

African Journal of Biotechnology Vol. 9(50), pp. 8566-8577, 13 December, 2010
Available online at <http://www.academicjournals.org/AJB>
DOI: 10.5897/AJB10.1105
ISSN 1684-5315 © 2010 Academic Journals

Full Length Research Paper

The preparation of 6x His-tagged granulocyte colony stimulating factor using an improved *in vitro* expression

Helmy, O. M.*, Hussein, M. M. M., Murad, F. E. and Shoeb, H. A.

Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Accepted 5 November, 2010

An improved *in vitro* expression system called the rapid translation system (RTS) was used in this study for the *in vitro* biosynthesis of 6x His tagged granulocyte colony stimulating factor (6x His-tagged granulocyte colony-stimulating factor (G-CSF)). This was done to overcome the problems associated with traditional cell based biotechnology. The study involved the preparation of template DNA for cell-free protein synthesis through gene amplification of open reading frame (ORF) of hGCSFb, cloning in pIVEX 2.4d vector and transformation of the produced construct in chemically competent *Escherichia coli* DH 5 α cells. A cell free protein synthesis system, RTS 100 *E. coli* HY kit, was tested for 6x His tagged G-CSF protein synthesis. Protein purification was done using Ni-NTA chromatography. Protein production was detected by two methods electrophoretically by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunologically by dot blotting immunodetection. The use of these methods yielded purified 6xHis-tagged G-CSF with a concentration of about 250 μ g/ml RTS reaction.

Key words: Granulocyte colony stimulating factor, *in vitro* expression system, RTS systems.

INTRODUCTION

Despite all its usefulness as a method for protein production, recombinant DNA technology has a lot of problems such as insoluble inclusion body formation, instability of recombinant proteins due to the presence of intracellular proteases and the toxicity of some recombinant proteins to host cells. A cell free protein synthesis system can overcome these problems because it is essentially free of cellular control mechanisms to ensure its survival (Kim and Choi, 1996). The *Escherichia coli* extract, rabbit reticulocyte lysate and the wheat germ extract are the three commonly used extracts for cell-free protein

synthesis. In the bacterial systems, the DNA species are added to the translation mixture, and the corresponding mRNA would be synthesized *in situ* by the endogenous RNA polymerase present in the bacterial extract or its supernatant fraction (Spirin, 2003). Significant progress has been made using a bacterial cell-free transcription-translation system from pure, individual components. These included recombinant His6-tagged translation factors (10 proteins), aminoacyl-tRNA synthetases (20 proteins), methionyl-tRNA transformylase, T7 RNA polymerase and the proteins of an ATP regenerating sub-system. In this system, bacterial ribosomes in the presence of appropriate plasmids produced correctly folded active proteins at a rate of about 0.1 mg/ml per hour (Shimizu et al., 2001). The invention of the continuous flow cell-free translation and coupled transcription-translation systems was a breakthrough as they overcame the main shortcoming of most test-tube translation and transcription-translation systems which was their limited lifetime and as a consequence, their low yield of proteins (Spirin et al., 1988).

The neutrophilic granulocytes are known to originate

*Corresponding author. E-mail: omneyamhelmy@gmail.com.
Tel (202) 23624917. Fax (202) 25316822.

Abbreviation: RTS, Rapid translation system; G-CSF, Granulocyte colony-stimulating factor; ORF, open reading frame; DMSO, dimethyl sulfoxide; MCS, multiple cloning site; RBS, ribosomal binding site; SAP, shrimp alkaline phosphatase; CECF, continuous exchange cell-free; HRP, horseradish peroxidase.

from multipotent stem cells of the bone marrow. The proliferation and differentiation of the progenitor cells for the granulocyte-macrophage lineage are regulated by a family of glycoproteins. These molecules are generally called colony-stimulating factors (CSFs) on the basis of morphology of the colonies stimulated. Granulocyte colony-stimulating factor (G-CSF) is almost specific for granulocytic lineage (Metcalf, 1985 and Nomura et al., 1986). G-CSF thus plays a critical role in the process of hematopoiesis, regulating the proliferation, differentiation and survival of neutrophils and neutrophilic progenitor cells (Bishop et al., 2001). Mouse granulocyte colony-stimulating factor (GM-CSF) was first recognized and purified in Australia in 1983 (Metcalf, 1985), and the human form was cloned by groups from Japan, Germany and United States in 1986 (Nagata et al., 1986 and Souza et al., 1986). G-CSF and GM-CSF are integral parts of the prevention of potentially life-threatening febrile neutropenia; however, only G-CSF has US FDA approval for use in chemotherapy-induced neutropenia. GCSF accelerate formation of neutrophils from committed progenitors and apart from granulopoiesis stimulation can also increase phagocytic and oxidative capacity of granulocytes after chemotherapy, thereby reducing the duration and severity of neutropenia (Dale, 2002). In addition, it is also used as support following autologous and allogenic bone marrow transplantation, and collection of CSF-mobilized peripheral blood progenitor cells (DiPersio and Pusic, 2008). G-CSF was reported to induce myocardial regeneration by promoting mobilization of bone marrow stem cells to the injured heart after myocardial infarction. G-CSF also reduced apoptosis of endothelial cells and increased vascularization in the infarcted hearts, further protecting against ischemic injury (Harada et al., 2005).

The aim of the present work is the use of rapid translation system (RTS), introduced by Roche applied science, which is a cell-free protein production system employing an enhanced *E. coli* lysate to perform coupled *in vitro* transcription-translation reactions for the production of 6x His tagged GCSF.

MATERIALS AND METHODS

Gene amplification of open reading frame (ORF) of hGCSFb

E. coli bacteria, strain GT 110 transformed by pORF 9-hGCSFb (an expression vector containing the human G-CSFb open reading frame) was obtained from Invivogen, France. The extraction of pORF9-hGCSFb was performed using a QIAprep Miniprep kit Qiagen, Hilden, Germany. The restriction digestion of the purified plasmid was done using Hind III restriction enzyme according to the manufacturer, Fermentas EU. This was followed by electrophoresis in 0.7% agarose gel / TAE at 70 volt and 90 mA (Sambrook and Russell, 2001). The DNA extraction from agarose gel was performed using a QIAquick gel extraction kit Qiagen, Hilden, Germany.

Polymerase chain reaction (PCR) reaction

Primers' design was performed using the fast PCR free software

<http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm>.

Forward primer I

5' → 3' CAGTGCA CATATGACAGTGCAGGAAGCCACC
The underlined represents NdeI restriction site.

Reverse primer

5' → 3' TCATGTCGAGCCCGGGTTCTGCTC
The underlined represents SmaI restriction site

Forward primer II

5' → 3' CAGTGCACATATGACA**ATCGAGGGACGC**↓ACC
The underlined represents NdeI restriction site, while the bold represents factor Xa protease cleavage site.

