعمال تقنية ال(ELISA). في هذه الدراسة تم قياس مستويات (mRNA) الموجودة في خلايا كبد الثدييات (HepG2) و التي تم نقل عدوى جين ال(hFIX) كمياً بوساطة (qPCR) بنجاح و ذلك باستخدام hACTB كجينات التدبير المنزلي. كما تم ملاحظة مستوى بروتين rFIXو التي تم ترجمتها باستخدام جهازELISA . كانت قيمة ΔCt و ΔΔCt للخلايا المصابة بالعدوى بالنقل (-١) و (-٢١) على التوالي ، في حين كان تركيز rFIX في هذه الخلايا ٥،٤٩٥ نانوغرام / مل ، والتي هي أكثر ب ٨٠٠ مرة بالمقارنة مع الخلايا السيطرة. التحقق من صحة مثل هذه الأساليب ، والتي تستخدم لتقدير كل من الجينات و البروتين المترجم، له أهمية كبيرة. وعلاوة على ذلك، دراسة وظيفة البروتين المستهدف هو أكثر أهمية نظرا للتحديات المرتبطة بهذه التقنيات.

Introduction:

Factor IX (FIX) is a vitamin K-dependent serine protease protein that is manufactured in the liver and has a major role in the coagulation process via activating factor X to the active form Xa which produce thrombin from its precursor prothrombin whereas the thrombin activate the fibrinogen to produce the fibrin mesh that ultimately resulted in producing blood clot that cease the bleeding[1].

This glycoprotein FIX is a functional enzyme that consists of 415 amino acid residues and catalyzes an important step in the blood coagulation process[2].

However hemophilia B (or alternatively called "Christmas disease") is observed when a deficiency and/or functionally defective types of hFIX (also known as Christmas factor) are encountered [3]. This disease was discovered for the first time in 1952 when a 5-years old boy named Stephen Christmas was deficient with FIX causing him hemophilia; hence the name Christmas disease was come from[4, 5]. To rectify this condition, the patients were treated by transfusing freshly prepared plasma, which contains the required FIX[6]. Nevertheless, this was not the perfect way as the transfusion may increase the risk of transmittance of potential viral infections like human immunodeficiency virus (HIV) and hepatitis type B virus (HBV). Also liver transplantation was performed, but many issues like histocompatibility and the use of immunosuppressant drugs to reduce the transplanted tissue rejection, have the major restrictions of performing such operations to heal the patients with hemophilia B[7]. In contrast, the utilization of the biotechnology and genetic engineering is considered as a superior alternative to treat these patients. This encompasses the encoding of a certain gene (gene of interest) into a foreign host cell using a particular vector. This newly inserted gen can be transcribed to produce mRNA whereas the mRNA can be translated in the endoplasmic reticulum (specifically the ribosomes) to the required protein[8].

In fact there are troublesome issues that associated with the production of required-recombinant protein. For instance the gene of interest can be lost from the host cell or any changes in the base pairs of the gene of interest may lead to the lack of gene expression and no recombinant protein would be observed. The above reasons enforce the utilization of quantitative technique to evaluate the effectiveness of the gene expression process [9, 10]. The first report on quantification of mRNA utilizing the polymerase chain reaction (PCR) was published in (1989) [11], which in turn was developed by Mullis in 1983 [12]. Porcher et al., (1992) followed this and described the quantitative PCR (qPCR), and then exponential utilization of qPCR has been reported[13]. The qPCR is a powerful, very sensitive, and robust technique that has been employed to estimate the level of mRNA after reversely transcribing this single strand mRNA to the double strand copy cDNA [14]. Real-time qPCR has been widely utilized in gene expression quantification-protocols due to many highlighted attributes. For example it is considered to be very sensitive, precise, highly productive, poses low limit of detection (based on fluorescence advances), can quantify the presence of templates even for single cell, finally its specificity and reproducibility allows the qPCR to be very accurate in quantifying either the number of genes or the mRNAs present in the sample [15, 16]. However, there are many limitations are associated with the qPCR protocol, so for the RNA extraction step the disadvantages encompasses the variability in efficiency of the extraction, which in turn cannot be specified, short half-life of mRNAs and the difficulty in assuring the absence of Rnases. Although the qPCR instrument itself has a few limitations including the difficulty in primers design to cope with the whole activity of the gene of interest particularly for the newly studied ones, also the presence of enzyme inhibitors and/or the nucleases in the extraction buffer may interfere with qPCR activity. Finally the challenges associated with housekeeping gene selection to normalize data and to reduce gene expression variability are considered other limitations of this technique [15, 17].

