Synthesis of human insulin gene i*n vitro* through computational methodology

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Abstract: Objective, still production of human recombinant insulin is big target for developing countries although it produced recombinant since 1982. **Material and Methods**: Human pro-insulin gene codons optimization with aid of a web based software called OPTIMIZER, where codons that rarely used by *E.coli* were replaced by abundant codons, without any changing in the amino acid composition. Pro-insulin long overlap oligos were assembly and amplified through two successive PCR reactions using different program and polymerase enzyme. The assembly step called Polymerase Cycling Assembly (PCA), where the second step was ordinary PCR reaction for the gene amplification. **Results**: The yielded synthetic gene was cloned into pCR2.1-TOPO cloning vector and transformed into TOP10F` competent *E.coli*. The correct sequenced cloned gene was subsequently digested and ligated into pET101/D/TOPO expression vector followed by transformation into BL21star (DE3) cells. The optimized strain has been cultivated in shack flask up to 0.6 OD before induction with IPTG. The pro-insulin expression yield and its characterization will be presented. **Conclusion**: to built target gene from synthetic overlap oligos, the optimized primers and cloning, expression vectors were harnessed and presented.

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Running title: synthetic human insulin

Introduction

Human Insulin is a 51-polypeptide hormone consisting of the A chain (21 Amino Acids) and the B chain (30 Amino Acids) of molecular weight of 5.8 kDa. Both chains are linked by two disulfide bridges (Cys7A–Cys7B/Cys20A–Cys19B) (**Jakubke et al, 2008**). Insulin is biosynthetically derived from the single-chain of 86-residue precursor, named p*ro-insulin* **(Fig 1)** of molecular weight about 9.5 kDa. After three disulfide bridges have been formed within *pro-insulin*, it is converted by proteolytic excision of the internal 31-residue C-chain (*C-peptide*) to form the two-chained (the B chain and A chain) active hormone that remain connected by disulfide bonds (**Jakubke et al, 2008**).

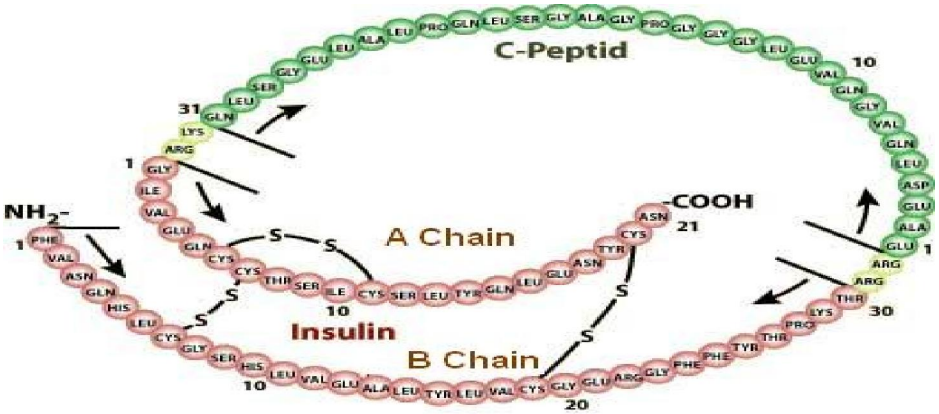

Fig 1: Structure of human pro-insulin. 82-polypeptide hormone consisting of the A chain (21 a.a.) and the B chain (30 a.a.) linked by two disulfide bridges (Cys7A–Cys7B / Cys20A–Cys19B). One more intrachain disulfide bond is located between Cys6–Cys11 within the A chain (**Murray et al, 2003**).

Human insulin hormone secreted from beta cells in the islets of Langerhans in the pancreas. It is passes directly into the bloodstream, where it play a crucial role in regulating the process of carbohydrate metabolism, so it consider the first responsible for the process of adjusting the level of blood glucose (Sanger *et al.,* 1955).