First PCR reaction mixture

0.5 µl pure Hind III digest (1.8 µg/µl), 10x PCR buffer, 2.5 mM dNTPs (Sigma Inc., St Louis, Mo, USA), Forward Primer I (100 pmole/ µl) (Qiagen, Operon), Reverse Primer (100 pmole/ µl), Q factor (5x), Taq polymerase enzyme (Qiagen Hilden, Germany) 2.5 units in a final volume of 50 µl. PCR was performed in a Techne Touchgene Gradient thermal cycler, USA. The cycling conditions used were one cycle of 95°C for 5 min, 35 cycles each of 95°C for 45 sec, 58°C for 45 sec (chosen from a gradient of annealing temperatures) and 72°C for 1 min and ending with a final extension at 72°C for 8 min. Samples were held at 4°C prior to analysis. The reaction mixture was analysed by gel electrophoresis through 0.7% (w/v) agarose in 1 x TAE buffer. The optimum PCR reaction was purified using QIAquick gel extraction kit.

The second PCR reaction

The purified 1st PCR product was used as template in the second reaction. Optimization of the second PCR reaction was carried out by changing annealing temperature, concentration of primers and addition of certain additives. The PCR reaction consist of 1 µl pure first PCR product, 10x PCR buffer, 2.5 mM dNTPs (Sigma Inc., St Louis, Mo, USA), Forward Primer II (10 pmole/ µl) (Qiagen, Operon), Reverse Primer (10 pmole/ µl), 25 mM MgCl₂, Q factor (5x), Taq polymerase enzyme (Qiagen Hilden, Germany) 2.5 units in a final volume of 50 µl. PCR was performed in a Techne Touchgene Gradient thermal cycler, USA. The cycling conditions used were one cycle of 95°C for 5 min, 35 cycles each of 95°C for 45 sec, 55°C for 45 sec and 72°C for 1 min and ending with a final extension at 72°C for 8 min. Samples were held at 4°C prior to analysis. Each reaction mixture was analysed by gel electrophoresis through 0.7% (w/v) agarose in 1x TAE buffer. The second PCR reaction product was purified using QIAquick gel extraction kit and was further purified by phenol: chloroform: isoamyl alcohol (25:24:1) at pH 8 (Sigma Inc., St Louis, Mo, USA) according to the method of Sambrook and Russell (2001).

Cloning in pIVEX 2.4d vector (Roche Diagnostics, Germany) and transformation of the produced construct in chemically competent *E. coli* DH 5 α cells

SmaI/NdeI (Roche Diagnostics., Germany) was used for the digestion of the second PCR product and pIVEX 2.4d vector

according to the manufacturer. The digested pIVEX 2.4d vector was further dephosphorylated using shrimp alkaline phosphatase (Roche diagnostics., Germany). The digested dephosphorylated vector and digested PCR product was purified using QIAquick gel extraction kit then further purified by phenol : chloroform : isoamyl alcohol (25:24:1) pH 8 (Sambrook and Russell, 2001).

Preparation of the ligation reactions

Molar ratios (1:5, 1:7, 1:10) of digested vector and insert were checked, 3 units T4 DNA Ligase and 30% PEG 8000 were used. The reactions were incubated overnight at 16°C (Sambrook and Russell, 2001). Chemically competent cells of *E. coli* DH 5 α were prepared and transformed according to the method of Pharmacia U.S.E mutagenesis manual. Testing the efficiency of transformation was done by using intact pIVEX 2.4d vector.

Preparation of minipreps from clones suspected to contain the pIVEX 2.4d-second PCR construct using QIAprep miniprep kit

The procedure was carried out according to the manufacturer and optimization of the protocol was carried out by testing incubation temperature of 30°C and addition of chloramphenicol.

A cell free protein synthesis system, RTS 100 *E. coli* HY kit (Roche Diagnostics., Germany), was tested for 6x His tagged G-CSF protein synthesis

The reaction solution was prepared according to the manufacturer and incubated at 30°C for 20 h. The reaction solution was stored frozen until purification or further processing.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for the RTS 100 *E. coli* HY reaction

Electrophoresis in 15% polyacrylamide gels was performed according to the method of Laemmli (1970). A BioRad mini-protein dual slab cell was used for running electrophoresis. The RTS samples were precipitated with acetone prior to the addition of SDS-PAGE sample buffer. The pellet was then dissolved in 70 μ l 1x SDS-PAGE sample buffer and heated for 5 min at 95°C. Samples were cooled before applying 25 μ l to gels. Electrophoresis was carried out at 90 volts and 40 mA for 90 min. Gels were stained for 30 min with 0.1% (w/v) coomassie blue R-250 in fixative whose composition is: 20% (v/v) methanol and 10% (v/v) glacial acetic acid and destained in 14.2% (v/v) methanol and 10% (v/v) glacial acetic acid overnight.

Protein purification was done using Ni-NTA chromatography (Qiagen Hilden, Germany)

Protein purification using Ni-NTA spin columns was carried out, according to the manufacturer. The eluted protein was precipitated with acetone prior to the addition of the SDS-PAGE sample buffer this was followed by SDS PAGE.

Determination of Ni-NTA spin column purified protein content

Protein purified from Ni-NTA spin kit was precipitated with acetone then resuspended in 10 μ l distilled H₂O. Protein content determination was carried out by coomassie blue dye assay according to the method of Bradford (1976).

Dot blotting immunodetection of 6xHis-tagged proteins with anti-His HRP conjugates (Chromogenic method) (Qiagen Hilden, Germany)

The RTS samples were precipitated with acetone prior to the addition of the denaturing buffer. The produced pellet was then dissolved in 200 μ l denaturing buffer, 10 μ l of the diluted protein sample was applied directly onto a nitrocellulose membrane. After applying the samples, the membrane was air dried at room temperature before proceeding with the detection process according to the manufacturer.

RESULTS

hGCSFb-gene synthesis by PCR of the Hind III digested pORF9-hGCSFb

The first PCR reaction

Hind III digested pORF9-hGCSFb was used as a substrate. Gradient of annealing temperatures ranging from 58 \rightarrow 69°C for 45 sec was tested. At the end of the PCR reactions, the different reactions performed using different annealing temperatures were screened using DNA agarose gel electrophoresis. The results of the first PCR reaction are shown in Figure 1. Annealing temperatures 58, 59.4 and 60.4°C showed high intensity bands of first PCR product of size 626 bp, annealing temperatures 61.5, 62.5, 63.5 and 64.3°C showed lower intensity bands of first PCR product. Thus, it can be concluded that the best annealing temperature for the first PCR reaction is 58°C.

The second PCR reaction

First PCR reaction product was used as a substrate. Gradient of annealing temperatures ranging from 58 \rightarrow 69°C for 45 sec were tested. No distinct bands of the second PCR product of size 626 bp were observed on agarose gel. The first PCR reaction product was used as a substrate. Another gradient of annealing temperatures ranging from 52 \rightarrow 58°C was tested. The results of the second PCR reaction are shown in Figure 2 where annealing temperatures ranging from 53.7 to 56.2°C showed intense bands of second PCR product of size 626 bp. Further optimization of the second PCR protocol was performed.