Scientists had studied the correlation of the quantity mRNA and multitude of the correspondent protein. This correlation is still unclear. For instance two genes transcribed into a similar mRNA may result in different multitudes of the proteins translated. Accordingly, for full assessment, the quantification of both the mRNA and the protein have to be performed. One of the most commonly used techniques to quantify a specific protein is the Enzyme-Linked Immunosorbent Assay (ELISA) [18].

The ease of operating, inconsiderable matrix effect, and the high sensitivity attributes of ELISA cause it to be the preferred tool to quantify proteins by the scientists in the biological field and its related disciplines. Moreover, sandwich ELISA is considered more sensitive than other types of ELISA as in this type the protein of interest (antigen) has two different epitopes that would be captured by two different antibodies [19]. This study aimed to quantify the level of the hFIX gene

expression utilizing the real time qPCR and to detect the translated rFIX protein employing the ELISA techniques.

Materials and Methods:

This study was performed on May-2013 at Sheffield Hallam University laboratories, Sheffield, UK.

Materials:

Instruments:

Veriti Thermal Cycler (PCR) instrument used was purchased from Applied Biosystems; StepOne™ Real-Time PCR Systems instrument was employed for the Real time (qPCR); finally, Infinite® 200 PRO series was used as Plate reader.

Reagents:

The reagents and the tools that were used human factor IX (hFIX) ELISA Kit were a 96 well FIX microplate (supplied with its seal) covered with polyclonal antibodies to fit with factor IX protein, standard hFIX lyophilized in a protein buffer, biotinylated polyclonal antibody that can attached to FIX protein, protein buffer diluent (EIA), surface active agent buffer (wash buffer), Streptavidin-Peroxidase Conjugate, tetramethylbenzidine as a chromogen-peroxidase substrate, and lastly 0.5 M HCl used as Stop Solution to terminate the enzyme-substrate reaction.

Methods:

Part 1: lysis of the HepG2 cells:

A $1x10^5$ HepG2 cell aliquot that was transfected with hFIX expression vector and a control of $1x10^5$ HepG2 cells aliquot were already provided by the Biomedical Research Centre BMRC of Sheffield Hallam University (Sheffield, UK). These two aliquots were lysed separately through the addition of 50 µl of lysis solution, which was prepared by combining of 1.1µl of DNase I and 109 µl of lysis solution. Then each prepared solution was mixed through pipetting the solution within the tubes five times. Then the solutions were incubated at laboratory temperature for 5 minutes. After that 5 µl of stop solution was added and mixed five times through pipetting up and down. Then an incubation of 2 minutes was performed at lab temperature and finally it was allocated in ice for the next step.

Part 2: ELISA section:

To quantify the level of the target protein gained (i.e. FIX), a standard curve was performed employing the following concentrations of the recombinant Factor IX (rFIX); (0.0, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0 and 100.0) ng/ml. These concentrations were prepared using serial dilution and a "Buffered protein base" as a diluent (this buffer was provided as x10 concentration and was diluted to x1 concentration to be used for dilution).

12 microplates were used, 8 of these were filled with 50 µl for the standards mentioned above and the other 4 microplates were filled with 50 µl of transfected HepG2 cells, transfected HepG2 culture medium, control HepG2 cell lysate, and culture medium (the 4 samples). These 12 microplates were sealed and incubated at laboratory temperature for 2 hrs. After the incubation period the microplates were washed five times with 200 µl each of the wash buffer (supplied as x20 and was diluted to x1). After the fifth washing process the rest of the washing buffer was removed, which was performed by tapping the microplates over a tissue paper. Then a 50 of the x1 biotinylated antibody (provided as x50 and was diluted with x1 EIA diluent buffer) against FIX protein was added to each one of the 12 microplates and was incubated for an hour. Then the antibody buffer solution was decanted and the microplates were washed 5 times with 200 µl each of the wash buffer exactly as above.

After that 30-µl aliquots of streptavidin-peroxidase conjugate (provided as x100 and was diluted to x1 with x1 EIA diluent buffer) were added to each one of the 12 microplates and incubated at the lab temperature for 20 minutes (instead of 30 minutes due to shortness of time). After that the solution was decanted. Then the microplates were washed 5 times with 200 µl each of the wash buffer exactly as illustrated above.

