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Construction of Synthetic Intact Human Parathyroid Hormone Gene and Testing the Transformation Efficiency and Expression of it in *E. coli* strains

Adnan Al-Badran[a], Rafeef A. Abdul-Jabbar[b*]

[a]*University of Basra, College of Science, Department of Biology, Basra, Iraq*

[b]*University of Basra, College of Pharmacy, Department of Clinical Laboratory Science, Basra, Iraq*

[b]*Email: rafeefamir@gmail.com*

Abstract

human parathyroid hormone gene (hPTH gene) is responsible for preproparathyroid hormone production (biologically inactive hormone) which further modified to form the biologically active hormone, a synthetic *hPTH* gene with 289bp which cod for active intact hPTH was constructed by Five long primers by splicing overlap extension PCR (SOE), the gene contain a de generated codon, and have two recognition site of restriction enzymes in both ends of the Gene, and a stop codon. The synthetic *hPTH* gene was transformed in two *E. coli* strains DH5α and BL21 (DE3) with transformation efficiency6.6$_*$ 10^6 and 3.4$_*$ 10^6 for both isolates respectively. And the transformation of gene was detected by extraction of constructed plasmid and amplification of cloning gene on it, the expression of the cloning gene in the BL21 (DE3) was detected by performing Real Time PCR which gave a Ct value of about (23.46). That makes the synthetic gene suitable for direct expression of human active protein inside the bacterial cells.

Keywords: synthetic gene; synthetic human parathyroid hormone gene; SOE PCR.

1. Introduction

Human Parathyroid hormone normal gene is located in the short (P) arm between positions 15.3 and 15.1 on chromosome 11 [1].

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* Corresponding author.

Its consists of 3 exons [2] Exon 1 is untranslated, exon 2 encodes a 25-amino acid signal peptide which is a part of the prohormone, and exon 3 encodes the remaining part of the prohormone (6 amino acids) and the whole PTH molecule (84 amino acids), this gene when transcribed to mRNA and the latter translated a larger polypeptide precursor called Pre-proparathyroid hormone (pre-proPTH) which is biologically in active containing 115 amino acids it was also identified as an early biosynthetic product during a studies using parathyroid gland slices the preproPTH undergoes two successive proteolytic cleavages inside the produced cell to yield active PTH with 84 amino acid. And because the using of the cDNA derived from mRNA for active hPTH production by recombinant DNA technology require post translational modification as described above, that make its cloning in bacterial system not useful because the latter are deficient to post translational modification as in higher organism [3].therefore for expression of this hormone in active form in bacteria required the using of synthetic PTH gene ,generally Synthetic genes are open reading frame sequences from publicly marked genes these sequence generated synthetically the synthetic gene is the product of choice if no natural clone is available or if available clones do not accomplish the specific requirements, like full sequence identity to a database sequence. Or for obtaining sequences verified-cloned DNA, especially when the source of biological material is not readily available (rare, lost or dangerous specimens) and when required DNA sequence optimization for maximization of protein yield (codons need to be optimized for expression in your preferred expression system, e.g. *E.coli* or yeast) [4]. Oligonucleotides are the building blocks for gene synthesis and Since the synthesis of the first synthetic gene till now there is a several methods for the synthesis and assembly of DNA sequences based on oligonucleotides have been described, a method for enzymatic ligation of oligonucleotides reported in the 1980s , the FokI method published in1988 [5] , self-priming PCR , the PCR assembly method [6], the template directed ligation(TDL) method, thermodynamically balanced inside-out (TBIO) PCR-based gene synthesis method[7] and improved version of the assembly PCR method described by Smith and his colleagues [8; 9] .Also there is a many other gene synthesis approaches like two-step total gene synthesis using both dual asymmetrical PCR (DA-PCR), PCR-based two-step DNA synthesis (PTDS) [9] , and overlap-extension PCR (OE-PCR) ,which uses a novel method of primer design to achieve high-fidelity assembly of long gene sequences [8] . PCR-based method of recombining DNA sequences [10] it is a simple, versatile technique for gene synthesis [11] described in a Biotechniques research report 27 years ago. And still attractive as one of the more important method for gene synthesis. The aim of current study was to use five long oligonucleotides for construction of active *hPTH* gene and detect the ability for gene transformation and expression in bacterial system.

2. Materials and Methods

The five oligonucleotides, random primer and all primers used in this study were supplied by Bioneer Company, Korea. The HindIII restriction enzyme was purchased from (Promega), USA, and KpnI from Invitrogen, USA, the pET32 a (+) vector as well as *E. coli* strain BL21 DE3 were purchased from (Novagen) USA and the other *E. coli* strain DH5α were purchased from (Invtrogen) USA, all other materials and kits supplied by Bioneer or Promega as pointed below.