Optimization of the second PCR reaction

Attempts to optimize the second PCR reaction were done by testing different variables including the testing of two different annealing temperatures, 54 and 55°C; the use of two different primers' concentration of 100 and 10 pmole/ μ l, supplementing the reaction with 25 mM MgCl₂ and testing 2 surfactants 5% dimethyl sulfoxide (DMSO)

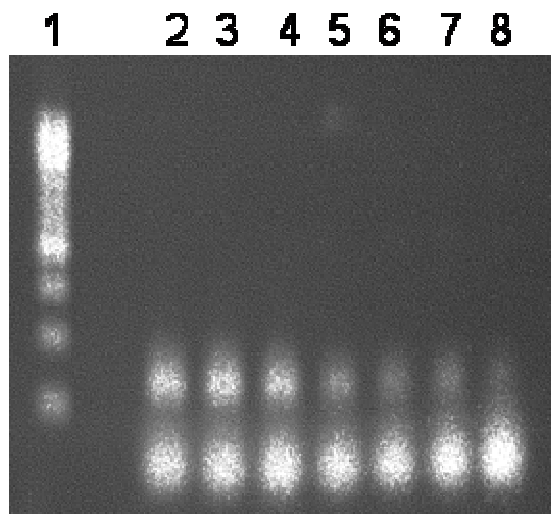


Figure 1. Agarose gel of first PCR reaction using gradient annealing temperatures from 58 to 64.3°C. Lane 1, DNA molecular weight marker XVII, Roche diagnostics., Germany, (500 base pair ladder from 5000 – 500 bp); Lane 2, PCR reaction with annealing temperature 58°C; Lane 3, PCR reaction with annealing temperature 59.4°C; Lane 4, PCR reaction with annealing temperature 60.4°C; Lane 5, PCR reaction with annealing temperature 61.5°C; Lane 6, PCR reaction with annealing temperature 62.5°C; Lane 7, PCR reaction with annealing temperature 63.5°C; Lane 8, PCR reaction with annealing temperature 64.3°C.

and Q factor. At the end of the PCR run, the 16 different reactions performed using these variables were screened using DNA agarose gel electrophoresis and the results are shown in Figures 3 and 4. The intensity of second PCR product band corresponding to each optimized second PCR reaction is recorded in Table 1.

The diluted primers reactions (I, K, M, O, J, L, P and N) gave better results than concentrated primers reactions (A, C, E, G, B, D, F and H). The sharpest intense band of the second PCR product was obtained when using an annealing temperature of 55°C, primers' concentration of 10 pmole/μl and supplementing the reaction with 25 mM MgCl₂ and Q factor.

Cloning in pIVEX 2.4d vector and transformation of the produced construct in chemically competent *E. coli* DH 5 α cells

Transformation of chemically competent *E. coli* DH 5 α cells

Examination of ampicillin Lysogeny broth (LB) agar plates inoculated with the three different transformation ligation reactions revealed that the ligation reaction with the highest vector to insert molar ratio (1:10) produced the largest number of colonies. One of these colonies contained the

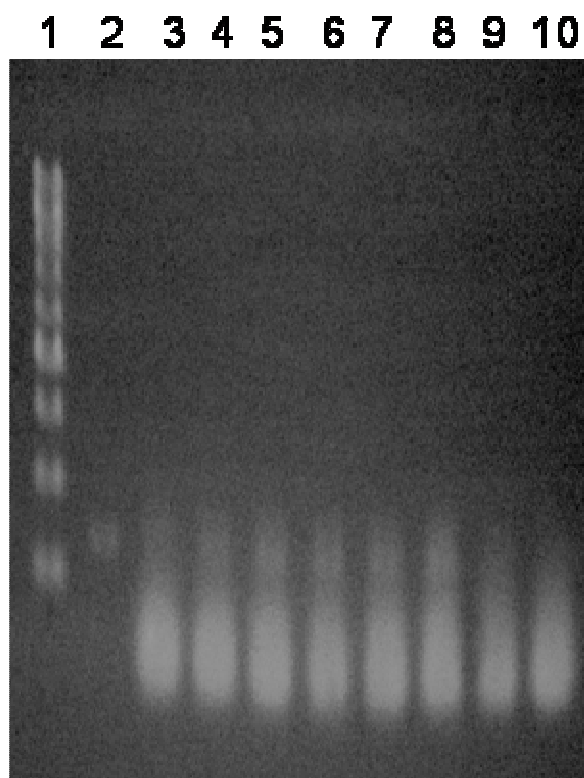


Figure 2. Agarose gel of second PCR reaction using gradient annealing temperatures from 52 to 58°C. Lane 1, DNA molecular weight marker XVII (500 base pair ladder ranging from 5000 bp – 500 bp); Lane 2, first PCR product; Lane 3, PCR reaction with annealing temperature 52°C; Lane 4, PCR reaction with annealing temperature 52.9°C; Lane 5, PCR reaction with annealing temperature 53.7°C; Lane 6, PCR reaction with annealing temperature 54.6°C; Lane 7, PCR reaction with annealing temperature 55.2°C; Lane 8, PCR reaction with annealing temperature 56.2°C; Lane 9, PCR reaction with annealing temperature 57.2°C; Lane 10, PCR reaction with annealing temperature 58°C.

desired vector-insert ligation product as shown in Figure 5.

Preparation of minipreps from clone suspected to contain pIVEX 2.4d-second PCR construct using QIAprep miniprep kit

Using the original protocol for the preparation of minipreps followed by agarose gel electrophoresis revealed plasmid DNA of the right molecular weight of 4148 bp but contaminated with chromosomal DNA. This chromosomal DNA contamination appeared as a background smear in the prepared gel as shown in Figure 6.

To overcome the contamination of the produced plasmid with chromosomal DNA, some modifications to the original protocol were done. These modifications include doubling the volume of the original culture, doubling the

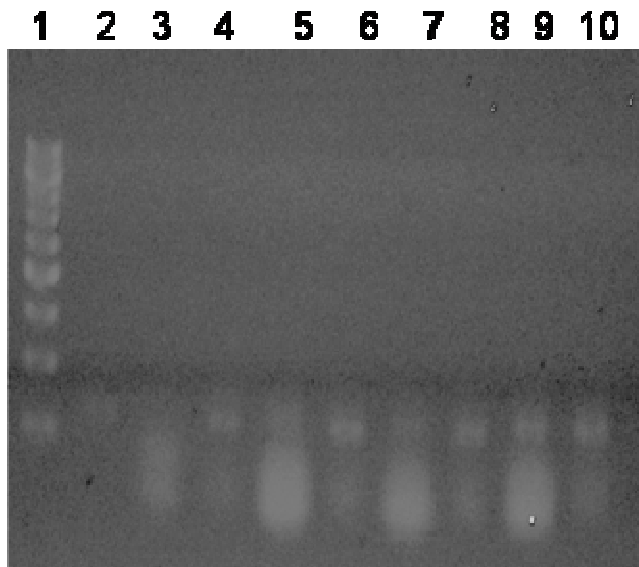


Figure 3. Agarose gel of optimized second PCR reactions using annealing temperature of 54°C. Lane 1, DNA molecular weight marker XVII (500 base pair ladder ranging from 5000 bp – 500 bp); Lane 2, First PCR product; Lane 3, Reaction A; Lane 4, Reaction I; Lane 5, Reaction C; Lane 6, Reaction; Lane 7, Reaction E; Lane 8, Reaction M; Lane 9, Reaction G; Lane 10, Reaction O.

concentration of ampicillin added to be 200 µg /ml, decreasing the incubation temperature to 30°C and the incubation time to 12 h without shaking and doubling the volumes of reconstitution, lysis and neutralization buffers. These modifications produced pure plasmid of molecular weight of 4148 bp, but with low yield. Concentration of the produced plasmid was performed by combining the yield of 2 miniprep reactions together and this was further purified and concentrated by mixing 100 µl prepared plasmid with 500 µl binding buffer and applying the produced mixture on a new column. This resulted in plasmid of high intensity and purity with molecular weight of 4148 bp. Adding chloramphenicol to a final concentration of 25 µg/ml to the starter culture together with the previously mentioned modifications resulted in plasmid with the highest intensity and purity and of molecular weight of 4148 bp as shown in Figure 7.