Then 50-µl aliquots of chromogen substrate conjugate were added to each microplate and incubate for 15 minutes. After that 50-µl aliquots of the stop solution were added to each microplate. The plates were allocated in a plate reader and the results were observed at 450 nm wavelength.

Part 3.A: Reverse Transcription (RT) section:

Three of 0.2 ml nuclease-free tubes were labeled as C (for the control HepG2 cells) and T (for the transfected HepG2 cells) and –RT (for the transfected HepG2 cells but without the addition of the reverse transcriptase enzyme). 25-µl aliquots of 2x reverse transcriptase buffer were added to each tube, while 2.5 µl of 20x reverse transcriptase mixture were added to each of the C and T tubes only. Then12.5 µl of the nuclease-free water was added to each of C and T tubes whereas 15 µl was added to the -RT tube. Finally, 10 µl of the RNA sample for the control HepG2 cells was added to tube C, in contrast 10 µl of the RNA sample for the transfected HepG2 cells was added to each of T and –RT tubes.

These three reaction tubes were mixed thoroughly and centrifuged for few seconds and were kept in ice. After that the tubes were allocated in a thermocycler for an hour at 37^{o}C at which the cDNA were synthesized and then five minutes at 95^{o}C to stop the function of the reverse transcriptase enzyme. Lastly the holding temperature was 4^{o}C and the three tubes with the cDNA were collected.

Part 3.B: real-time qPCR section:

The collected cDNA were amplified using two Cocktails: the hFIX Cocktail and the housekeeping gene, which is the human Beta-actin (hACTB). These two Cocktails were similar in containing 10µl of 2x TaqMan Universal PCR mastermix and 3 µl of nuclease-free water, however these Cocktails differed in the last 1 µl to make the final volume 14 µl. The 1 µl was 20x TaqMan gene expression assay (hFIX) that was used to prepare hFIX Cocktail while it was 20x TaqMan gene expression assay (hACTB) that was used to prepare hACTB Cocktail. Each Cocktail was prepared for 8 reactions. 14 µl of each Cocktail was placed in 2 different series of 8 PCR reaction tubes. Then for each 8-series tube the following buffers were added as 6µl volume as follows: Control-cDNA for the 1st and 2nd tubes; Transfected-cDNA for the 3rd and the 4th tubes; Reverse transcriptase negative_cDNA for the 5th and the 6th tubes; for the 7th tube it was the cytomegalovirus plasmid encoded with human factor IX or phFIX-CMV (10ng); and finally the 8th tube contained the nuclease free water. Then the reactions were mixed well and centrifuged for short period that assured the contents were in the bottom of the tubes the bubbles were get rid or reduced to their minimum.

Finally the reaction tubes were allocated in the fast-capable real-time qPCR machine that was programmed as step 1; one cycle of 20 seconds for enzyme activation at 95^{o}C. Then for step 2; forty cycles every 1second at 95^{o}C were performed, followed by 20 seconds at 60^{o}C.

Results:

A. Quantification of the transcribed hFIX gene:

The hFIX gene was successfully transcribed in both the control and the transfected HepG2 cells in which the hACTB gene was considered as house keeping gene (reference gene). The following row data was observed as show in table 1.

Table 1: shows the qPCR data observed with the StepOne™ Real-Time qPCR Systems instrument. The above data was of the transcription of both hFIX and hBACT genes. Duplicates of control (C), transfected (T), and reverse transcriptase negative (-RT) -cDNA copies were used. The plasmid was used as positive control while the nuclease-free water was used as negative control.

hFIX primers	Observed qPCR data	hACTB primers	Observed qPCR data
C-cDNA	**32.300**	**C-cDNA**	**12.565**
C-cDNA	**32.172**	**C-cDNA**	**12.828**
T-cDNA	**10.827**	**T-cDNA**	**12.655**
T-cDNA	**11.620**	**T-cDNA**	**12.815**
-RT-cDNA	**Undetermined**	**-RT-cDNA**	**Undetermined**
-RT-cDNA	**Undetermined**	**-RT-cDNA**	**Undetermined**
Plasmid	**11.250**	**Plasmid**	**22.991**
Nuclease-free water	**Undetermined**	**Nuclease-free water**	**35.959**

The observed data in table 1 was simplified and the mean of the duplicates was calculated and the Ct values were obtained. The net Ct, ΔCt, and ΔΔCt values are illustrated in table 2.