Diabetes mellitus describes a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both pregnancies (**Rother, 2007**). Most cases of diabetes mellitus fall into three broad categories: a) Insulin-dependent diabetes mellitus (IDDM) also known as type I diabetes which characterized by loss of the insulin-producing beta cells of the islets of Langerhans of the pancreas by a T-Cell mediated autoimmune attack, leading to insulin deficiency, causes approximately 5 % of diabetes mellitus cases, b) Non–insulin dependent diabetes mellitus (NIDDM) also known as type II diabetes is the most common form of diabetes accounts for about 90% to 95% of all diagnosed cases of diabetes. It is characterized by disorders of insulin action and secretion, c) Gestational diabetes mellitus (GDM) is a form of glucose intolerance diagnosed during pregnancy pregnancy (**CDC report, 2008, Rother, 2007**).

Diabetic complications can be classified broadly as microvascular or macrovascular disease. Microvascular complications include neuropathy (nerve damage), nephropathy (kidney disease) and vision disorders (e.g. retinopathy, glaucoma, cataract and corneal disease). Macrovascular complications include heart disease, stroke and peripheral vascular disease (which can lead to ulcers, gangrene and amputation). Other complications of diabetes include infections, metabolic difficulties, impotence, autonomic neuropathy and pregnancy problems. But people with diabetes can lower the occurrence of these and other diabetes complications by controlling blood glucose, blood pressure, and blood lipids (**www.healthinsite.gov.au/topics/Complications_of_Diabetes) (2011).**

Recently, in the international diabetes federation survey for ten countries which have diabetes patients worldwide, Egypt occupied the ninth position of 10 with 8 million patients; this will grow to be 10 million by 2025. The Arab Emirates Union, Saudi Arabia, Bahrain, Kuwait, Oman states occupied the second, third, fourth, fifth, and sixth positions worldwide, respectively**.** The world population is growing at a rate of 1–2%, the annual reported increase the incidence of insulin-dependent diabetes can be as high as 5–6%.The increasing demand for insulin per patient can be 0.5–1 gram per year **(IDF, 2010)**.

Insulin was discovered in 1921 by Fredrick Banting and Best **(Banting et al, 1922)**, and was purified and crystallized by (**Abel, 1926)** five years later. The elucidation of the primary structure was described by (**Sanger et al, 1955)**, Dorothy Crowfoot-Hodgkin determined the spatial conformation of insulin in 1969 (**Jakubke et al, 2008)**.

The initial approach taken by the scientists at Genentech entailed inserting the nucleotide sequence coding for the human insulin A and B chains into two different *E. coli* cells (K12). It was followed by several large companies in the production recombinant version of this hormone at industrial level, such as Novo Nordisk and Eli Lilly **(Chance et al, 1999)**. In spite of this, the number of published research in this area does not explain the construction methods or its industry. Since this product is economically importance, which made us think about starting to implement of recombinant insulin production technology in Egypt first at the laboratory scale. Egypt, Arab and African countries considers between the World's largest importers of this strategic product in the world.

Protein engineering applications involving mutagenesis and expression of proteins from recombinant DNA. Synthetic genes offer many advantages over the naturally occurring genes. The potential problems include high G/C or A/T content, codon bias, and complex intron/exon structures (**Redwan, 2006**). Briefly, this study depends upon designing of the suitable gene sequence for the human pro-insulin, genetic codons optimized using computer program to be compatible with abundant codons used by *E.coli* in which the protein produced (**Redwan, 2006**). Human pro-Insulin gene were assembled and amplified using PCR based techniques, then synthesized gene were inserted into cloning vector and transformed into *E.coli,* which were chosen carefully for the cloning step. The cloned gene was isolated and purified then ligated into the expression vector to express the pro-insulin protein.

Material and Methods

Codon Optimization

Gene **o**ptimization of human pro-Insulin gene codons to be easly used by *E.coli* was performed using OPTIMIZER; a web server for online optimizing the codon usage of DNA sequences (**Garcia, 2007) (**http://genomes.urv.es/optimizer/), through replacing the rare codons with the most abundant codon used by *E.coli* to obtain the most suitable human Pro-Insulin gene sequence of 261 nucleotide for 87 amino acid residues.