A cell free protein synthesis system, RTS 100 *E. coli* HY kit, was tested for 6x His tagged G-CSF protein

SDS-polyacrylamide gel electrophoresis for the RTS 100 *E. coli* HY reaction

Electrophoresis in 15% polyacrylamide gels was performed. A band corresponding to the 6xHis-tagged GCSF with a molecular weight of 21,978 kDa was produced as shown in Figure 8. The 6xHis- tagged GCSF molecular weight was determined using protein molecular

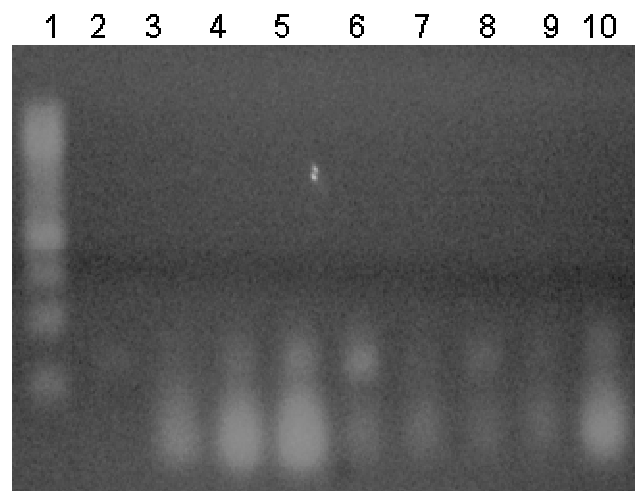


Figure 4. Agarose gel of optimized second PCR reaction using annealing temperature of 55°C. Lane 1, DNA molecular weight marker XVII (500 base pair ladder ranging from 5000 – 500 bp); Lane 2, First PCR product; Lane 3, reaction B; Lane 4, reaction C; Lane 5, reaction D; Lane 6, reaction L; Lane 7, reaction P; Lane 8, reaction N; Lane 9, reaction J; Lane 10, reaction H.

weight calculator (<http://www.sciencegateway.org/tools/proteinmw.htm>).

SDS-PAGE for the Ni-NTA purified 6xHis- tagged GCSF protein

Ni-NTA metal affinity chromatography matrices (Ni-NTA Spin columns) were used for the purification and extraction of the 6xHis- tagged GCSF from RTS 100 *E. coli* HY reaction. Electrophoresis in 15% polyacrylamide gels was performed. A band corresponding to 6xHis- tagged GCSF with a molecular weight of 21,978 kDa was observed as shown in Figure 9.

Ni-NTA spin column purified 6xHis-tagged GCSF protein content

Protein purified from a single Ni –NTA Spin column reaction, corresponding to one 50 µl RTS reaction was used for determination of protein content. The 6xHis- tagged GCSF produced from a 50 µl RTS reaction is equal to 12.7 µg, approximately 250 µg/ml RTS reaction.

Dot blotting immunodetection of 6xHis-tagged proteins with anti-His HRP conjugates (Chromogenic method)

Anti.His HRP conjugate was used for direct detection of 6xHis-tagged GCSF protein by chromogenic method. A

Table 1. The intensity of second PCR product band corresponding to each optimized second PCR reaction.

Reaction Number	Variable							Intenisty of second PCR product band
	100 pmole/ μ l Primers	10 pmole/ μ l Primers	0.5mM Mgcl2	5 % DMSO	Q factor	Ta 54 $^{\circ}$ C	Ta 55 $^{\circ}$ C	
A	•				•	•		+
B	•				•		•	+
C	•		•		•	•		++
D	•		•		•		•	+++
E	•			•		•		++
F	•			•			•	++
G	•		•	•		•		+++
H	•		•	•			•	++
I		•			•	•		+++
J		•					•	+
K		•	•			•		+++
L		•	•		•		•	++++
M		•		•		•		+++
N		•		•			•	+
O		•	•	•		•		+++
P		•	•	•			•	+

•, Presence of the variable.

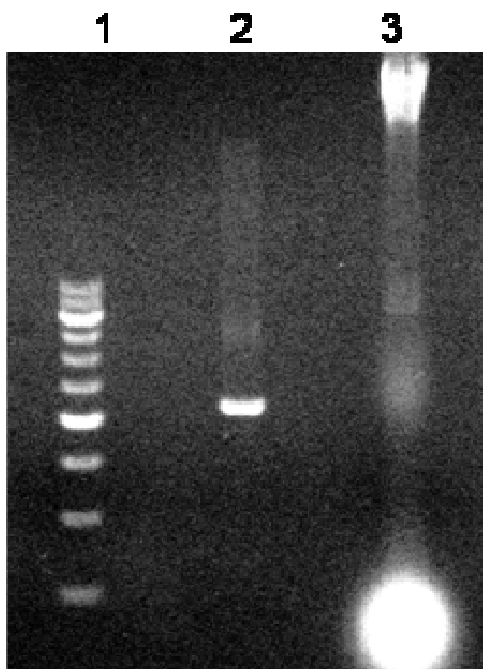


Figure 5. Comparison between pIVEX 2.4d vector and plasmid extracted from a colony of transformed cells with the vector to insert molar ratio (1:10) prepared by toothpick minipreparation method. Lane 1, DNA molecular weight marker XVII (500 base pair ladder 5000 – 500 bp); Lane 2, pIVEX 2.4d vector of molecular weight of 3583 bp; Lane 3, plasmid from the transformed colony of molecular weight of 4148 bp.

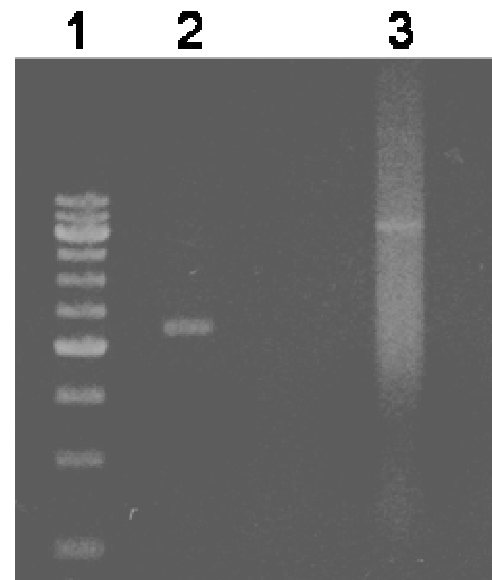


Figure 6. Agarose gel of miniprep of the transformant clone. Lane 1, DNA molecular weight marker XVII (500 base pair ladder ranging from 5000 bp – 500 bp); Lane 2, pIVEX 2.4d vector; Lane 3, Miniprep of transformed clone.

