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A simple and cost Effectively Method for Production of Recombinant of Full Length of Human Placenta-Specific Protein using *E. coli* BL21 strain

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Abstract

Placenta-specific protein 1 (PLAC1) is one of the most significant cancer/testis antigens with restricted expression in the placenta and testis and high expression in a wide range of human cancers. PLAC1 has fundamental roles in cancer progression and is suggested as a diagnostic biomarker and therapeutic target for many cancers. However, producing full length of recombinant PLAC1 (rPLAC1) in *E. coli* BL21 strain as soluble form with maintaining its efficacy is still limited. Yet, major issues such as inclusion bodies (IBs) formation must be overcome. Here, we try to address this issue by optimizing rPLAC1 expression conditions. We purified rPLAC1 by immobilized metal affinity chromatography (IMAC) without using urea and detected the production of rPLAC1 by SDS-PAGE and western blot (WB). Our results demonstrated that the optimum conditions for the production of the full length of rPLAC1 in *E. coli* BL21 are induction at the early-log phase of growth ($O.D_{600nm}$=0.3-0.5) using 0.5 mM IPTG at induction temperature of 22 °C for 7-8 hours. Moreover, we indicated that optimization of induction conditions probably increases protein-soluble form yield without needing to use urea or any denaturing buffer in purification later. Taken together, we have introduced a simple and cost effectively method for the production of the full length of human rPLAC1 and increasing its soluble form without using urea.

Keywords: Placenta-specific protein 1; Cancer/Testis antigen; Recombinant protein; *E.coli* BL21.

1. Introduction:

Placenta-specific protein 1 (PLAC1) is a type II membrane protein with 212 amino acids (aa) (Cocchia et al., 2000; Koslowski et al., 2007). It consists of three main parts: a short N-terminal intracellular component as a conserved signal peptide about 23 aa, a single transmembrane domain from 5-22 aa, and a large extracellular domain from 23-212 aa (Cocchia et al., 2000; Roldán, 2012; Mahmoudian et al., 2019). The extracellular domain contains a homolog part to the N-terminal sub-domain of the zona pellucida (ZP3) glycoprotein that ranges from 29–119 aa (Mahmoudian et al., 2019). PLAC1 is a cell membrane-associated protein (Fant et al., 2007; Koslowski et al., 2007; Silva et al., 2007; Liu et al., 2008; Ghods et al., 2014a; Ghods et al., 2014b; Nejadmoghaddam et al., 2017; Wu et al., 2017). It could be cytoplasmic (Liu et al., 2008; Ghods et al., 2014b; Liu et al., 2015; Wu et al., 2017; Yin et al., 2017) or nuclear protein (Liu et al., 2014; Liu et al., 2015). However, PLAC1 has different molecular weights, which range from 24 kDa to 30 kDa, depending on multiple factors like posttranslational modification (PTM) especially glycosylation (Mahmoudian et al., 2019; Mahmoudian et al., 2020). At the gene level, the PLAC1 gene is located on Xq26.3 containing 6 exons, the last exon (number 6) contains an open reading frame (ORF) with a length of about 639 bp (Cocchia et al., 2000; Chen et al., 2011; Caballero and Chen, 2012; Devor et al., 2014; Devor, 2016). PLAC1 is concerned with restricted expression type on the apical region of Syncytiotrophoblast, the too limited expression on Cytotrophoblast (CTB) and testis (Fant et al., 2007; Silva et al., 2007; Fant et al., 2010; Roldán, 2012; Wagner, 2014; Chang et al., 2016; Mahmoudian et al., 2019). PLAC1 is not detectable in other normal cells in normal status (Ghods et al., 2014a; Ghods et al., 2014b; Mahmoudian et al., 2019). In addition, it is considered an essential component for proper placental and embryonic development (Jackman et al., 2012; Devor, 2014; Chang et al., 2016; Mahmoudian et al., 2019). Nevertheless, PLAC1 is an important member of the cancer/testis antigens (CTAs) family (Cocchia et al., 2000). Recent studies confirmed its expression in more than 74 cancer cell lines (Silva et al., 2007; Mahmoudian et al., 2019). Including prostate (Ghods et al., 2014a; Nejadmoghaddam et al.,

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Abbreviations used in this article: aa; amino acids, APS; Ammonium persulfate, bp; Base pair, E. coli; Escherichia coli, sfGFP; super folding Green Fluorescent Protein, IB; Inclusion Body, IPTG; Isopropyl-β-D-Thiogalactothiopyronoside, ORF; Open reading frame, PBS; Phosphate-buffered Saline, PCR; Polymerase Chain Reaction, PLAC1; Placenta specific-protein1, plac1; Placenta specific-protein1 coding gene, SDS; Sodium Dodecyl Sulfate, SDS-PAGE; SDS-Poly Acrylamide Gel Electrophoresis, PTM; posttranslational modification, TBST; Tris-buffered Saline-Tween20, TEMED; Tetramethylethylenediamine, WB; Western Blot.