Primer design

Long primers were designed using DNAWorks software; a computer program that automates the design of oligonucleotides for gene synthesis. The software was used for designing oligonucleotide sets (primers) for gene assembly by both Polymerase Cycling assembly (PCA) and polymerase chain reaction (PCR) (http\helixweb.nih.gov/dnaworks/), **(Hoover et al, 2009).**

Human pro-insulin gene assembly and amplification

The assembly and amplification of double stranded DNA fragments using the Polymerase Chain Reaction (PCR) Technique was performed in separate PCR (GeneAmp 9700 Thermal Cycler, Perkin- Elmer, USA) reactions were performed using the ten long overlap primers, and four Taq polymerases; a (Finzeme (non-proof reading, Finzyme, Finland), b) *pfu* (proof-reading and high fidelity, Stratagen, USA), c) Red Hot Star (non-proof reading, ABgen, USA), and d) GC-RICH PCR system (it contains mixtures of polymerases enzymes; Taq DNA polymerase in combination with *Tgo* DNA polymerase a thermostable enzyme with proof- reading activity. to assemble and amplify the human pro-insulin gene.

The primers were added to a final concentration of 20 pM/each primer in assembling step, while the amplification step was run with 2-3 µl of the assembled products with 20 pM of the outermost primers of each strand. The PCR was carried out in a 50 µl reaction, in Finzeme, *pfu,* Red Hot Star buffer and 200 mM dNTPs with 5U of each polymerase. The PCR profile of 35 cycles at 94 °C for 30s, 56 °C for 2 min, 72 °C for 2 min and final extension 72 °C for 10 min for all enzymes except the GC- RICH PCR system, the company recommended profile (1X of 95 °C for 3 min, 35X of 95 °C for 30s - 65 °C for 30s - 68 °C for 45s, 1X of 72 °C for 7 min) was used. The PCR products were run on 2% agarose gel electrophoresis to visualize the PCR products (**Mohammed et al., 2012**).

Human pro-insulin gene cloning

The PCR product was cloned in 5 minutes pCR2.1 TOPO TA cloning vector (Invitrogen, USA) according to the company instruction manual. Briefly, 2 µl of cloning reaction was transformed into TOP10F` ultracompetent *E.coli*. Cells were plated onto LB plates (2% Tryptone, 1% NaCl, 2% yeast extract, 2% Agar) containing X-gal (20 µg/ml), IPTG (32 µg/ml) and ampicillin (100 mg/ml). Miniprep was performed using Qiagen miniprep kit (Qiagen, USA) from overnight culture of white colonies, and then panned by PCR and *Eco RI* digestion.

Cloned human pro-insulin gene in pCR2.1 TOPO cloning vector was isolated and amplified via ordinary PCR reaction using two short outermost primers include the 4 base sequences (CACC) at the forward primer as recommended by the company. The PCR product was cloned into 5 minutes pET101/D-TOPO expression vector (Invitrogen, USA). About 2 µl of cloning reaction was transformed into BL21 Star (DE3) competent *E.coli*. The cells were plated onto LB plates containing ampicillin (100 mg/ml). on the next day, 9 colonies out of about 150 colonies grown on the plate were picked up into separate tube and cultured overnight in LB medium. Miniprep was performed using Qiagen miniprep kit (Qiagen, USA) for the nine samples and then panned by X*baI* and S*acI* Restriction digestion to evaluate the insert before sending to the sequencer.

Human pro-insulin protein expression

Five of the liquid cultures of BL21 *E.coli* containing pET101/D/TOPO-proINS were divided into two volumes, one of them left and the other undergoes induction using 1 µl/ml culture medium of (1M IPTG), for 5 hours with vigorous shaking (250 rpm) at room temperature. SDS-PAGE was performed for both "induced" and "non-induced" bacterial cultures using minivertical protein electrophoresis (BioRad), Coommassie blue stained gels were destained thoroughly before imaged using gel documentation system (Alpha Innotech, USA) (**Redwan et al., 2006, 2012**).