2.1. Construction of Synthetic Recombinant Human Parathyroid Hormone (1-84) gene (shPTH)

The gene was constructed by gene splicing by overlap extension PCR (SOE PCR) using the following primers which given by Liu and his colleagues [12]:

Primer 1:
5' CTGGGTACCGATGACGATG AC
AAGTCTGTGAGTGAAATTCAGCTGATGCATAACCTGGGGAAACATCTGAACT-3',
Primer 2:
5'AA
ATTGTGCACATCCTGCAGCTTCTTACGCAGCCATTCTACACGCTCCATCGAGTTCAGATGTTTCCCC
AGG3,
Primer 3:
5'TACGCGGACGCTGGGAACCAGCATCGCGCGGACCAGCGGGGCACCCAGGGCAACAAAATTGTGC
ACATCCTGCA-3',
Primer 4:
5'TCTGCC TCGCCCAG GCTTTTTTCATGGCTCTCAACCA AGACATTGTC TTCCT TTTTACGCGG
ACG CTGGGAACCA-3',
Primer5:
5'-CGCAAGCTTATCACTGGGATTTAGCTTTAGTTAATACA
TTCACATCGGCTTTGTCTGCCTCGCCCAGGCTTTT-3'.

At this primers for the preferential codons of *E. coli*, were used and designed with termination codon and HindIII recognition site at C-terminus as well as a KpnI recognition site and an Enterokinas separation site at N-terminus, and also two primers were used to amplify the whole gene after construction of full length of gene, F primer 5`-CTGGGTACCGATGACGATG-3' and R primer 5'CGCAAGCTTA TCAC GGGATTTA-3'.

The primers or oligonucleotide designed to form the gene with four PCR cycles in the first PCR cycle, the first primer and second primer were used as sense and antisense primers separately. 73 nucleotides of primer 1 were similar to the designed nucleotides (1–73) of the gene. While, nucleotides sequence of second primer was complementary to the designed nucleotides (54–126). 20 nucleotides of the first primer and second primer from 3' terminus were complementary (overlapping region). So, they could be used as a template and primer to each other. The sequence of the first PCR production was identical to the designed nucleotides (1–126). Again, in second PCR, the first PCR product and the third primer were used as a template and primer to each other and produced the designed nucleotides (1–181). And the relaxation PCRs may be deduced by the same way. Finally, the designed oligonucleotide (1–289) was gained.

2.1.1. Construction and amplification of shPTH gene

In the building of gene the first PCR product gained by using GoTaq® Green Master Mix Cat. # M7122 (Promega)**.** The PCR reaction tube for each PCR cycle contain the 12.5µl of Gotaq® Green Master Mix as well as sufficient amount of Nuclease-free water to complete the volume to 25µl the other PCR tube contents for the first PCR cycle about 0.6µl from primer1 and primer2 were added, while in the second PCR cycle about 1µl of

the first PCR product with 0.6µl of primer3 and 1µl of F primer were added also in the third PCR cycle about 1µl of the second PCR product with 0.6µl of primer 4 and 1µl of F primer were added. Finally, in the fourth PCR cycle about 1µl of the third PCR product with 0.6µl of the fifth primer and 1µl of the F primer. The PCR reaction for the above PCR cycles was proceeding to thermal cycling with the initial Denaturation temp. 95c° for 2 min and 30 cycles of 20sec. at 95c°, 20sec. at 55c°, 40sec. at72 c° and final extension at 72c° for 5min. one time After that the PCR products loaded in the wells of 2% Agarose gel electrophoresis.

2.2. Purification of Recombinant shPTH Gene

After detecting the desired *shPTH* gene by running it in Agarose gel the gene was purified from Agarose gel by using MEGA quick-spin™ Total Fragment DNA Purification Kit from (Intron) Korea. And the concentration of the dsDNA for purified *shPTH* gene measured by using the Optizen POP Nano BIO Spectrophotometer.

2.3. Construction of recombinant pET32a-hPTH vector

The pET 32a (+) vector Cat. NO.69015 was used in this study, the constructing pET 32a (+) - hPTH can be achieved by Digestion of both the *shPTH* gene and the pET 32a (+) plasmid by endonuclease restriction enzymes primarlly by HindIII Cat. No. R6041 and then by KpnI Cat. NO.15232010, the procedure of restriction reactions was prepared according to the protocols of supplied companies. Ligation of the restricted *shPTH* gene and restricted pET 32a plasmid was achieved by using the AccuPower® Ligation PreMix Cat. No. K-7103, and as the protocol provided with the ligation premix.

2.4. Transformation of Competent Cells

The constructed vector pET32a-shPTH was transformed into two genetic engineered *E. coli* strains primarily in to DH5α strain and then transformed into the BL21(DE3) strain, the transformation methods were done according to the protocols provided with the Novagen and Invitrogen companies.

