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Research Article

# Comparison of Human Factor IX Abundance and mRNA Expression Levels In Stable Insect and Mammalian Cells

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## Abstract

In the production of recombinant proteins, the selection of an expression system is very important. Although CHO cells are used as mammalian expression system, the ability of insect Schneider line 2 (S2) cells as a new expression system for the high production of many human proteins were confirmed. It is suggested that high copy number of an introduced gene and so high transcription in these cells can be regarded as one of the reasons for high expression of recombinant proteins. Therefore, the present study aimed to evaluate the correlation of recombinant human coagulation factor IX (hFIX) abundance and mRNA expression. The amount of hFIX mRNA by quantitative real-time PCR as well as hFIX protein in cell lysate and cultured media by Elisa was analyzed. The results of data analysis indicated 6 fold increases in mRNA level in S2 cells in comparison to CHO cells. Furthermore, S2 cell line indicated 5.5 and 7-fold increase in total and secreted protein level, respectively, compared to CHO cell line. The data demonstrated the correlation of mRNA and protein abundance and indicate that S2 cell lines are superior in producing the recombinant proteins.

**Keywords:** Drosophila S2 cells, CHO cells, Coagulation factor IX, Real-time PCR, Elisa

## Introduction

Gene expression is a process by which information from a gene is used in the synthesis of a functional gene product. Several steps in the gene expression process may be modulated such as DNA-RNA transcription step to post-translational modification of a protein (Myhre et al., 2013). Moreover, in the production of recombinant proteins, expression of a foreign gene is influenced by several factors such as copy number of introduced gene, and the ability of cell line in transcription and translation (Brown, 2016; Wikibooks, 2017). Therefore, using an expression system is important in the regulation of gene expression including a wide range of mechanisms to increase the production of specific gene products.

Among the various protein expression systems, a cell system used increasingly for the expression of various recombinant proteins is based on *Drosophila melanogaster* Schneider line 2 (S2) cells, which offers a straightforward approach to stably produce high quantities of a functional protein (Moraes et al., 2012). The S2 cells were developed as a plasmid-based and non-lytic integration system, capable of stably expressing high levels of recombinant proteins (Bernard et al., 1994; Kirkpatrick and Shatzman, 1999). The S2 expression system has the advantage of proliferating without requiring CO2, being easily adapted to large-scale fermenters, being capable of long-term continuous culture, having a null expression background, and having the appropriate post-translational machinery (Moraes et al., 2012; Vatandoost et al., 2012). Correct post-translational modification of a recombinant protein especially in the case of hFIX influence on production of protein and so indicated as an important factor in the selection of an appropriate host cell. Moreover, the Drosophila metallothionein (Mtn) promoter is shown to be tightly regulated and capable of driving high-level heterologous transcription upon induction by metals such as Cu or Cd ions (Maroni et al., 1986; Otto et al., 1987). Furthermore, Drosophila cells can integrate up to 1000 copies of an expression cassette in a single transfection event while protein expression remains tightly regulated even at high copy numbers with low basal expression, maintained in the absence of an inducer (Kim et al., 2008). By considering these advantages, it seems that insect S2 expression

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system has the mechanisms to increase the production of recombinant proteins. Using this system, a wide variety of proteins that are appropriately processed and are able to maintain biological activity have successfully been expressed in large scale (Moraes et al., 2012). The reason for high expression is still in question in spite of demonstrating a high expression of recombinant proteins in S2 cells. It is believed that high copy number of introduced gene and so high mRNA abundance can play a significant role and is correlated with the expression of recombinant proteins. By considering all this issues, the expression of hFIX as a recombinant protein at the mRNA and protein level was evaluated in the present study. In other hand, a new expression system, *Drosophila melanogaster Schneider* line 2 (S2) cells, was taken into consideration in comparison with mammalian CHO cells.

**Materials and Methods**

All the enzymes used for the molecular biology, the kits for PCR purification and plasmid isolation, RNA preparation, reverse transcriptase (M-MuL V), and other chemicals such as protease inhibitors were purchased from SinaClon in Tehran, Iran. Oligonucleotides were synthesized by Denazist (Mashhad, Iran) and Bioneer (Daejeon, Republic of Korea). The pcDNA3, pMT-V5-HisA and pCoHygro plasmids and all cell culture reagents were got from Thermo Fisher Scientific, except Schneider's insect medium, penicillin G and streptomycin (Sigma-Aldrich). Further, Geneticin (G418), hygromycin, vitamin K and protease inhibitors were purchased from Roche and the enzyme-linked immunosorbent assay (ELISA) specific for human FIX (AsserachromhFIX:Ag, Stago, France) and activated partial thromboplastin time (aPTT) reagents were purchased from Diagnostica Stago (Bern, Switzerland).