2017), breast (Koslowski et al., 2007; Li et al., 2018), uterus (Devor et al., 2014), cervix (Chen et al., 2021), ovary (Devor et al., 2017). In addition to, lung (Yang et al., 2018), liver (Wu et al., 2017), colon (Ren et al., 2020), gastric (Liu et al., 2021), pancreatic cancers (Yin et al., 2017), nasopharyngeal carcinomas (Lin et al., 2021), melanoma (Mahmoudi et al., 2020), and osteosarcoma (Yu et al., 2021). PLAC1 has important roles in cancer progression and maintenance including transforming normal cells into cancer cells, growing, resistance to apoptosis, immortalization, proliferation, migration, invasion, metastasis, and angiogenesis (Koslowski et al., 2007; Li et al., 2018; Mahmoudian et al., 2019; Ma et al., 2020; Ren et al., 2020; Roldán et al., 2020). PLAC1 has differential expression in many cancers such as prostate cancer, which shows a correlation of PLAC1 expression level with the Gleason score (Ghods et al., 2014a). Furthermore, PLAC1 is an important diagnostic and prognostic biomarker for multiple cancers and an attractive candidate target for cancer immunotherapy especially prostate cancer (PCa) (Nejadmoghaddam et al., 2017).

Recombinant protein (RP) production technology is one of the most important pharmaco- medico-biotechnology techniques that aim to use engineered biological tools for the production of pharmacological benefit proteins (Rosano and Ceccarelli, 2014; Pham, 2018; Puetz and Wurm, 2019). The most remarkable class of RPs is recombinant membrane proteins like PLAC1 that form about 20–30 % of all genes encode products (Schlegel et al., 2014). Nevertheless, isolating membrane proteins from their natural sources suffers from many difficulties due to their low abundance leading to a low isolated amount, which does not meet the needs of structural and functional studies (Schlegel et al., 2014). In addition, the use of natural sources excludes the potential of genetically manipulating proteins to ease their detection and/or purification (Schlegel et al., 2014). *E. coli* strains are the most widely used bacterial host to produce RPs, thus 80% of proteins with their solved three-dimensional structures were submitted to the protein data bank (PDK), and more than 29 engineered antibodies were produced by *E. coli* strains (Frenzel et al., 2013; Kaur et al., 2018). However, many challenges are facing it in the production of multiple proteins, especially membrane proteins, like miss-folding, expression of proteins in insoluble form owing to its hydrophobic nature and tendency to aggregate in inclusion bodies (IBs), and digestion by proteases (Gopal and Kumar, 2013; Rosano and Ceccarelli, 2014; Nazari et al., 2017; Kaur et al., 2018). Several studies have produced rPLAC1 since some of them have used eukaryotic systems (Mahmoudian et al., 2020). However, the expression in eukaryotic systems is very expensive compared to prokaryotic systems. Others have used different prokaryotic systems mainly focused on *E. coli* strains. Thus, most of them have employed expensive genetically modified strains and molecular tools (Silva et al., 2007; Dong et al., 2008; Nazari et al., 2017; Nejadmoghaddam et al., 2017). In addition, others have used the *E.coli* BL21 strain, which is known for its cost-effectivity with different gene constructions (full length or the truncated form/without the Transmembrane part) (Nazari et al., 2017). Nevertheless, they have not obtained the expression of this recombinant protein (rPLAC1), neither the soluble form nor insoluble form in this strain, unless fusing it with high relative molecular weight protein tags (Nazari et al., 2017), which may affect the protein functional three-dimensional structure (Gopal and Kumar, 2013; Kaur et al., 2018). In addition, the main challenges facing the production of full length rPLAC1, in other genetically modified *E.coli* strains, have not been solved completely, especially the formation of insoluble proteins and aggregation in IBs (Silva et al., 2007; Nazari et al., 2017; Nejadmoghaddam et al., 2017)**.** However, optimization studies have indicated the importance of optimizing the expression conditions to obtain the correct form of recombinant protein (Gopal and Kumar, 2013; Rosano and Ceccarelli, 2014; Kaur et al., 2018). Therefore, we focused in our present study on optimization production conditions of rPLAC1 in full length, using specific strain (*E.coli* BL21), as using this efficient expression system in the expression of rPLAC1 has multiple advantages especially easy to handle and cost/time effective(Gopal and Kumar, 2013; Rosano and Ceccarelli, 2014; Kaur et al., 2018). We used simple strategies depending on the reference studies (Gopal and Kumar, 2013; Kaur et al., 2018). First, we have induced the BL21 at decreased-gradient induction temperatures for increased-gradient induction periods in the first log phase with 0.5 mM IPTG (phase-1). Second, we tested the optimum condition from phase-1 using gradient optical densities (O.D) at the early-log phase of the bacterial growth curve, followed by testing it using gradient concentrations of inducer and time course expression. Here, we have produced rPLAC1 in full length and soluble form (as half amount of produced rPLAC1) in the BL21 strain by optimization of four-cultivation conditions (inducer concentration, bacterial-growth phase, temperature, and time course expression) without using any urea or denaturing agents in purification later.