2.5. Screening for cloning isolates

To determine the presence of target gene in the transformed competent *E. coli* strains (which growing after transformation on the selective LB plate with Ampicillin), a single colony was cultured in 10 *ml* LB broth containing Ampicillin (50 µg/ml) overnight at 37c°, then from 2 to 5ml of the bacterial broth was used for plasmid extraction by AccuPrep® Plasmid Mini Extraction Kit Cat. No.: K-3030**.** The target gene *shPTH* which is found in the pET32 a (+)-hPTH (extracted plasmid) amplified by using whole gene amplified (F and R primers), and the PCR tube contain 12.5µl of Gotaq® Green Master Mix, 1 µl of each primer, 3.5µl of extracted plasmid and complete the volume to 25 µl with Nuclease free water. The thermal cycler program was 2 min at 95c° one time, 30 cycles of 30sec. at 95c°, 30 sec. at 57.8c°, 40 sec. at 72 c° and final extension at 72c° for 5min.

2.6. Testing for cloning shPTH gene expression by Real Time PCR

A real-time PCR is a laboratory technique of molecular biology based on the (PCR). It monitors the amplification of a targeted DNA molecule during the PCR, in this test the Real-Time PCR used as Gene Expression Analysis Techniques and that is carried out by multiple steps: **First step**: **isolation of RNA**, the RNA of cloning Bl21 (DE3) strain was extracted by taking 2ml of bacterial broth after induction by adding (1mM) of IPTG as a final concentration to the LB broth containing the transformed bacteria and then the bacteria was grown with shaking for 4 an hours at 30c°.the RNA extraction was carried out manually by using AccuZolTM cat No. (K-3090). And The concentration of the extracted RNA was determined by measuring the absorbance at 260 nm. by using the spectrophotometer an absorbance of one unit at 260 nm. agrees to 40µg of RNA per ml [13].At this way for measuring RNA concentration about 10µl of the extracted bacterial RNA was diluted with TE buffer until the total volume reach 1000µl (990µl of the TE buffer was added) (1:100) buffer. **Second step: Optimize Primers**; the primers already design for specific *shPTH* gene amplification. **Third step: Reverse Transcription,** the extracted RNA converted to cDNA by used GoScript™ Reverse Transcription System kit Cat. # A5000 and Random Primer (0.5µg/reaction) for cDNA synthesis. **Fourth step: Amplify cDNA using** AccuPower® GreenStar™ qPCR PreMix from Bioneer, and the cDNA amplification was carried out by addition of 1µl of each F and R primer (10pmol) with 6µl of cDNA and completes the volume to20µl DEPC-distilled water. the real-time PCR tube was sealed by The Optical adhesive film , vortex and spin. **Five steps: Run Reactions**, Real-time PCR instrument was Start and loaded. The **Real Time** PCR setting was Programmed.as 5min. of denaturation at 95C°, 45 cycles of 35sec. at 95C°, 1min. at 55 C°, scan, 1min at 72 C° and final extension for 7min. at 72 C° Melting and 2 min. incubation at 25 C°. After reaction was completed, data analysis was performed.

2.7. Calculation of Transformation Efficiency

To measure the transformation efficiency for each of the competent strain, the following procedure was used (Invitrogen): Each of the competent bacterial strain was transformed by the 5fold dilution of the ligation reaction as described by the protocol of transformation for each bacterial strain supplied company. Then 50µl to 100µl of each transformed cells were plated on LB agar containing Ampicillin, the plates were incubated at 37°C, overnight. The plates with single colonies (10 - 100) were designated for the calculation of the efficiency of transformation. The transformed colonies were counted and the following equation was used:

$$\text{Transformation Efficiency (cfu/µg DNA)} = \frac{\text{colony forming unit}}{\text{pg of vector used}} \times \frac{1*10^6\,\text{pg}}{\text{µg}} \times \frac{1\text{ml}}{0.1\text{ml plated}} \times \text{df}$$

df:

dilution factor

cfu: colony forming unit

3. Results

3.1 Construction of Synthetic Recombinant Human Parathyroid Hormone (1-84) gene (shPTH)

A construction of *shPTH* gene was performed by splicing overlap extension PCR and detected by Agarose gel

electrophoresis the Figure (1) show the products of the four PCR cycles carried out during construction of gene.

3.2. Cloning of Synthetic Human Parathyroid Hormone (shPTH) Gene

3.2.1. Construction of pET32a-shPTH Vector

The purified *shPTH* gene as well as the pET32a (+) plasmid were restricted by two endonuclease restriction enzymes in the sit (8 bp for KpnI and 281bp for HindIII of the *shPTH* gene) and sit (238bp for KpnI and 173bp for HindIII of pET32a (+)) then ligated to form pET32a-shPTH vector as in Figure (3) which show the pET32a-shPTH vector with 6116 bp in accordance with 5900bp of pET32a (+) plasmid control. After purification of the restricted *shPTH* gene and pET32a (+), the concentration of Restricted pET32a (+) and *shPTH* gene was (145and115) ng/µl respectively.