Results

Optimization results

Computational codon optimization using OPTIMIZER: a web server for online optimizing the codon usage of DNA sequences **(Garcia, 2007)** (http://genomes.urv.es/OPTIMIZER/), that converts the amino acid sequence of the human pro-insulin polypeptide into nucleotides sequence that are abundant use by *E.coli* to obtain the most suitable human pro-insulin gene sequence of 261 Nucleotide for 86 amino acid residues, with addition of start codon ATG of the methionine amino acid, resulting in the following sequence with as minimal as 55.8% GC content :

ATG TTT GTG AAC CAA CAC CTG TGC GGC AGC CAC CTG GTG GAA GCT CTC TAT CTG GTG TGC GGC GAA CGT GGC TTC TTC TAC ACC CCG AAG ACC CGC CGT GAG GCA GAG GAC CTG CAG GTG GGC CAG GTG GAG CTG GGC GGC GGC CCG GGT GCA GGC AGC CTG CAG CCG TTG GCC CTG GAA GGC TCC CTG CAG AAG CGT **GGC ATT GTG GAA CAA TGC TGT ACC AGC ATC TGC TCC CTG TAC CAG CTG GAA AAC TAC TGC AAC TAG**

Human pro-insulin gene assembly and amplification results

261 nucleotides were undergoes processing using DNAWorks software for designing ten long overlapping primers of 42 base length for each primers (Five primers for forward direction and another backward five primers) for human pro-Insulin gene assembly using Polymerase cycling Assembly (PCA) Technique.

Assembly and amplification of depending upon special designed PCR program using four different DNA polymerases (Red hot star, Finzyme, *pfu* and GC-Rich system) as demonstrated in the **(Fig 2)**, all of the three enzymes of (Red hot star, Finzyme, *pfu*) were failed to amplify the correct full length of the human pro-insulin gene, they were assembled but several bands were amplified. However, the GC –Rich polymerase system was successfully assembled and amplified the correct size and sequence of human pro-insulin gene.