**Cell culture and Preparation of Stable Clones**

*Drosophila Schneider* (S2) and CHO cells as a kind gift from Dr. A.R. Zomorodipour, NIGEB, Iran were cultured and transfected as described previously (Haddad-Mashadrizeh et al., 2009; Vatandoost et al., 2012). In summary, the CHO cells were grown in a 5% $CO_2$ atmosphere at 37°C, sub-cultured at a density of $2 \times 10^5$ cells in a volume of 2 ml in 6-well plates, and were transfected with 2 µg pcDNA3-hFIX by using FuGene–6. Individual clones were expanded in the presence of 450 µg/ml geneticin. Expression media containing 6 µg/ml of vitamin K1 was added to ~70% confluent cells, upon which the individual clones were screened for FIX production. S2 cells were kept at 28ºC without CO2 under the normal atmosphere in Schneider's insect medium, supplemented with penicillin G (50 units/mL) and streptomycin (50 µg/mL). One day before transfection, $3\times10^6$ cells were seeded in a volume of 3 ml in 6-well plates, upon which the cells were allowed to loosely adhere. The S2 cells were transfected with pMT-hFIX and the pCoHygro plasmid, including the hygromycin resistance gene by employing the calcium phosphate co-precipitation method with minor modifications (3). After 48 hours, individual clones were selected in the presence of 300 µg/ml hygromycin B. The clones were screened for FIX expression, followed by the induction with 0.5 mM CuSO4 in the presence of 6 µg/ml vitamin K1.

**Analysis of hFIX Transcript Levels with Quantitative Real-time PCR.**

The total RNA was prepared from the induced cells by using RNX-Plus Kit (SinaClon, Iran), based on the manufacturer's instructions and was reversely transcribed by using random primers and revertAid M-MuLV. The cDNA synthesis was done by using specific primers, hFIX-F (5'GAATGTTGGTGTCCCTTTGG3') and hFIX-R (5'AATGGCACTGCTGGTTCAC3'). Using a total of 100 ng of RNA from each cell line, real- time PCR was performed, using SYBR green method and premix Amplicon kit on an ABI 7500 real-time PCR system. The ribosomal protein L32 (RPL 32) for S2 cells and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for CHO cells, were considered as the internal control. A 165 bp and 123 bp fragments of RPL 32 and GAPDH were amplified through using oligonucleotides RPL-F (5' ATCGGTTACGGATCGAACAA 3')/RPL-R (5' GACAATCTCCTTGCGCTTCT3') and GAPDH-F (5'-AGGTCGGTGTGAACGGATTTG-3)/ GAPDH-R (5'-TGTAGACCATGTAGTGGTCA-3'), respectively. The real-time PCR conditions consisted of a pre-denaturation step at 95°C for 5min, followed by 40 cycles at 95°C for 15s and 58°C for the 20s.

**Quantification and Activity Analysis of Recombinant hFIX by ELISA and aPTT**

Human FIX was quantified in conditioned media by employing an ELISA based on the procedure provided by the manufacturer. In the procedure, strip wells were pre-coated with goat polyclonal antibody to hFIX.

Then, the samples were diluted and 100 µl of text sample were applied to the wells. After 30 minutes of incubation to the present hFIX, antigen binds to

the coated antibody, unbound material washing away and, 100 µl peroxidase-labeled FIX detecting antibody were applied and allowed to bind to the captured hFIX for 30 minutes. Then, the wells were rewashed and a solution of TMB (100 µl of peroxidase substrate tetramethylbenzidine) was applied and allowed to react for 10 minutes. A blue color is developed and changed to yellow upon quenching the reaction with 100 µl of 0.2 M sulphuric acid.

Finally, the color formed is measured spectrophotometrically in a microplate reader at 450 nm. The absorbance at 450 nm is directly proportional to the concentration of hFIX, based on the standard curve as stated in ng/mL. The assay is calibrated by using the calibrator plasma provided in the kit. Moreover, the results obtained after the subtraction of non-specific absorbance were determined on cultured media from the non-transfected cells. In addition, intracellular accumulated hFIX was assessed, for which the cells were pelleted by centrifugation at 100 g for 5 min, upon which they were resuspended in 500 µL of ice-cold lysis buffer (100 mM KCl, 2 mM MgCl2, 10 mM, HEPES pH 7.5, 0.5% Triton X100), including an antiprotease mixture combined of completed Protease Inhibitor (Roche). Subsequently, the lysate was centrifuged at 12,000 g for 10 min at 4 ºC and the supernatant was assessed for hFIX.