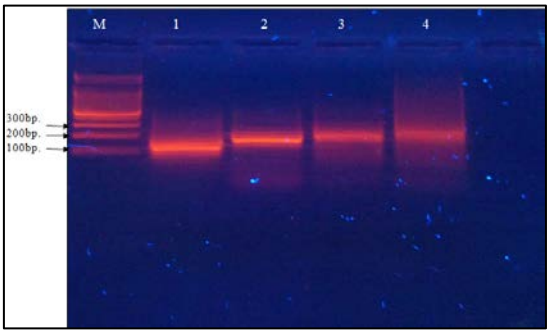

Figure 1: The 2% Agarose gel electrophoresis for construction of shPTH gene. Lane L (100bp DNA ladder) and lane1-4 the products of the four PCR cycles used in the construction of the gene (1st product size (126bp), 2nd (181bp), 3rd (236bp) and 4th (289bp)).

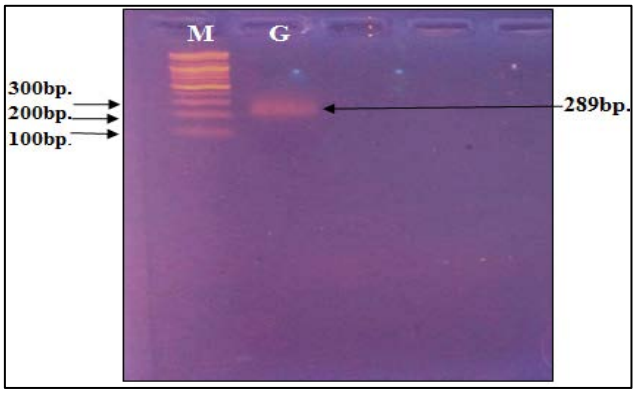

Figure 2: The 2% agarose gel Electrophoresis for purified *shPTH* gene lane G and lane M 100bp DNA ladder.

3.2.2 Transformation of competent bacterial cell

The transformed colonies were grown in selective LB plate supplied with 50 or 100µg/ml of Ampicillin as shown in Figure (4).

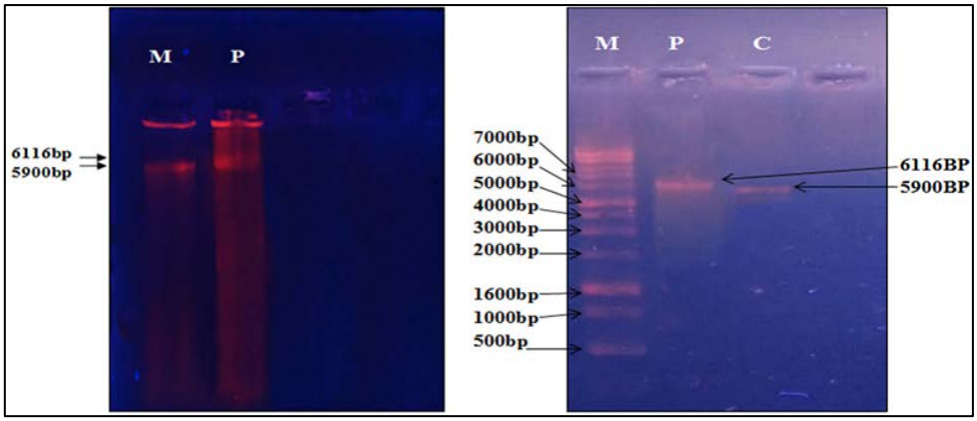

Figure 3: The 0.8% Agarose gel electrophoresis for constructed pET32a-*shPTH* with (6116bp) in lane P and lane C show the pET32a (+) control with (5900bp) in the right. And in the left lane M represent 1Kbp. Ladder and lane C and P represent the pET32a (+) control and constructed pET32a-*shPTH* respectively.

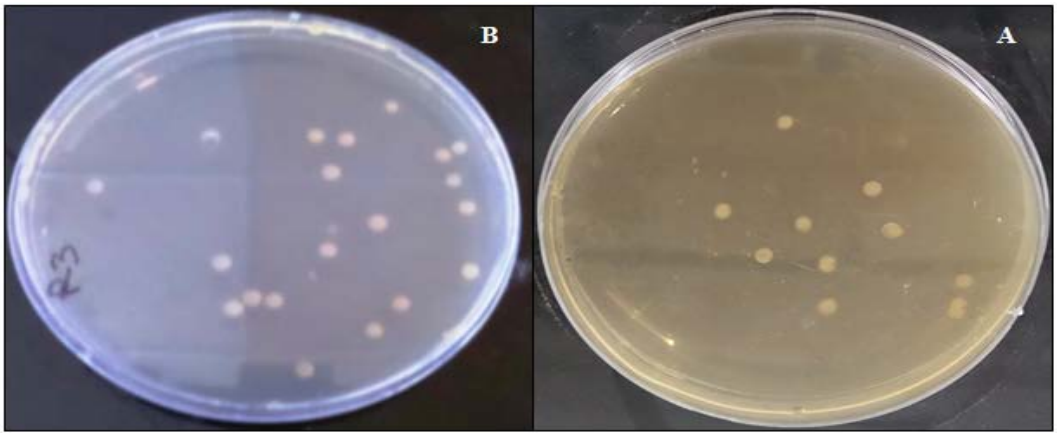

Figure 4: The transformed (A)DH5α and (B)BL21(DE3) colonies. (**a**) ; (**b**)