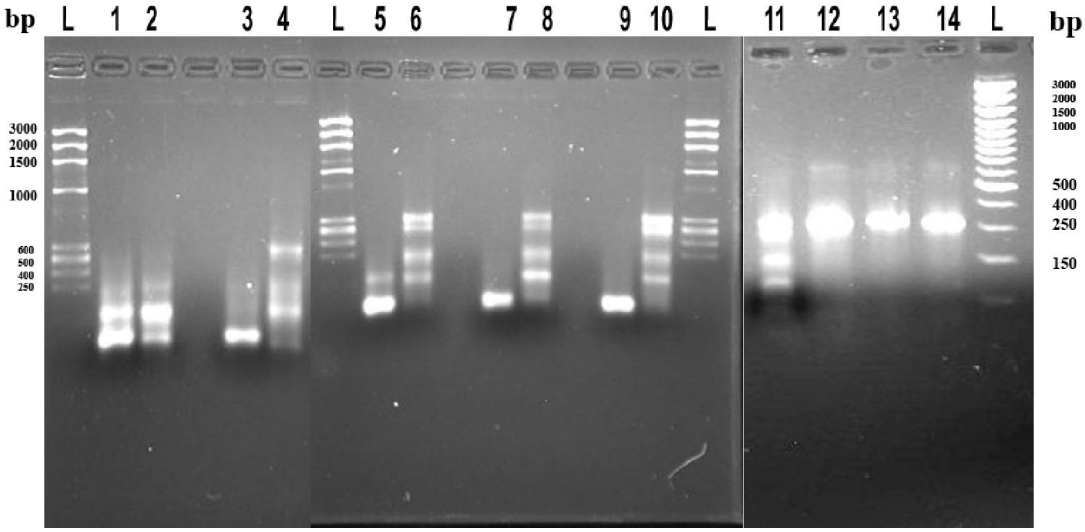

Figure 2: Assembling and amplification of the human pro-insulin synthetic gene products were run on 2% Agarose gel. Lanes 1, 3, 5 and 7 are primers assembling reactions using Red-hot star, Finzyme, *pfu* and GC-Rich enzymes, while lanes 9 and 11 are same reaction of lane 7 but with different denaturation enzyme buffer or different PCR program, respectively. Lanes 2,4,6,8,10,12,14 represent the full length gene amplified with the short primers using the same enzymes respectively. Lanes 8 and 9 gene amplified with GC-Rich enzymes but with different molar of denaturation buffer. Lastly, lanes 12–14 were the amplification with same enzyme but with different amplification program. L pointed the DNA ladder100 bp range.

Gene cloning

White and Blue colonies appear on LB plates that used during the plating of the transformation reaction products of *E.coli* transfected with cloning vector pCR2.1-TOPO (3931 bp) **(Fig 3)** that carry pro-Insulin gene, i.e. pCR2.1-TOPO–pINS **(Fig 4).** Purified plasmid from the overnight cultured were enzymatic digested with E*coR1* restriction enzyme to gives two distinct separated bands one for the cutted plasmid and the other for pro-Insulin gene fragment at about 278 bp shown in **(Fig 5)**.

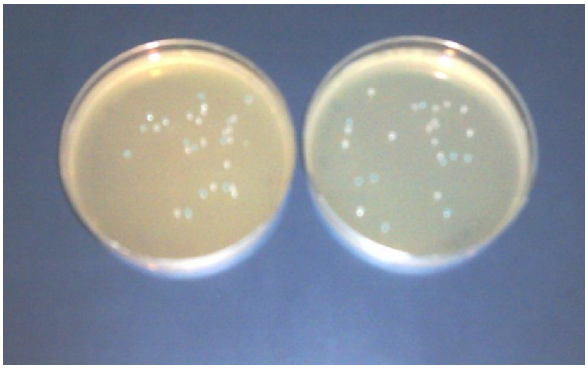

Figure 3: White / blue colonies appear in the LB plates, white colonies reflect the positive results, where the blue ones describe the negative results.

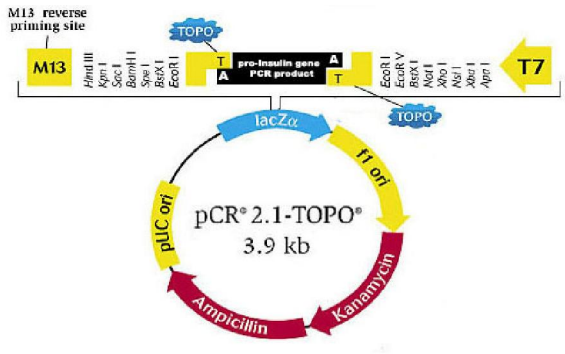

Fig 4: Diagram depicted the location of synthesized pro-insulin gene insert at the cloning site in the pCR2.1-TOPO, using Topoisomerase I enzyme.

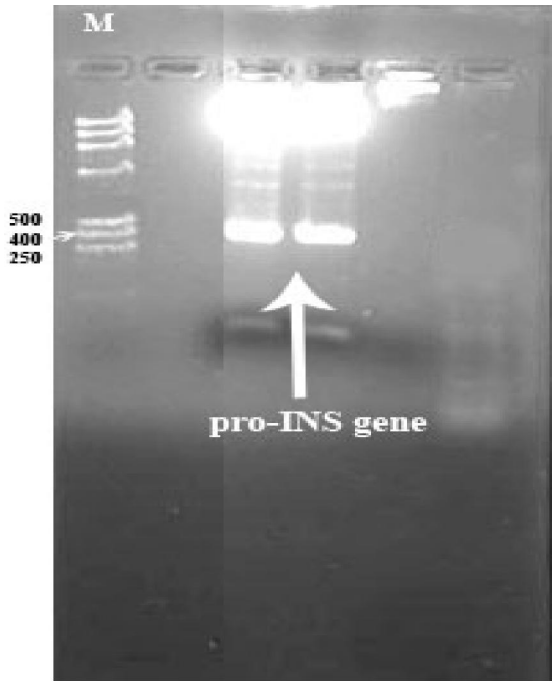

Fig 5: DNA gel electrophoresis showing the digested plasmid pCR2.1 TOPO vector from two different clones and the pro-Insulin gene fragments after digestion by *Eco R1*.