The functional activity of recombinant hFIX was examined using an aPTT assay (Otto et al., 1987). First, human plasma immuno-depleted of FIX (100 µL) was mixed with conditioned media (100 µL) and aPTT reagent (100 µL). After 3 min of incubation at 37 ºC, 100 µL of a prewarmed CaCl2 solution (25 mM) was added to the mixture, and the clotting time was recorded. Then, the activity of the expressed hFIX was calculated based on the standard curve of normal human plasma (Iranian blood transfusion organization), and one unit of FIX activity corresponded to the amount of FIX in 1 ml of normal plasma (~ 5 µg/ml).

**Data Analysis**

All expression analysis of the experiments was carried out in duplicates or triplets, and the generated data were presented as the mean ± SD. ANOVA was used for data analysis, followed by a Tukey post-hoc test. $P<0.05$ was considered as the level of significance.

**Results**

In this study, mRNA level and expression of the hFIX in stable hFIX producing CHO and S2 cells were investigated. After induction, the presence of FIX mRNA in both cell types was confirmed by employing RT-PCR. Following determining of quantity, purity and integrity of extracted RNA using spectrophotometry and electrophoresis, PCR for hFIX, GAPDH and RPL32 genes was performed. Observation of single bands for hFIX, RPL32 and GAPDH gene which was 182 bp, 165 bp and 123 bp, respectively, indicated the accuracy of amplification of the target gene (Fig. 1)

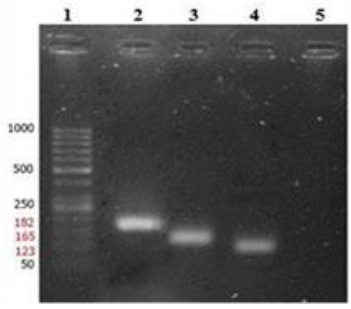


**Figure 1**. Gel electrophoresis of RT-PCR products of hFIX, RPL32 and GAPDH. Electrophoresis of PCR products obtained by RT-PCR on 2% agarose gel electrophoresis. Lane 1, 50 bp DNA ladder (Thermo Scientific); lanes 2, 3 and 4, the amplification product of hFIX (182 bp), RPL32 (165 bp) and GAPDH (123 bp); lane 5, negative control.

Real-time PCR analysis showed that both of cell types were able to express hFIX effectively, and the hFIX transcript level in S2 cells was higher than that of the CHO cells (Fig. 2).

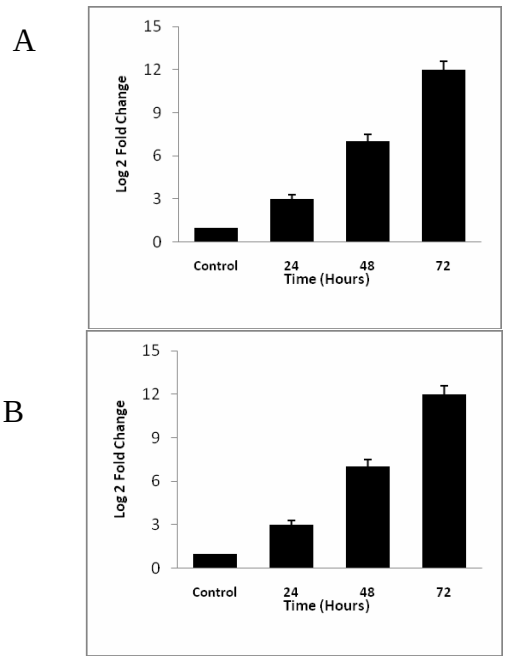


**Figure 2**. Real-time PCR analyses for the assessment of hFIX. The hFIX mRNA levels in stable S2 (A) and CHO (B) cells during 72h after induction assessed by real-time RT-PCR. Data are mean ± SEM

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Compared to non-transgenic cells, there was a significant increase in gene expression during 72 hours in both cells (P <0.05). Further, RNA expression levels were reached to 12 times after 72 hours in comparison to the control gene, which was 2 fold in case of CHO cells. Moreover, the comparison of RNA expression levels showed 4.5 fold increases in S2 cells than CHO cells. To evaluate the hFIX expression in hFIX producing stable CHO and S2 cells, cell lysate and conditioned media were obtained and subjected to ELISA to measure the hFIX concentration. Detectable hFIX levels were achieved in both cells although there was a significant difference in hFIX levels between the hFIX producing stable S2 and the CHO cells ($P$>0.05). The highest total hFIX content in 72h was 502 and 91 ng/ml/$10^6$ cells in S2 and CHO cells, respectively, through which, 432 and 63 ng/ml/$10^6$ cells were secreted to media (Fig 3). As the secretion efficiency of a particular protein is defined as the ratio between the secreted fraction and its total amount, the ratio of secreted hFIX to total hFIX protein was calculated. The highest secretion efficiency of S2 cells was 86%, in comparison to 68% in CHO cells.