3.3. Screening for cloning isolates by Amplification of the target gene by specific primers

To screen for expression isolates the transforming isolates (both *E. coli* BL21 (DE3) and DH5α) were detected to have the desired recombinant pET32a-shPTH vector by amplifying the target *shPTH* gene which inserted in that vector, the later was extracted from transformed colonies and used as a template for *shPTH* gene amplification as shown in the Figure (5), and (6) which show the Agarose gel electrophoresis of amplifying *shPTH* gene.

3.4. Detection of gene expression by real time PCR

The RNA of transformed isolates was extracted after induction period of the transformed isolates by IPTG. And the RNA concentration was 10 µg/ml**.** The extracted RNA was converted to cDNA by using GoScript™ Reverse Transcription System and random primer in the reaction tube. And then Real time RT PCR was carried out which show a high *shPTH* gene expression as shown in Figure (7) of the fluorescent (threshold cycle) curve with Ct value of about (23.46) and Figure (8) for melting curve and the gene.

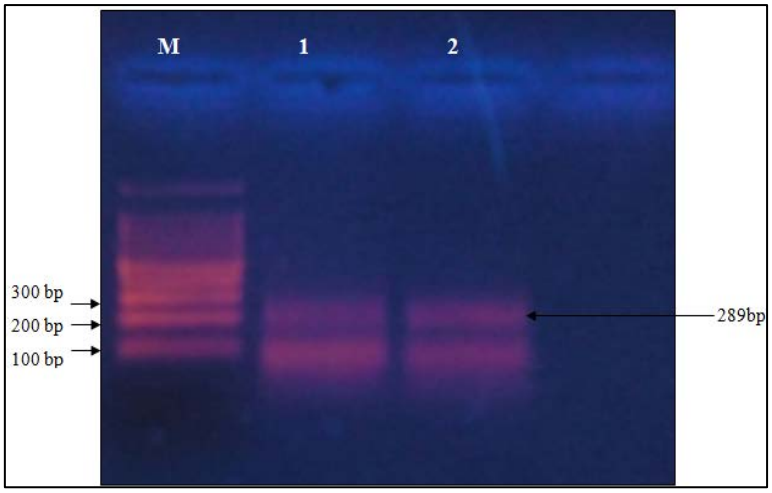

Figure 5: The Agarose gel electrophoresis for amplified *shPTH* gene. This amplified from recombinant pET32a-shPTH vector extracted from transformed BL21 (DE3). The lane M represent 100bp DNA ladder and lane1, 2 represent the amplified *shPTH gene.*

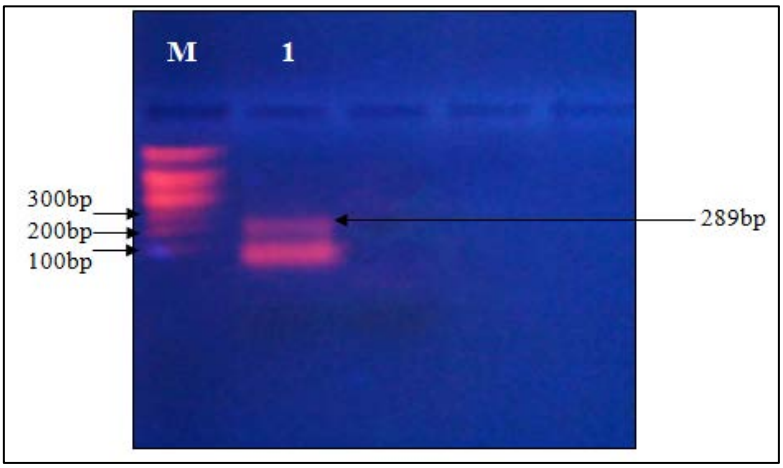

Figure 6: The Agarose gel electrophoresis for amplified *shPTH* gene. This amplified from recombinant pET32a-shPTH vector extracted from transformed DH5α. The lane M represent 100bp DNA ladder and lane1 represent the amplified shPTH gene.

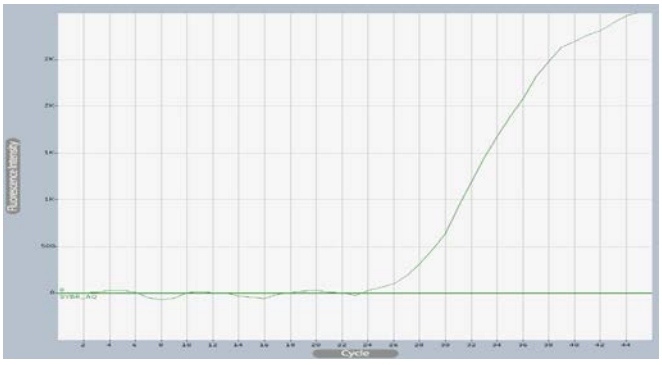

Figure 7: The fluorescent Ct curve for *shPTH* gene.