Table 2: shows the Ct, ΔCt, and ΔΔCt values for the gene of interest (GOI) hFIX and the reference gene hACTB in both control and the transfected mammalian HepG2 cells.

GOI (hFIX)	Ct-values (cycles)	Reference gene (hACTB)	Ct-values (cycles)	ΔCt	ΔΔCt
C-cDNA	33	C-cDNA	13	20	- 21
T-cDNA	12	T-cDNA	13	- 1	

B. Quantification of the translated FIX protein:

FIX protein has been successfully expressed in both the mammalian hepatic cell-line (HepG2 cells) and the growth media. The plate reader showed an increase in the absorbance at 450nm wavelength corresponding to an increase in the FIX concentration as shown in table 3 below and plotted in figures 3 and 4 respectively.

Table 3: The absorbance of a serially diluted [FIX] and log [FIX]. The absorbance was recorded by Infinite® 200 PRO series plate reader at 450nm wavelength and 9nm bandwidth. The absorbance values were listed after subtracting the blank value (0.26).

FIX (ng/ml)	Log ([**FIX] (ng/ml)**)	Absorbance
100.00	2.00	0.92
50.00	1.70	0.7
25.00	1.40	0.53
12.50	1.10	0.37
6.25	0.80	0.2
3.13	0.49	0.09
1.56	0.19	0.05
0.00	N/A	0

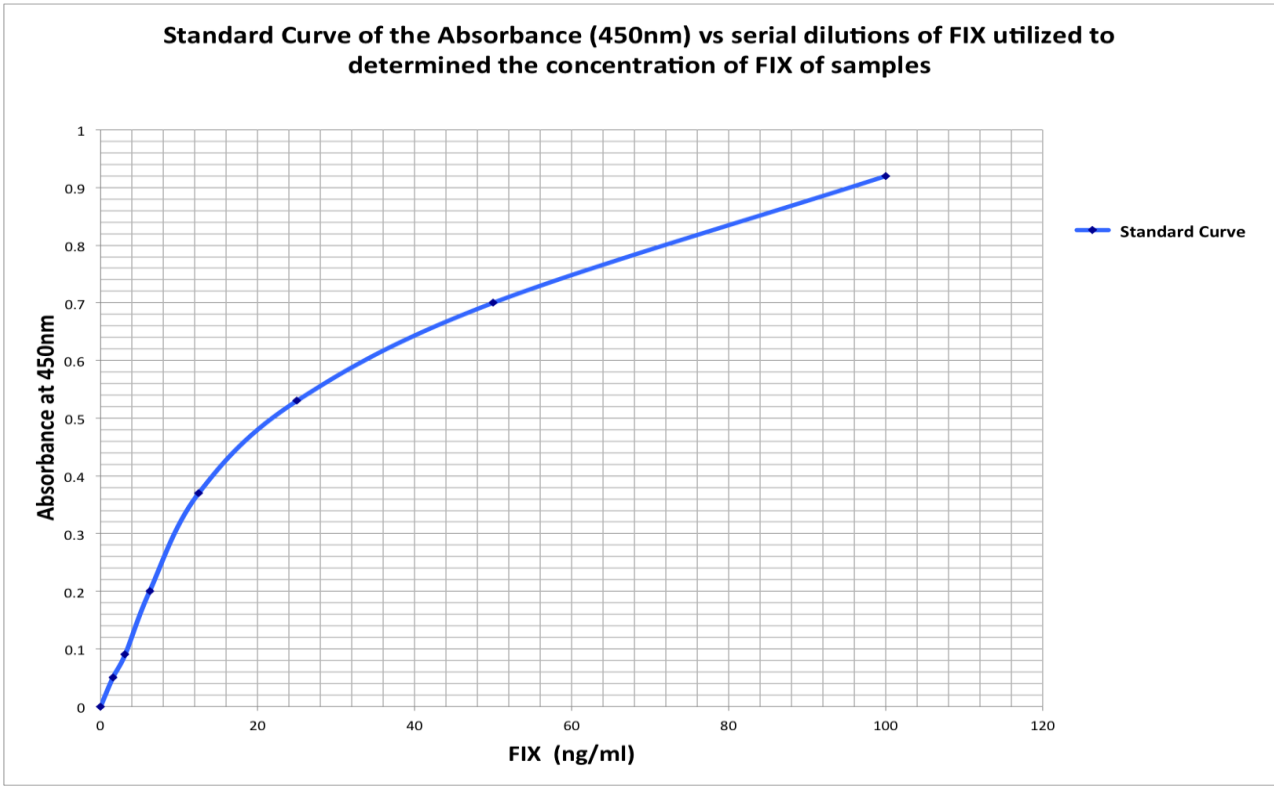

Figure 3: shows the standard curve of absorbance at 450nm (observed with plate reader) versus the concentration of FIX ng/ml (obtained utilizing sandwich type ELISA technique).