coloured precipitate was observed on the nitrocellulose strip at the site of application of the sample as shown in Figure 10. This confirmed the production of the protein of interest, 6xHis- tagged GCSF protein by RTS 100 *E. coli*

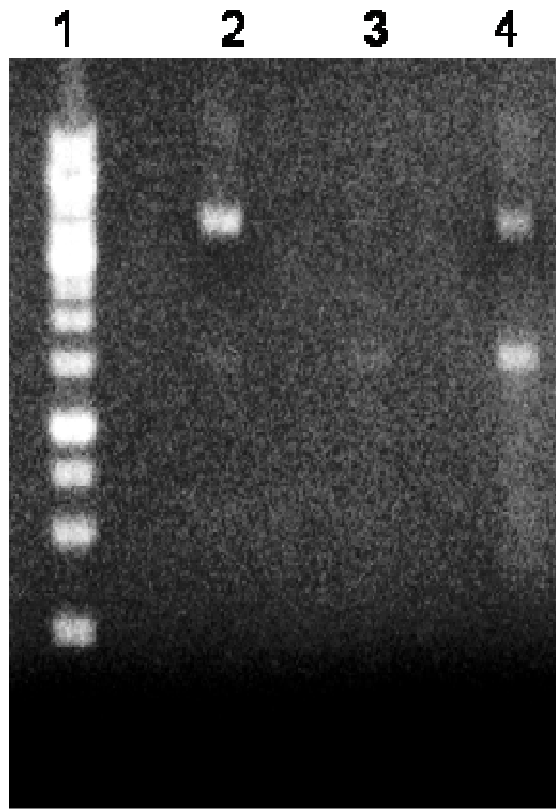


Figure 7. Comparison between diluted and concentrated extract of pIVEX 2.4d-second PCR construct miniprep with that extracted after the addition of chloramphenicol. Lane 1, Gene Ruler™ 1 Kb DNA ladder (showing 14 bands 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500 and 250 bp); Lane 2, Miniprep of transformed clone prepared by the addition of chloramphenicol to the starter culture; Lane 3, Miniprep of transformed clone prepared with the modified extraction protocol; Lane 4, concentrated plasmid of transformed clone.

HY kit.

DISCUSSION

Recombinant G-CSF has wide pharmaceutical applicability. In this study, we explored the expression of recombinant G-CSF in rapid translation system. RTS was invented and manufactured by Roche company. It is one of the simplest systems to express target proteins *in vitro* to overcome the problems associated with traditional protein production using recombinant DNA technology (Zhou et al., 2003) This study involved preparation of template DNA for cell free protein synthesis, protein expression in RTS 100 *E. coli* HY kit and protein purification. Confirmation of G-CSF production by RTS 100 *E. coli* HY kit was done by electrophoretic and immunodetection methods.

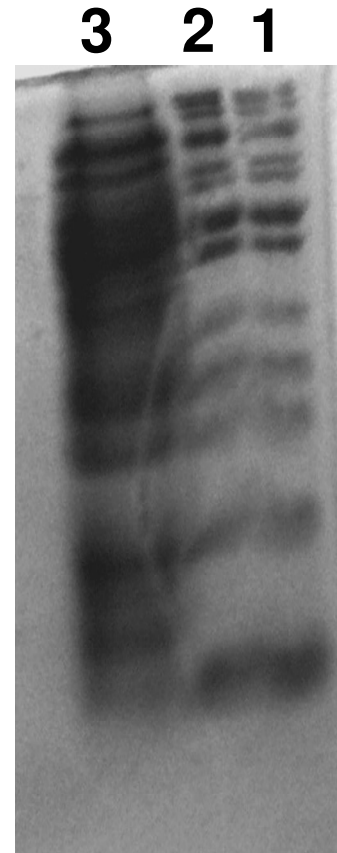


Figure 8. SDS-PAGE for the RTS 100 *E. coli* HY reaction. Lane 1-2, PAGE Ruler™ unstained protein ladder showing 14 bands 200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25, 20, 15 and 10 kDa, respectively; Lane 3, RTS reaction showing the protein of interest 6xHis-tagged GCSF.

Template DNA for cell-free protein synthesis gene amplification by PCR hGCSFb gene primers' design

Many plasmid vectors carry promoters derived from bacteriophages such as T3, T7 and SP6 adjacent to the multiple cloning site (MCS). Foreign DNA inserted at restriction sites within MCS can be transcribed *in vitro* when the recombinant plasmid is incubated with the appropriate DNA-dependent RNA polymerase (Sambrook and Russell, 2001). Some vectors carry two bacteriophage promoters in opposite orientations, located on each side of the MCS. This organization allows RNA to be synthesized *in vitro* from either end depending on the type of RNA polymerase used (Short et al., 1988). Vectors carrying the T7 promoter can be used to express cloned DNA sequences in bacteria or *in vitro* systems containing T7 RNA polymerase (Tabor and Richardson, 1985). The target gene in the vector to be used in RTS system should be under the control of a T7 promoter located downstream of a ribosomal binding site (RBS) sequence, the distance between T7 promoter and start codon (ATG)

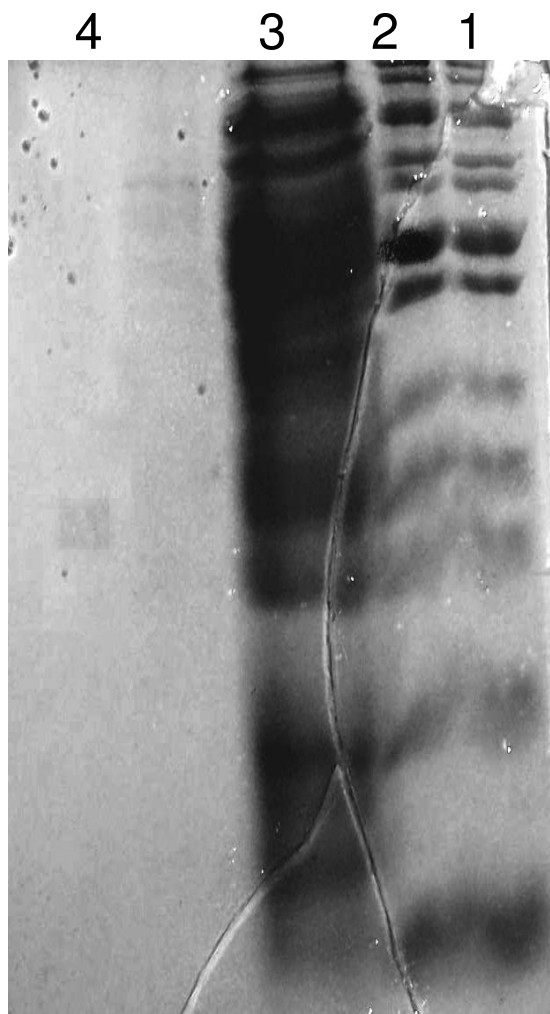


Figure 9. SDS-PAGE for Ni-NTA purified 6xHis-tagged GCSF protein. Lane 1-2, PAGE Ruler™ Unstained protein ladder showing 14 bands 200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25, 20, 15 and 10 kDa, respectively; Lane 3, RTS reaction showing the protein of interest 6x His-tagged GCSF; Lane 4, Ni-NTA purified 6xHis-tagged GCSF protein.

should not exceed 100 base pairs, the distance between the RBS sequence and start codon (ATG) should not be more than 5–8 base pairs, T7 terminator sequence should be present at the 3' end of the gene as recommended by the manufacturer of the RTS 100 *E.coli* HY kit.