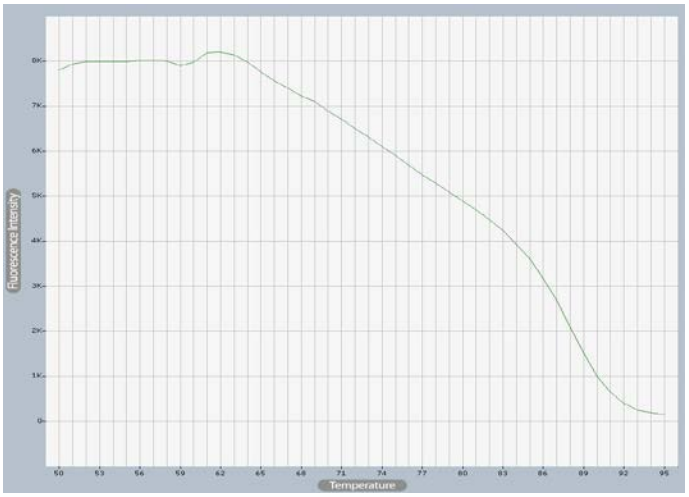

Figure 8: The melting curve of Real Time RT PCR for *shPTH* gene.

3.5. Transformation Efficiency

The transformation efficiency for *E. coli* BL21 (DE3) and DH5α was $6.6_{*}\ 10^{6}$ and $3.4_{*}\ 10^{6}$ respectively.

4. Discussion

The *hPTH* gene mRNA is translated to give a prePro-hormone with 115 amino acid (biologically in active hormone) therefore cloning of cDNA of the *hPTH* gene was not attractive for the researcher who seek for hormone activity. Since 1983, Vasicek and his colleagues found the *hPTH* gene in the a λ phage gene library and determined the sequence of each parts of the gene, the active gene sites was restricted from the total gene or gene cDNA which inserted in a multiple plasmid like pSSHPTH10[2] vector [14]. All that led the researcher as well as the drug production company to use a recombinant hPTH cDNA or used a synthetic gene which code for production of the active hormone form (84 amino acid). For the above reason a synthetic recombinant human parathyroid hormone gene was chosen for our trail to produce a full length active Hormone (1-84) which produced by using five long primers that constructed together by splicing overlapped extension PCR. The *shPTH* gene was design by using degenerated codon, degenerated codon means multiple codons can cod for the same amino acid [15] , *E. coli*-like' codons were used to design the recombinant *shPTH* gene that's lead to 8.3% of the bases (21.4% of the codons) being different from those in the cDNA of human PTH [14]. In unicellular organisms, high frequency usage codons correlate with abundant cognate tRNA molecules and have evolved to optimize translational efficiency [16], therefore The more preferred codon for bacteria were used in order to increase the recombinant hPTH expression.

The *shPTH* gene was constructed by chemically synthesis five long oligonucleotides using splicing overlap extension PCR method, in this method Initial PCRs generate overlapping gene segments that are then used as template DNA for another PCR to create a full-length product [11]**,** Chemical synthesis of nucleotide sequences can deliver a powerful molecular means for genes modification, revealing gene functions and studying the relationship between the structure of each protein part and its function [17].

Also, the *shPTH* gene was constructed with a two restriction sites, the restriction sites in the start and the end of gene (kpnI in 3` end of the gene and HindIII in 5`end), both of these restriction-enzyme cut the double stranded DNA to form a sticky end. Sticky ends are more useful in molecular cloning because they ensure that the gene fragment is inserted into the plasmid in the right direction [18]. as well as the sites of restriction enzyme , the *shPTH* gene contain a stop codon before HindIII restriction site at 5`end ,the stop codon is a nucleotide triplet within messenger RNA that signals a termination of translation of the proteins [19].

The expression of recombinant human Parathyroid hormone was carried out by growing the transformed isolate BL21(DE3) in the LB medium because the BL21 (DE3) isolate was used for gene expression because it's a lysogen of λDE3, and therefore carries a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter Such strains are suitable for production of protein from target genes cloned in pET vectors or other protein under the control of T7 promoter [20], while there was no ability for using the other transformed isolate (DH5α) for the production that's because the genotype of DH5 α made it a suitable strain for plasmid maintenance and growth without modification because it has *recA* mutation and lacks some endonucleases which might digest the plasmids during the isolation procedure , The DH5α strain has a mutations in its genotype make it a best strain in laboratory cloning procedures [21] , Also the DH5α isolate do not have T7 RNA Polymerase on its genomic DNA therefore it cannot express the target gene which express under the control of T7 promoter, the latter have only the ability to bind with T7 RNA Polymerase and not any other RNA Polymerase.