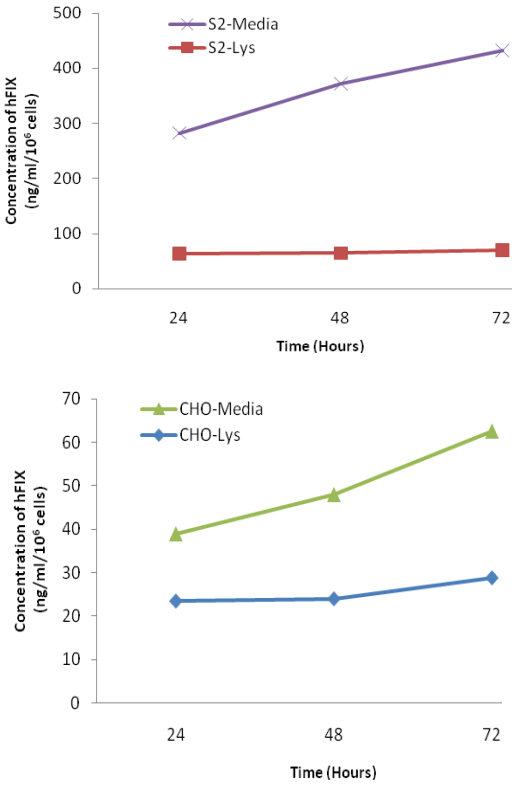


**Figure 3.** Evaluation of the hFIX expression in stable CHO- hFIX and S2- hFIX cells. hFIX levels in stable CHO (B) and S2 (A) cells were determined by Elisa at various post-induction times. ELISA performed on samples taken from both the culture supernatant and the cellular fraction. Data are mean ± SEM

## Discussion

Optimizing the generation of the recombinant proteins in laboratory expression systems is important for the purpose of the reduction of the production cost since the treatment of many of diseases including Hemophilia-B is done by replacement therapy of recombinant proteins. There are many ways to increase expression and the activity of the recombinant proteins in expression systems (Vatandoost and Pakdaman, 2016) such as the expression systems with the capability of more active protein production. In recent years, the S2 cells with various advantages including high growth rate have been introduced as an efficient expression system. Because of the capability of these cells to integrate multiple copies of expression plasmids in a transfection event (Cherbas and Cherbas, 2007; Moraes et al., 2012), it was expected that the number of the transcribed mRNAs would be high. The comparison of the expression of coagulation factor IX in S2 and CHO cells showed that the amount of the expression of FIX mRNA in S2 cells is about 6 times higher than CHO cells. Regarding the reasons, we can refer to the high copy number of the integrated plasmid in S2 cells in comparison with CHO cells, and the power of the used promoter in vectors. It seems that the Mtn promoter is more powerful although the CMV promoter in mammalian vectors is a constitutive powerful promoter. Further, it is tightly regulated (Johansen et al., 1989) and this is, especially crucial when expressing proteins that may have a metabolic board to growing cells.

Protein levels are influenced by the regulation of transcription, translation and protein stability (Myhre et al., 2013). Based on FIX mRNA level in S2 and CHO cells, it is also expected that the amount of produced protein will be about 6 times. However, the results of the total amount of protein in two cells indicated that this ratio is about 5.5 times. In consistent with the previous results (Myhre et al., 2013), the results of the present study indicated that mRNA expression was correlated significantly to protein abundance. Therefore, the more mRNA in S2 cells results in more protein production although there is another mechanism like translation and translocation to ER that may be effective in this process. In addition, the amount of secreted proteins to cultured media is important since the purification simplicity of the recombinant proteins is a significant factor in their production process. The assessment of secreted FIX to cultured media indicated that while FIX was secreted at levels up to 63 ng/ml/$10^6$ on day 3 post-induction in CHO cells,

the highest secreted FIX in S2 cells reached to 432 ng/ml/$10^6$ that was seven-fold more. Furthermore, although high densities of S2 cells rather than CHO cells can have an influence on the amount of FIX in cultured medium, in comparison to one million cells, they showed that high expression in S2 cells might be affected by factors such as the transcribed mRNA level, translation and translocation capability. It was expected that higher mRNA and so higher produced FIX might result in more secretion. However, the results indicated that by increasing FIX expression, the secretion efficiency of FIX in S2 cells increased during 24 to 72 hours. Furthermore, the secretion efficiency of FIX in S2 cells with more protein expression was about 15% higher than CHO cells.

In conclusion, the S2 cells were superior to mammalian cells in expression of recombinant proteins from mRNA to protein level.

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