In this study, pIVEX 2.4d vector contained all the regulatory elements necessary for *in vitro* expression including a T7 promoter (T7-P), a ribosomal binding site, a start codon (ATG) upstream of a multiple cloning site for the insertion of the target gene together with a stop codon and a T7 terminator (T7-T). In addition the pIVEX2.4d vector also contained a region coding for His6 tag and a factor Xa protease cleavage site sequence



Figure 10. Dot blotting immunodetection of 6x His-tagged proteins with anti-His HRP conjugates by the chromogenic method.

upstream of the multiple cloning site to allow for the purification of expressed proteins via Ni-NTA matrices (http://www. rocheappliedscience.com/sis/ protein expression/literature/vector_pivex2.4d.htm). The portion coding for the protein, GCSF, was intended to be cloned in pIVEX2.4d vector satisfying the requirements previously discussed.

Taking into consideration that the RTS system cannot introduce post-translation modification as signal sequence cleavage, in order to produce a protein with no additional bases at the N- terminus a protease cleavage site, factor Xa protease, was introduced directly in the forward primer upstream of the target gene sequence. Proteases that have been commonly employed to cleave fusion tags include tobacco etch virus (TEV) protease (Carrington et al., 1989 and Rocco et al., 2008), factor Xa (Smith and Johnson, 1988 and Jenny et al., 2003) and enterokinase (LaVallie et al., 1993; Lasnik et al., 2001). Factor Xa was chosen in this study as there was no factor Xa restriction site within the protein sequence. This promoted the production of intact pure protein. The sequence encoding the signal peptide of hGCSFb started at position 570, the sequence encoding the mature peptide started at position 661, while the sequence encoding the stop codon was at position 1184 of pORF9-hGCSFb.

According to our cloning strategy, a restriction enzyme NdeI sequence was introduced in the forward primer and a restriction enzyme SmaI sequence was introduced in the reverse primer. A NdeI restriction site sequence together with a factor Xa protease cleavage site sequence introduced in the forward primer in one step means the introduction of 12 proper base substitution mutations. This will render the primer unstable and of low quality. So in order to correct for this difficulty, we introduced these site directed mutations in two sequential PCR reaction.

PCR reactions

Choosing the optimum annealing temperature requires repeating PCR reactions with a different temperature each time which increases the cost and waste time. Thus, we performed a series of PCR reactions with a gradient of annealing temperatures in one run for both the first and second PCR reactions.

Optimization of the second PCR reaction

The primer and Mg^{2+} concentration in the PCR buffer and annealing temperature of the reaction may need to be optimized for each primer pair for efficient PCR (Ling et al., 1991 and Forbes et al., 1993). In addition, PCR efficiency can be improved by additives that promote DNA polymerase stability. Q-solution according to the manufacturer will often enable or improve suboptimal PCR systems caused by templates that have a high degree of secondary structure or that are GC-rich.

Attempts to optimize the second PCR reaction were done by testing 16 different variables combinations, including the testing of two different annealing temperatures 54 and 55°C, the use of two different primers' concentration 100 and 10 pmole/ μ l, supplementing the reaction with 25 mM $MgCl_2$ and testing 2 surfactants 5% DMSO and Q factor. It was shown that an annealing temperature of 55°C, using the primers' concentration of 10 pmole/ μ l and supplementing the reaction with 25 mM $MgCl_2$ and Q factor produced sharp intense bands of the second PCR product. Thus, using diluted primers is shown to increase the specificity of the PCR reaction and supplementing the reaction with 25 mM $MgCl_2$ greatly improved the yield (sensitivity) of the second PCR reaction. This result is consistent with that stated in Roux (1995).

Preparation of the pIVEX 2.4d vector-second PCR construct Restriction digestion of pIVEX 2.4d vector and second PCR product

Restriction digestion was performed in this study for both the second PCR reaction product and the pIVEX2.4d vector. Improvement of the digestion reaction was done by increasing the incubation periods of the digestion reactions to 2½ h instead of 1½ h. Also, the inactivation of restriction enzymes at the end of the reaction by heating at 65°C for 15 min enhanced further processing of the digested products. This result was consistent with that reported in <http://www.neb.com/nebecomm/products/faqcategory1.asp#663>. Restriction digestion with *Sma*I produced blunt ends while restriction digestion with *Nde*I produced sticky ends.

In order to prevent religation of blunt ended linearized digested plasmid DNA, the digested vector was further dephosphorylated with shrimp alkaline phosphatase (SAP). SAP has advantage over calf intestinal phosphatase in that it is completely and irreversibly inactivated by heat treatment for 15 min at 65°C. SAP is active on either 5'-protruding or blunt ends.

Preparation of the ligation reaction

To achieve optimum results in ligation, different ratios of digested second PCR and digested pIVEX 2.4d vector were checked. It is recommended in T4 DNA ligase

instruction manual to use a molar ratio vector: insert of 1:5 in case of blunt end ligation. The insert to vector molar ratio can have a significant effect on the outcome of a ligation and subsequent transformation step. Molar ratios can vary from a 1:1 insert to vector molar ratio to 10:1. It may be necessary to try several ratios in parallel for best results (DNA ligation, http://openwetware.org/wiki/DNA_ligation). In our study, it was observed that the molar ratio of vector: insert of 1:10 produced the desired ligated product.

In order to increase the chance of blunt end ligation in this study, 15% w/v PEG 8000 was added to the ligation reaction. This result was consistent with that determined by Pfeiffer and Zimmerman (1983); they found that the addition of 15% w/v PEG 8000 to ligation reactions increase the chances of blunt end ligation by more than 95%. The addition of high concentrations of polyethylene glycol 8000 to the reaction mixture causes macromolecular crowding, which strongly enhances blunt- and cohesive-end ligations and prevents intramolecular circularization (Pfeiffer and Zimmerman, 1983).

Preparation of chemically competent cells

Transformation of chemically competent *E. coli* DH5 α cells

By testing different ligation variables, it was found that ligation reaction with the highest vector: insert molar ratio (1:10) produced the largest number of colonies. Screening of all the obtained colonies, from the different tested ligation reactions, for the transformed plasmid revealed a clone containing a plasmid with the expected molecular weight of 4148 bp. In contrast to recommendations by T4 DNA ligase instruction manual ligation reaction using 1:5 molar ratio of vector: insert, revealed no plasmid while 1:10 molar ratio of vector: insert produced the desired plasmid.

Optimization of the QIAprep miniprep kit's protocol

Using the original miniprep kit's protocol for extraction of plasmid pIVEX 2.4d vector-second PCR construct from *E. coli* DH5 α cells, yielded plasmid DNA of the correct molecular weight of 4148 bp but contaminated with sheared chromosomal DNA. This represented a problem as the purity of the plasmid preparation is essential for *in vitro* protein synthesis. This problem was not simply solved by purifying DNA fragments from agarose gels, as this treatment inhibits *in vitro* protein synthesis as recommended by the manufacturer of the RTS system. The prepared lysates in this study were too viscous to promote gentle handling. The increased viscosity of lysates caused genomic DNA shearing (Sambrook and Russell, 2001).