In order to get the mRNA of the cloning gene. Plasmid in the transformed bacterial isolate must induced for mRNA production by addition of IPTG to the bacterial broth and incubates it for several hours 4-5 hours at 25-30c°, at that time the lac I in both the plasmid and genomic DNA was repressed by Binding with IPTG molecules that lead to T7RNA polymerase expression in the genomic DNA of the bacterial and bind to T7 promoter in the pET32a-shPTH plasmid ,that permit to the target gene to transcribed and form mRNA which translate to form the target protein [22]. At this point the total bacterial RNA was extracted by TRIZOL Reagent is a ready-to-use reagent for the isolation of total RNA manually from cells and tissues [23], The concentration of RNA should be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml [24]. The extracted RNA was converted to cDNA by using GoScript™ Reverse Transcription System and random primer Primers which are a mixture of oligonucleotides representing all possible sequence for that size. Random Primers can be used to cDNA synthesis. from total RNA or messenger RNA (mRNA) [25; 26]., the RT PCR or revers transcription PCR is a technique commonly used in molecular biology to detect RNA expression, the RT- PCR is abbreviation of Real Time quantitative reverse Transcription PCR) is a major development of PCR technology that enables reliable detection and measurement of products generated during each cycle of PCR process and the real time RT PCR with SYBER ® GREEN detection produce a faster and dependable result [27], also the Figure (8) show the melting curve of RT RealTime PCR for the *shPTH* gene, the Tm is defined as the temperature at which 50% of the helices are dissociated. Typically, the thermal cycler being used to run the qPCR is programmed to produce the melt curve after the amplification cycles are completed. At the end of the qPCR run, the thermal cycler starts at a preset temperature (usually above the primer T_m; e.g., 60°C) and measures the amount of fluorescence. The temperature of the sample is then

increased incrementally as the instrument continues to measure fluorescence. As the temperature increases, dsDNA denatures becoming single-stranded, and the dye dissociates, resulting in decreasing fluorescence as shown in Figure (8) [28]. And the Figure (7) show a high Ct of qRT Real-Time PCR of the target gene, Ct mean The spot where the reaction curve intersects with threshold line (a line in the graph that represents a level above background fluorescence) is the Ct, or "threshold cycle." This spot shows the number of cycles it took to detect a real signal from the samples. Generally for high gene expression the Ct must be below 30 cycles, and its (23.46) for the target gene which indicate a high gene expression [29].

5. Conclusions

The cloning and expression of normal human parathyroid hormone gene in bacterial strain led to production of biologically inactive hormone because the bacteria lack post translational modification that present in higher cell organism. Therefore, in the current study, a synthetic recombinant human Parathyroid hormone gene that's code only for the active hormone form was constructed by five long primer using SOE PCR, and then expressed in *E. coli* strain.

6. Recommendation

We recommended using the cloning isolate with *shPTH* gene for production of the human parathyroid hormone to estimate the chemical and biological features for the produced hormone.

Supplementary Materials:

The following are available online at https://www. thermofisher. com/iq/en/home/life-science/pcr/real-time-pcr/real-time-pcr-applications/gene-expression-using-real-time-pcr.html. Gene Expression Analysis Using Real-Time PCR and http:// classroom.synonym.com/advantages-using-sticky-end-enzymes-17470.html for The Advantages of using sticky end enzyme.

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Author Contributions

Dr. Adnan Al-Badran contributed to the conception and design of the study, Rafeef Abdul-Jabbar contributed to the conception and design of the study, performed experimental work and participated in data analysis. Both authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Reference

[1] Bilezikian, J. P, Marcus, R., Levine, M. A., Marcocci, C, Silverberg, S.J, Potts, J. T. The Parathyroids: Basic and Clinical Concepts. Academic Press.3rd edition, Print Book ISBN: 9780123971661. EBook ISBN: 9780123977908, 2014. Pages: 946.

[2] Goswami, R., Mohapatra, T., Gupta, N., Rani, R., Tomar, N., Dikshit, A., & Sharma, R. K. Parathyroid hormone gene polymorphism and sporadic idiopathic hypoparathyroidism. Journal of Clinical Endocrinology and Metabolism, 89(10), 4840–4845, 2004.

[3] Khow, O., & Suntrarachun, S. Strategies for production of active eukaryotic proteins in bacterial expression system. Asian Pacific Journal of Tropical Biomedicine, 2(2), 159–162. 2012.

[4] Marsic, D., Hughes, R. C., Byrne-Steele, M. L., & Ng, J. D. PCR-based gene synthesis to produce recombinant proteins for crystallization. BMC Biotechnology, 8, 44. 2008.

[5] Li, L., Wu, L. P., & Chandrasegaran, S. Functional domains in Fok I restriction endonuclease. Proceedings of the National Academy of Sciences of the United States of America, 1992. 89(10), 4275–9.