White colonies appear on LB agar plate that used in the plating of the transformation reaction of TOP10 transformed with ligated reaction of correct sequenced of the pINS-pET101/D expression vector. The purified miniprep of the pINS-pET101/D was subsequently transformed into for BL21 Star (DE3) **(Fig 6)** and **(Fig 7).** On the next day, nine clones were picked up and cultured overnight. Nine out of nine clones has been demonstrated a pINS-pET101/D insert **(Fig 8)** after digested with enzymes *SacI* and *XbaI* restriction Endonucleases.

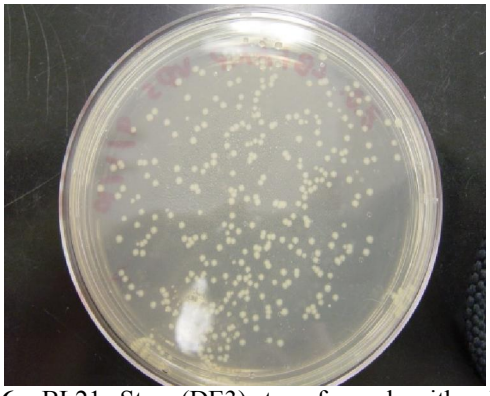

Fig 6: BL21 Star (DE3) transformed with pINS-pET101/d vector appears on LB amp resistance agar plate.

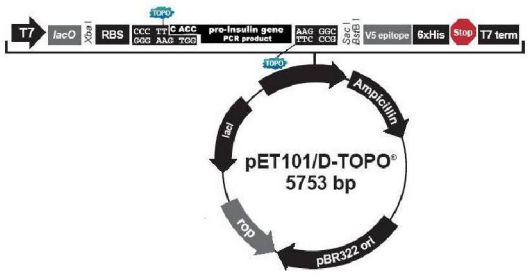

Fig 7: synthesized pro-Insulin gene insert location in the cloning site of pET101/D-TOPO.

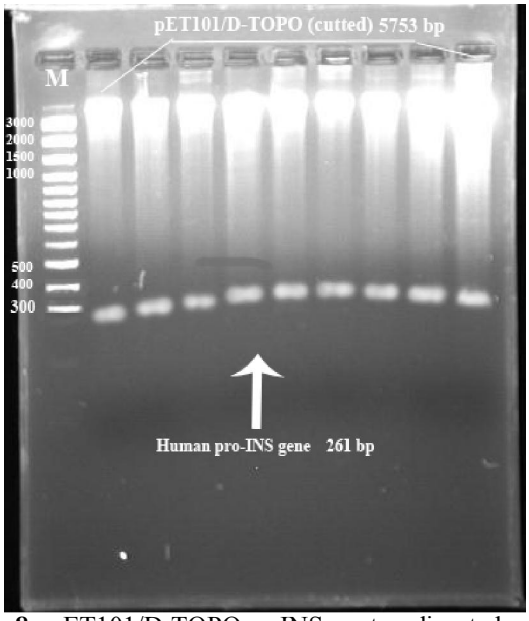

Fig 8: pET101/D-TOPO– pINS vector digested with SacI/XbaI run on 2 % agarose gel. All 9 lanes of the digested vector contains two bands one for empty vector (~ 5700 bp) and the other for the insert (261 bp), the first lane in the left refers to 100 bp ranged DNA ladder.

SDS-PAGE for expressed human pro-Insulin protein in bacterial cultures

After confirming that all nine colonies contain the expression vector, five out of nine has been cultured in LB contain amp. On the next day, 0.6 OD cultures has been with 1 µl/ml (1 M IPTG). Protein gel electrophoresis (SDS-PAGE) has been run to check the protein expression . Figure 9 shown a pro-insulin band with correct calculated size (9.5kDa) in comparison with non-induced culture protein profile.