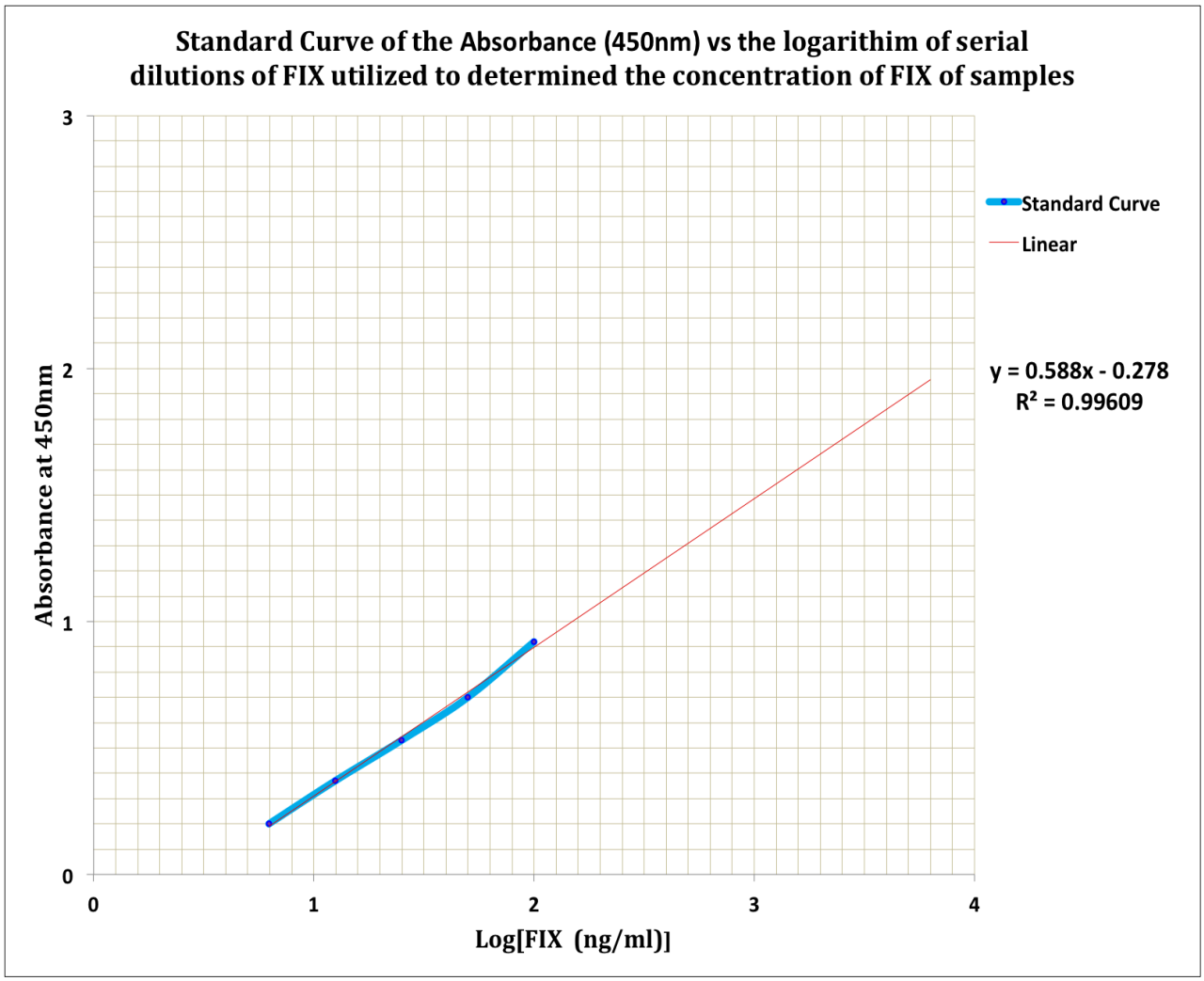

Figure 4: shows the linearity of the correlated absorbance at 450nm (observed with plate reader) versus the logarithm of [FIX] ng/ml. The slope was found to be (0.588) while the intercept is (-0.278) as shown in the linear equation (y= 0.588x – 0.278). The R-squared value is 0.996.