In order to overcome this problem, modifications to the

original Qiagen protocol were tested. Culturing the single colony in double the amount of LB broth and doubling the volumes of reconstitution, lysis and neutralization buffers used were tested. These modifications reduced the viscosity of lysates and promoted gentle handling of lysates.

The pVEX 2.4d vector has an ampicillin resistant marker. Ampicillin is an unstable antibiotic and is rapidly depleted in growing cultures due in part to the β -lactamase secreted by resistant bacterial cells. Daughter cells that do not receive plasmids will replicate much faster than plasmid containing cells and can quickly take over the culture. Doubling the concentration of ampicillin sodium salt to 200 $\mu\text{g/ml}$, in this study will maintain the transformant clones always under stress and prevent them from losing the plasmid (Qiagen plasmid resource-center, <http://www1.qiagen.com/Plasmid/BacterialCultures.aspx#tab2>).

In order to prepare fresh cultures with no lysed cells or degraded DNA, we reduced the incubation temperature to 30°C and the incubation time to 12 hours without shaking as overgrown cultures contain lysed cells and degraded DNA (Qiagen plasmid resource center, <http://www1.qiagen.com/Plasmid/BacterialCultures.aspx#tab2>).

The previously mentioned modifications combined together gave rise to a plasmid with high purity and of the expected molecular weight of 4148 bp, but with low concentration. To further purify and concentrate the prepared minipreps, each 2 miniprep reactions were pooled together and were further purified and concentrated by mixing 100 μl prepared plasmid with 500 μl binding buffer and reapplying the produced mixture to a new column. This treatment yielded pure concentrated plasmid with the expected molecular weight of 4148 bp.

Although the plasmid can be purified in large yield from bacterial cultures that are allowed to grow to saturation in the absence of chloramphenicol, the treatment with sub-minimal concentration of chloramphenicol is beneficial. Chloramphenicol inhibits protein synthesis and blocks host DNA synthesis, but has no effect on the replication of plasmids. This reduced the bulk and viscosity of bacterial lysates, without affecting plasmid replication (Sambrook and Russell, 2001). Thus, in this study chloramphenicol was added to a final concentration of 25 $\mu\text{g/ml}$ in the overnight culture to reduce the viscosity of lysates. The addition of chloramphenicol yielded plasmid DNA of high purity, with the expected molecular weight of 4148 bp and of high concentration.

Protein expression in RTS 100 *E. coli* HY kit

The RTS *E. coli* HY lysate used in this study contains all the machinery needed to drive coupled transcription and translation of a DNA template in a single reaction vessel. The RTS 100 *E. coli* HY kit was tested for protein synthesis using the purified pVEX 2.4d-second PCR construct as template DNA. A band corresponding to the

protein of interest His6 tag-GCSF with a molecular weight of 21,978 kDa was visualized after coomassie blue staining of the polyacrylamide gel. Similar studies using RTS system for cloning and expression of the HIV protein in *Escherichia coli* cell-free system was reported by Chen et al. (2007).

Protein purification using Ni-NTA spin column

The vector pVEX 2.4d contains a region coding for His6 tag upstream of the multiple cloning site. A hexa-histidine tag was added to the N-terminus of the protein by cloning the gene coding GCSF in pVEX2.4d vector. In order to achieve one step separation and exact processing, His6 tag-GCSF purification from the RTS reaction was performed in this study by using Ni-NTA spin columns. Similar studies involving purification of His6 tagged proteins by Ni-NTA are discussed in Lasnik et al. (2001), Beeler et al. (2004), Jianbo et al. (2007) and Bogomolovas et al. (2009).

A band corresponding to the purified protein His6 tag-GCSF with a molecular weight of 21,978 kDa was detected on the polyacrylamide gel after coomassie blue staining. This confirmed the production of His6 tag-GCSF by RTS 100 *E. coli* HY reaction.

Ni-NTA spin column purified 6xHis- tagged GCSF protein content

Determination of the His6 tag-GCSF protein content from a single Ni-NTA spin column purification reaction using coomassie blue dye assay according to the method of Bradford (1976) was done. The concentration of His6 tag-GCSF produced from a 50 μl RTS reaction is equal to 12.7 μg approximately 250 $\mu\text{g/ml}$ RTS reaction. The protein yield from the traditional batch mode expression RTS 100 *E. coli* HY Kit approaches 400 $\mu\text{g/ml}$ RTS reaction for the highly soluble and efficiently expressed proteins. The protein yield from a continuous exchange cell-free (CECF) reaction is up to 5 mg per ml using the RTS 9000 *E. coli* HY Kit. This is consistent with the fact that in the traditional batch mode expression, cell-free protein synthesis plateaus after approximately 2 to 4 h as substrates become exhausted and inhibitory reaction by-products accumulate which accounts for the lower expression levels. In contrast, the RTS CECF format enables protein expression to continue for up to 24 h, maximizing protein yield. This is possible because the CECF system does not allow the build up of inhibitory products or the exhaustion of substrates (Spirin, 2003).

Dot blotting immunodetection of His6 tagged proteins with anti-His HRP conjugate (chromogenic method)

Protein detection using the dot blot protocol is similar to

western blotting in that both methods allow for the identification and analysis of proteins of interest. Dot blot methodology differs from traditional western blot techniques by not separating protein samples using electrophoresis. Sample proteins are instead spotted onto membranes and hybridized with an antibody probe. Semi-quantitative measurements can be made for the spots. This is the fastest and simplest immobilization method where dilutions of the RTS reaction containing the His6 tag-GCSF in denaturing buffer were pipetted directly on a nitrocellulose membrane. Anti-His HRP conjugate was used for simple, direct detection of His6 tag-GCSF. They comprise the tetra His antibody that recognizes the (His) 4 epitope. Tetra His antibody binds to proteins that have His6 tag regardless of the surrounding amino-acid context. The anti-His HRP conjugate also contain a horseradish peroxidase (HRP) moiety that allows direct detection by chromogenic methods without the requirement of a secondary antibody (QIAexpress detection and assay handbook, 2002, third edition, Qiagen). Chromogenic substrates give rise to insoluble, colored products that form a precipitate on the nitrocellulose membrane consistent with the expected results in literature (QIAexpress detection and assay handbook, 2002, third edition, Qiagen). Similarly, dot blotting technique was used by Chapple et al. (2006) for screening of protein production in mammalian cells. This provided a further confirmation of the production of the protein of interest, His6 tag-GCSF protein, by RTS 100 *E.coli* HY kit.

Future investigation suggestions

- (1) Further process optimization by protein expression in RTS 9000 *E. coli* HY Kit would most certainly further increase the yield to mg/ml range.
- (2) The optimization of the protein yield, the removal of the His6 tag and further characterization of the produced protein will quantitatively define the process to a level which could be tested on a pilot scale and hence suites the design of an industrial process.

ACKNOWLEDGMENT

This work was funded by the National Academy of Science Egypt.