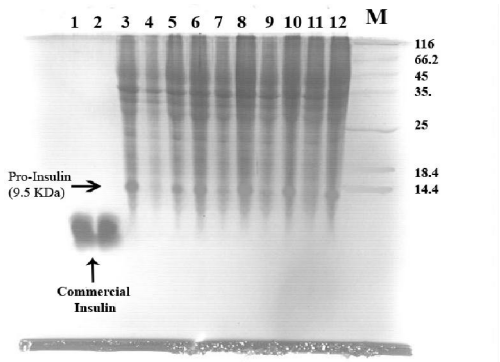

Fig 9: 15 % SDS-PAGE showing the commercial insulin at lane 1&2. Lane 3,6,8,10,12 induced and non induced lanes 4,5,7,9,11. Protein marker (M) at the right side.

Discussion

There is an increased world demand for the human insulin Protein, requiring highly productive efficient productive systems; *E.coli* based production of the human Insulin offering several advantages such as relatively high correct cloned vector number per bacterium and high yield of the secreted recombinant proteins in addition to low cost production process. Although the use of naturally occurring genes might appear to be the quickest approach, many such genes will prove to be suboptimal for cloning and overexpression in heterologous systems like *E.coli* or yeast. The potential problems include high G/C content, codon bias and complex intron/exon structures. An approach to overcoming the complications in cloning is gene synthesis. In this approach, the protein coding sequence can be directly optimized for the expression system of choice. Variants of this strategy include oligonucleotide ligation **(Heyneker et al, 1976)**, the *FokI* method **(Mandecki et al, 1998)** and self-priming PCR **(Dillon et al, 1990).** A particularly appealing method, due to its inherent simplicity, is assembly PCR which used in this research **(Stemmer et al, 1995).** This involves generating overlapping oligonucleotides which, when assembled, form the template for the gene of interest. The oligonucleotides are then repetitively extended by PCR, to assemble the full-length gene in a single step.

Our main point was directed to using bioinformatics in codon optimization of human pro-insulin gene sequence to suit the *E.coli* translation machinery and polymerase cycling assembly (PCA). Computational Optimization using OPTIMIZER: a web server for online optimizing the codon usage of DNA sequences using pre-computed codon usage tables from a predicted group of highly expressed genes from more than 150 prokaryotic species under strong translational selection, was resulted in alteration of 43 codon to suit *E.coli* translation machinery where the GC content after optimization were 55.81 % **(Garcia et al, 2007).**

The primer sequence determines several things such as the length of the product, its melting temperature and ultimately the yield. Un optimized primer design will results in little or no product due to non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation **(Abd-Elsalam, 2003)**.

Many Programs have been developed for gene synthesis to automatically design oligonucleotides based on user specific hybridization temperature and oligonucleotide length such as DNA Works (**Hoover et al,2002)**, Gene2Oligo **(Rouillard et al, 2004)**, Assembly PCR Oligo Maker **(Rydzanicz et al, 2005)**, GeMS (**Jayaraj et al, 2005)**, GeneDesign (**Richardson et al, 2006)** and TmPrime **(Bode et al, 2009)**.

Only DNAWorks program which able to design gapped PCR-based gene assembly, DNAWorks a computer program that automates the design of oligonucleotides for gene synthesis (Hoover et al, 2002). DNAWorks program requires simple input information, i.e. amino acid sequence of the target protein and melting temperature (needed for the gene assembly) of synthetic oligonucleotides. The program outputs a series of oligonucleotide sequences. Those oligonucleotides are characterized by highly homogeneous melting temperatures and a minimized tendency for hairpin formation. Using this program we have successfully designed and constructed our gene of interest human pro-insulin gene. The approach presented here may simplify the production of proteins from a wide variety of organisms for genomics-based studies.