The plate reader instrument precision (standard deviation) for the observed data is found to be (0.04). Also the lower limit of detection and the lower limit of quantification were found to be 0.12 and 0.4 respectively.

Accordingly, the concentration of factor IX was calculated based on the linear equation gained from the plot on figure 4. The concentration of FIX inside the transfected cells is found to be 6.8 ng/ml, in contrast its concentration is much more in the transfected medium, which is calculated and found to be 5495 ng/ml, which is approximately 800-fold. The observed results are listed in table 4 below.

Table 4: This table reveals the calculated concentrations of FIX that are corresponding to the observed absorbance.

Samples	Absorbance at 450nm	[FIX] (ng/ml)
Transfected cells	0.21	6.8
Transfected cell media	1.92	5495
Control cells	Below the limit of detection	N/A

Control cell media	Below the limit of detection	N/A

Discussion:

Scientists worked on pseudotyped adeno-associated virus vectors to express hFIX. The expressed gene was not evaluated, while the final produced hFIX protein was evaluated utilizing ELIZA technique[20]. In this study, the transcribed hFIX gene was evaluated employing the RT-qPCR technique. It has been reported that the utilization of the 463bp β-actin reference gene was as a measure of quality control (QC) and as a standard that allows for the normalization of the number of cells, efficiency of the RNA prepared and the cDNA synthesized[21]. In this study the employment of β-actin gene was of such importance as can be seen in table 1, the observed qPCR data for β-actin gene was approximately the same for all the control and transfected cells. On the other hand, regarding the gene of interest (hFIX gene), the number of qPCR cycles was markedly reduced in the transfected cells as compared with the control cells as shown in table 1. Moreover, the reverse-transcriptase negative controls showed "undetermined" data for both hFIX and hACTB primers. This indicates that the absence of the reverse transcriptase enzyme urge that no mRNA would be reversibly transcribed to cDNA, so that if any amplification occurs in the –RT tubes then it should be due to contaminant DNA. Fortunately, there is no contamination as revealed by the –RT rows in table 1.

Furthermore, the use of the nuclease free water as a negative control has a particular importance. This can be explained as if there are any nucleases then they would destroy the cDNA as well as any possible contaminant then a false negative result would be observed. In contrast if the nuclease-free water was used then no reaction should be observed, as there is no added cDNA otherwise if any signal would be observed then this definitely means the sample was contaminated. In this study no contaminant was encountered with the hFIX while with the hACTB it was observed in the nuclease-free water negative control as can be seen in table 1.

The established precision for the plate reader instrument (SD= 0.04), besides the relatively high R-squared value (R^2 = 0.996), both of these indicate a reliable data observed as shown in figure 4. In previous study, the level of the hFIX protein produced has been reported to be 100 times its basal level[20]. In this study more improvement was performed so that the produced hFIX protein was found to be more than 800 times as revealed in table 4.

Nevertheless, TaqMan was used to obtain a reliable data. The only restriction of using TaqMan is that it is relatively more expensive than SYBR green, so if the work is huge or used frequently then SYBR green would be economically a good choice, otherwise for more sensitive/reliable results then TaqMan is the best choice[22].

In fact as the functional rFIX can successfully catalyze the coagulation process; it is logically if the made rFIX would be added (in a concentration similar to that in the plasma) to a plasma deficient with FIX (from a diseased patient), then it has to perform coagulation and hence this also could be a way to know if the recombined FIX is functional or not.

Conclusion:

In this study the quantification of hFIX gene expression was successfully performed utilizing two worldwide used techniques namely RT-qPCR and ELISA. In future work, it is highly suggested to evaluate the quality (function) alongside with the quantity of the target product. Spending one moment thinking how are millions of people are suffering from a particular genetic disease (e.g. Christmas disease) calls for more efforts, time, and investment that are wealthy to be spent to develop the available techniques of gene expression quantification like qPCR and ELISA as well as the arising field of gene gun technology are wealthy to be researched to reduce/stop the suffering of many life-threatening diseases and to improve the quality of life.