[6] Stemmer, W.P., Crameri, A., Ha, K.D., Brennan, T. M. and Heyneker, H. L. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxy ribonucleotides. Gene. 1995, 16; 164(1):49-53.

[7] Gao, X., Yo, P., Keith, A., Ragan, T. J., & Harris, T. K. Thermodynamically balanced inside-out (TBIO) PCR-based gene synthesis: a novel method of primer design for high-fidelity assembly of longer gene sequences. Nucleic Acids Research, 31(22), 143-146. 2003.

[8] Rouillard, J. M., Lee, W., Truan, G., Gao, X., Zhou, X., & Gulari, E. Gene2Oligo: Oligonucleotide design for in vitro gene synthesis. Nucleic Acids Research, 32(WEB SERVER ISS.), 176–180. 2004.

[9] Xiong, A. S., Yao, Q. H., Peng, R. H., Li, X., Fan, H. Q., Cheng, Z. M., & Li, Y. A simple, rapid, high-fidelity and cost-effective PCR-based two-step DNA synthesis method for long gene sequences. Nucleic Acids Research, 32(12): 98-104. 2004.

[10] Horton, R. M., Cai, Z., Ho, S. N., and Pease L. R. Gene Splicing by Overlap Extension: Tailor-Made Genes Using the Polymerase Chain Reaction. Reprinted from BioTechniques. 8(5):528-535. 1990.

[11] Heckman, K. L., & Pease, L. R. Gene splicing and mutagenesis by PCR-driven overlap extension. Natural Protocol, 2(4), 924–932. 2007.

[12] Liu, Q., Lin, J., Liu, M., Tao, X., Wei, D., Ma, X., & Yang, S. Large scale preparation of recombinant human parathyroid hormone 1-84 from Escherichia coli. Protein Expression and Purification, 54(2), 212–219. 2007.

[13] Salih A. A., Mehdi K. H., A.-B. A. I. Cloning of Human Insulin Gene in Bacterial Expression System. LAP LAMBERT Academic Publishing, 2013.

[14] Kronenberg, H. M., Samuel, R. Nussbaum, T. D. Recombinant DNA method for production of parathyroid hormone. 1990. WO 1990014415 A1. Patent WO1990014415A1.

[15] Wei, X., Kuhn, D. N., & Narasimhan, G. Degenerate primer design via clustering.. Proceedings of the 2003 IEEE Bioinformatics Conference, CSB,2003, 75–83.

[16] Angov, E. Codon usage: Nature's roadmap to expression and folding of proteins. Biotechnology

Journal, 6(6), 650–659. 2011.

[17] Naimov S., Dukiandjiev S. and de Maagd R. A. A rapid pcr based method for gene synthesistrol. Proceedings Of The Balkan Scientific Conference Of Biology In Plovdiv (Bulgaria) From, 2005(May), 277–282.

[18] Nguyen D.H. The Advantages of Using Sticky End Enzymes. Synonym.com. 2016.

[19] Griffiths AJF, Miller JH, Suzuki DT, et. An Introduction to Genetic Analysis. 7th edition. W. H. Freeman and Company. 2000.

[20] Novagen. competent cells. User Protocol TB009 Rev. F 0104 © EMD Biosciences, Inc.,1-23, 2004.

[21] Felthauser, A. Preparation of DH5a competent cells. Solutions, 2002.10–12.

[22] Rosano, G. L., & Eduardo, C. A. Recombinant protein expression in Escherichia coli: Advances and challenges. Frontiers in Microbiology, 5(APR), 1–17. 2014.

[23] Sigma Aldrich. TRI reagent protocol. User Manual - Sigma Aldrich, (10814), 1–2, 2014.

[24] White, P. Evaluating Concentration and Purity of RNA. Functional GENOMIC CORE, 2004. 6–7.

[25] QIAGEN. October For fast and highly sensitive one-step RT-PCR Sample & Assay Technologies QIAGEN Sample and Assay Technologies, 2012 (October).

[26] Bustin, S. a. Real-Time Reverse Transcription PCR. Encyclopedia of Diagnostic Genomics and Proteomics, 2005. 718–726. http://doi.org/10.1081/E-EDGP.

[27] Pfaffl, M. Livestock Transcriptomics: Quantitative mRNA Analytics in Molecular Endocrinology and Physiology. Accessed at February, 2006. (January). Retrieved from Retrieved from http://www.sigmaaldrich.com/technicaldocuments/protocols/ biology/tri-reagent.html.

[28] Downey, N. Interpreting melt curves: An indicator, not a diagnosis Researchers. 2014. Retrieved from http://eu.idtdna.com/pages/decoded/decoded-articles/core-concepts/decoded/2014/01/20/interpreting-melt-curves-an-indicator-not-a-diagnosis

[29] Vermasvuori, R.. Production of recombinant proteins and monoclonal antibodies - Techno-economical evaluation of the production methods. English, 2009. 1–117.