The quality and quantity of PCR-based gene synthesis are influenced by several factors, such as annealing temperature, concentration of oligonucleotides, DNA polymerase, concentration of monomers and number of PCR cycles, Using DNAWorks and adjusting reaction parameters and gives ten long primers about 42 nucleotides, used in the first step of gene synthesis (PCA), the long primers were very Convergent in Tm (melting temperature) where between 57 and 58 ^{o}C, and the overlapping lengths were not spaced they were ranging between 14

and 19 nucleotides, Also two short (24 and 26 nucleotides) outermost primers for the ordinary Human pro-Insulin gene amplification**.**

We tried to minimize oligonucleotide cost, it is possible to leave gaps between oligonucleotides, so that the number of nucleotides used is less than the length of the DNA. Moderate gaps and overlaps between the oligonucleotides result in suitable lengths of the synthesized oligs and therefore cheaper cost. (OE-PCR) of these fragments lead to the assembly of the full-length DNA. Unfortunately, although the major bands after the assembly step appeared to be the correct size for all genes synthesized with all four polymerases enzymes used (Red hot star, Finzyme, *pfu* and GC-Rich system), after gel cleaning, only GC-Rich system over other polymerases which give the correct size band of the 261 bp pro-Insulin gene, while the other three reactions contained between 1-3 incorrect size bands.

Two sources of error may be identified during the construction of synthetic DNA molecules: (1) errors from the phosphoramidite synthesis of the oligodeoxyribonucleotides, (2) editing errors that occur during DNA polymerase-catalyzed enzymatic copying, and (3) errors that result from thermal damage to DNA. We used synthetic 5` hydroxylated oligonucleotides obtained from commercial vendor **(MWG Biotech, Germany)** and the Oligos were purified using HPSF technology to be gene synthesis grade. However, shorter synthetic oligomers have fewer errors – for that reason, we limited their size to ~ 42 nucleotides. The second source of error may stems from the enzyme editing mistakes when annealed oligomers are enzymatically extended. Extension errors depend on the fidelity of the polymerase. Extension errors can be reduced with a high fidelity polymerase enzyme and optimization of the biochemical reaction conditions during DNA extension using GC-Rich system in combination with proofreading Polymerase **(GC-RICH system, Roche)**.

Several reagents have been used to disrupt the base pairing or to isostabilize DNA like DMSO, Formamide, Glycerol, TAMAC and Betaine, Betaine improves the amplification of these genes by reducing the formation of secondary structure caused by GC-rich regions and, therefore, may be generally applicable to ameliorate the amplification of GC-rich DNA sequences **(Wolfgang et al, 1997),** another study by **(Baskaran et al, 1996)** in which Betaine is applied in combination with DMSO for the uniform amplification of a mixture of DNA with varying GC content.

Unfortunately, the non-proofreading enzymes (Red-Hot star, Finzyme) and proofreading (*pfu* clone) were failed to correctly amplify the full-length of the human pro-insulin gene. They were assembled but several bands were amplified. However, the GC-Rich system successfully assembled and amplified the correct size of the human pro-insulin gene (261 bp). The GC-Rich system has been used with resolution buffer included (Formamide, TAMAC and Betaine) **(Wolfgang et al, 1997),** in addition to the DMSO and glycerol in enzyme buffer. Designing our PCR outermost primers was accompanied with addition of single 3´ Adenine overhangs for efficient PCR ligation into TOPO vector **(Shuman, 1994).** The isolated plasmids pCR2.1TOPO-pINS was checked for correct size of the human pro-Insulin gene insertion by enzymatic digestion using E*coRI* restriction Endonucleases, which cut cohesively down and up streaming the gene inserted, about 278 bp discrete isolated band were appeared.

Using two short forward and backward outermost primers, where the forward primers include the 4 base pair sequences (CACC) necessary for directional cloning on the 5′ end and to under T7 promoter regulated expression.

The induction of the transformed BL21 star cells containing pINS-pETet101/d vector were expressed the correct protein. A well distinct band had been appeared at 9.5 kDa. It represents the correct calculated molecular weight of the human pro-insulin protein.

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