References:

1. RUTLAND, Joshua (2008). *Studies of methods for the purification of the activation peptide of blood clotting factor X*. ProQuest, UMI Dissertations Publishing.
2. STIRLING, D. and LUDLAM, C. A. (1993). Haemophilia B and factor IX mutations. *Lancet,* **341** (8853), 1127.
3. CHAVALI, Sreenivas, GHOSH, Saurabh and BHARADWAJ, Dwaipayan (2009). Hemophilia B is a quasi-quantitative condition with certain mutations showing phenotypic plasticity. *Genomics,* **94** (6), 433-437.
4. BIGGS, R. and M ACFARLANE, R. G. (1962). Christmas disease. *Postgraduate medical journal,* **38** (435), 3-12.
5. BMJ PUBLISHING GROUP (1952). Christmas disease. *British medical journal,* **2** (4799), 1405-1406.
6. WANG, Lili; ZOPPE, Monica; HACKENG, Tilman; GRIFFIN, John; LEE, Kuo-Fen and VERMA, Inder (1997). A factor IX-deficient mouse model for hemophilia B gene therapy, *Proceedings of the national academy of sciences of United States of America*, 94(21), 11563–11566.
7. ARMENTANO, D., et al. (1990). Expression of human factor IX in rabbit hepatocytes by retrovirus-mediated gene transfer: Potential for gene therapy of hemophilia B. *Proceedings of the national academy of sciences of the united states of america,* **87** (16), 6141-6145.
8. GUSTAFSSON, Claes; GOVINDARAJAN, Sridhar and MINSHULL, Jeremy (2004). Codon bias and heterologous protein expression, *TRENDS in Biotechnology*, 22 (7), 83-92.
9. FOGOLIN, Mariela' EBERHARDT; Marcos; KRATJE, Ricardo and ETCHEVERRIGARAY, Marina (2002). Choice of the adequate quantification method for recombinant human GM-CSF produced in different host systems, *Electronic Journal of Biotechnology* [Online], 5 (3), 15-16.
10. STRACHAN, Tom and READ, Andrew (2011). Human molecular genetics, 4th edition, New York : Garland Science/Taylor and Francis Group.
11. BECKER-ANDRÉ, M. and HAHLBROCK, K. (1989). Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). *Nucleic acids research,* **17** (22), 9437-9446.
12. MULLIS, K., et al. (1986). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol. 51: 263-273.
13. PORCHER, C., et al. (1992). A simplified method for determination of specific dna or rna copy number using quantitative pcr and an automatic dna sequencer. *Biotechniques,* **13** (1), 106.
14. VANGUILDER, Heather D., VRANA, Kent E. and FREEMAN, Willard M. (2008). Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques,* **44** (5), 619-626.
15. FREEMAN, W. M., WALKER, S. J. and VRANA, K. E. (1999). Quantitative RT-PCR: Pitfalls and potential. *Biotechniques,* **26** (1), 112-112.
16. SALEH-LAKHA, Saleema, et al. (2005). Microbial gene expression in soil: Methods, applications and challenges. *Journal of microbiological methods,* **63** (1), 1-19.
17. SALEH-LAKHA, Saleema, et al. (2011). Challenges in quantifying microbial gene expression in soil using quantitative reverse transcription real-time PCR. *Journal of microbiological methods,* **85** (3), 239-243.
18. OSTLUNDA, Gabriel and SONNHAMMERA, ERIK L.L. (2012). Quality criteria for finding genes with high mRNA–protein expression correlation and coexpression correlation, *Gene*, **497** (2), 228–236.
19. REMICK, Daniel G. and NATARAJAN, Sudha (2007).The ELISA Standard Save: Calculation of sample concentrations in assays with a failed standard curve, *Journal of Immunology Methods*, 336(2), 242–245.
20. GRIMM, Dirk, et al. (2003). Preclinical in vivo evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. *Gene therapy*, **102** (7), 2412-2419
21. CHOUDHARY, I., et al. (2013). Single step detection of HIV-1 proviral DNA and housekeeping beta-actin gene from dried blood spots by a monoplex polymerase chain reaction. *Journal of virological methods,* **187** (1), 203-206.
22. PONCHEL, Frederique, et al. (2003). Real-time PCR based on SYBR-green I fluorescence: An alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. *BMC biotechnology*, **3** (1), 18-18.