REFERENCES

- Beeler JA, Tang WJ (2004). Expression and purification of soluble adenylyl cyclase from *Escherichia coli*. *Methods Mol. Biol.* 237: 39-53.
- Bishop B, Koay DC, Sartorelli AC, Regan L (2001). Reengineering granulocyte colony-stimulating factor for enhanced stability. *Sep. 7; J. Biol. Chem.* 276(36): 33465-33470.
- Bradford MM (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
- Bogomolovas J, Simon B, Sattler M, Stier G (2009). Screening of fusion partners for high yield expression and purification of bioactive viscotoxins. *Protein Exp. Purif.* Mar. 64(1): 16-23.
- Carrington JC, Cary SM, Parks TD, Dougherty WG (1989). A second proteinase encoded by a plant polyvirus genome, *EMBO J.* 8:365-8370.
- Chapple SDJ, Crofts AM, Shadbolt SP, McCafferty J, Dyson MR (2006). Multiplexed expression and screening for recombinant protein production in mammalian cells *BMC Biotechnology* 6:49, complete electronic version at <http://www.biomedcentral.com/1472-6750/6/49>.
- Chen H, Xu Z, Yin X, Cen P (2007). Cloning and expression of the HIV protein in *Escherichia coli* cell-free system. *Appl. Microbiol. Biotechnol.* 77: 347-354.
- Dale DC (2002). Colony-stimulating factors for the management of neutropenia in cancer patients. *Drugs*, 62 Suppl 1: 1-15.
- DiPersio JF, Pusic I (2008). The use of growth factors in hematopoietic stem cell transplantation. *Curr. Pharm. Des.* 14(20): 1950-1961.
- Forbes BA, Hicks KE (1993). Direct detection of mycobacterium tuberculosis in respiratory specimens in a clinical laboratory by polymerase chain reaction. *J. Clin. Microbiol.* 31(7): 1688-1694.
- Harada M, Qin Y, Takano H, Minamino T, Zou Y, Toko H, Ohtsuka M, Matsuura K, Sano M, Nishi J, Iwanaga K, Akazawa H, Kunieda T, Zhu W, Hasegawa H, Kunisada K, Nagai T, Nakaya H, Yamauchi-Takahara K, Komuro I (2005). G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. *Nat. Med.* Mar.11(3): 305-311.
<http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm>
http://www.roche-applied-science.com/sis/proteinexpression/literature/vector_pivex24d.htm.
- QIAexpress detection and assay handbook, 2002, third edition, Qiagen
Qiagen plasmid resource center, http://www1.qiagen.com/Plasmid/Bacterial_Cultures.aspx#tab2
- Jenny RJ, Mann KG, Lundblad RL (2003). A critical review of the methods for cleavage of fusion proteins with thrombin and factor Xa. *Protein Express. Purif.* 31: 1-11.
- Jianbo L, Limei X, Feng Y (2007). Expression and characterization of recombinant thermostable alkaline phosphatase from a novel thermophilic bacterium *Thermus thermophilus* XM, *Acta Biochim. Biophys. Sin.* 39: 844-850.
- Kim DM, Choi CY (1996). A semicontinuous prokaryotic coupled transcription/ translation system using a dialysis membrane. *Biotechnol Prog.* Sep-Oct; 12(5): 645-649.
- Laemmli U (1970). Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.
- Lasnik MA, Porekar VG, Stalc A (2001). Human granulocyte-colony stimulating factor (hG-CSF) expressed by methylotrophic yeast *Pichia pastoris*. *Pflugers Arch. Eur. J. Physiol.* 442(suppl 1): R184-R186.
- LaVallie ER, Rehemtulla A, Racie LA, DiBlasio EA, Ferenz C, Grant KL, Light A, McCoy JM (1993). Cloning and functional expression of a cDNA encoding the catalytic subunit of bovine enterokinase. *J. Biol. Chem.* 268: 23311-23317.
- Ling LL, Keohavong P, Dias C, Thilly WG (1991). Optimization of the polymerase chain reaction with regard to fidelity: modified T7 Taq and vent DNA polymerases. *Genome Res.* 1: 63-69.
- Metcalf D (1985). The granulocyte-macrophage colony-stimulating factors. *Science*, 229: 16-22.
- Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, Hirata Y, Kubota N, Oheda M, Namoura H, Ono M (1986). Molecular cloning and expression of cDNA for the human granulocyte-colony stimulating factor. *Nature*, 319(6052): 415-418.
- Nomura H, Imazeki I, Oheda M, Kubota N, Tamura M, Ono M, Ueyama Y, Asano S (1986). Purification and characterization of human granulocyte colony-stimulating factor (G-CSF). *EMBO J.* 5: 871-876.
- Pheiffer BH, Zimmerman SB (1983). Polymer-stimulated ligation: enhanced blunt- or cohesive-end ligation of DNA or deoxyribonucleotides by T4 DNA ligase in polymer solutions, *Nucleic Acids Res.* pp. 17853-7871.
- Rocco CJ, Dennison KL, Klenchin VA, Rayment I, Escalante-Semerena JC (2008). Construction and use of new cloning vectors for the rapid isolation of recombinant proteins from *Escherichia coli* *Plasmid*, 59: 231-237.

- Roux KH (1995). Optimization and troubleshooting in PCR. *Genome Res.* April 4:S185-S194 (<http://genome.cshlp.org/content/4/5/S185.full.pdf>)
- Sambrook J, Russell DW (2001). *Molecular cloning: a laboratory manual*. 3rd ed., New York: Cold Spring Harbor Laboratory Press.
- Shimizu Y, Inoue A, Tomari Y, Suzuki T, Yokogawa T, Nishikawa K, Ueda T (2001). Cell-free translation reconstituted with purified components. *Nat. Biotechnol.* Aug. 19(8): 751-755.
- Short JM, Fernández JM, Sorge JA, Hose WD (1988). I-ZAP: A bacteriophage I expression vector with in vivo excision properties. *Nucleic Acids Res.* 16: 7583-7600.
- Smith DB, Johnson KS (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase *Gene* Vol. 67, Issue 1, 15 July, pp. 31-40.
- Souza LM, Boone TC, Gábrilove J, Lai PH, Zsebo KM, Murcock DC, Chazin VR, Bruszewski J, Lee H, Chen KK, Barendt J, Platzer E, Moore MAS, Mertelsmann R, Welte K (1986). Recombinant human granulocyte-colony stimulating factor: effects on normal and leukemic myeloid cells. *Science*, 232: 61-65.
- Spirin AS, Baranov VI, Ryabova LA, Ovodov SY, Alakhov YB (1988). A continuous cell-free translation system capable of producing polypeptides in high yield. *Science*, Nov 25; 242(4882): 1162-1164.
- Spirin AS (2003). Cell free protein synthesis, Early history of cell free translation systems. Introduction, pp. 7-12, RTS application manual for cell-free protein expression (http://www.roche-applied-science.com/sis/proteinexpression/literature/manual/cell_free.htm).
- Tabor S, Richardson CC (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes *PNAS* February 1, 82(4): 1074-1078
- Zhou J, Li X, Zhang H, Tang Y, Wang X (2003). Cloning and gene expression of G protein competitive inhibitory polypeptide and its prophylactic effects on myocardial hypertrophy *in vitro*. *Acta Pharmacol. Sin.* 24(11): 1108-1112.