2. Materials and Methods

2.1. Bacterial strain, growth media, plasmid, and ladders.

E. coli strain; top10 (Invitrogen, USA) and BL21 (DE3) (Novagen, Germany) were used in cloning and protein expression, respectively, and transformed with the pRSET vector (Invitrogen). For general maintenance, pre-culturing, cloning, and protein expression, *E. coli* was cultured in Luria Bertani Broth (LB Broth) (Sigma-Aldrich, USA), and LB Agar (Sifin, Germany) with 100 µg/ml ampicillin (amp) (Cytogen, Korea), in an orbit-rotating incubator at 37 °C. Two DNA ladders; 1kb Ladder and 100 pb Ladder (Vivantis, Malaysia), and two protein molecular weight ladders (INTRON Biotechnology, Korea) were used too.

2.2. Plasmid construction and cloning ORF plac1.

ORF plac1 was amplified from human genomic DNA by PCR using two specific primers; plac1-for-*Xho1* (5`-AT ATA CTC GAG CAA AGT CCA ATG ACT GTG CTG TG-3`) and plac1-rev-*Kpn1* (5`-A TAT GGT ACC TCA CAT GGA CCC AAT CAT ATC ATC-3`). These primers were designed for amplifying ORF plac1 without signal peptide coding sequence (ss), and to add *XhoI* restriction site at the 5` end and *KpnI* at the 3` end of the PCR amplicon (inserted gene) (Figure 1A). The PCR amplification program was been optimized. Thus, it

consisted of 5 min of denaturation step at 94 °C followed by 35 cycles of short denaturation step at 94 °C for 30 sec, annealing at gradient temperature of melting for 30 sec, and final extension at 72 °C for 2 min. Finally, 72 °C for 5 min. In addition, the amplification was done using PFU polymerase (Thermos scientific, USA). Both amplified ORFplac1 fragment and the pRSET vector (Figure 1B) were digested with *XhoI* and *KpnI* restriction enzymes (New England Biolabs, USA). Then, they were ligated with each other using DNA Ligase T4 (Thermo scientific). *E. coli* top10 was transformed with this recombinant vector pRSET–ORF plac1 (Figure 1C), by heat shock. The transformed colonies with recombinant vector (positive colonies) were screened by colony-PCR using T7 universal primers. After that, the extracted plasmids from these positive colonies were digested with the same enzymes and were confirmed by sequencing. Then the confirmed construction was cloned in *E. coli* BL21 using the same procedure. In addition to transformation BL21with pRSET-sfGFP construction as a positive control (Al-Homsi et al., 2012; Al-jaghasi et al., 2021). *Geneious v4.8* software was used to design recombinant construction schematics.

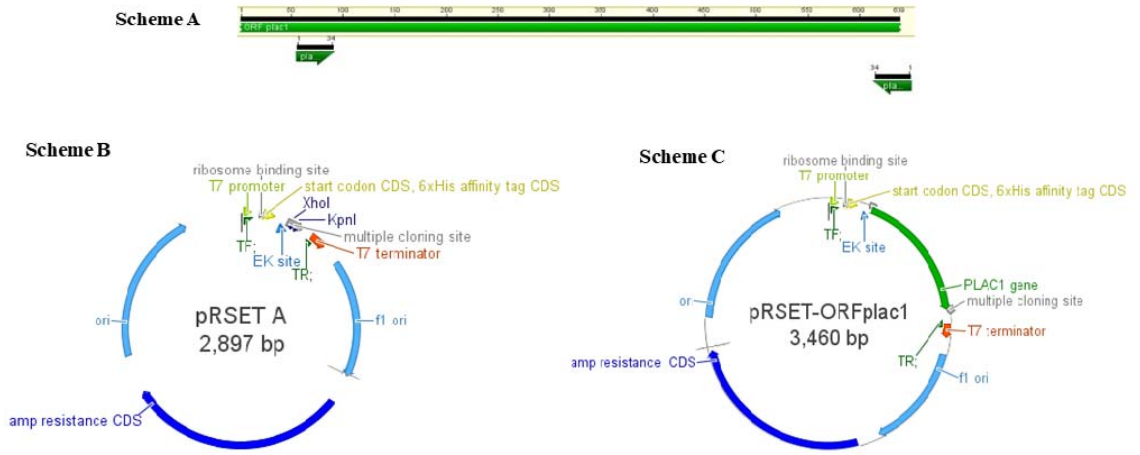

Figure 1: Schematic of ORFplac1 and pRSET before and after ligation. Scheme A; ORFplac1 with specific primers, Scheme B; pRSET construction, &Scheme C; pRSET-ORFplac1 construction.

2.3. Expression and purification of rPLAC1 protein.

Fresh transformed *E. coli* BL21 with recombinant vector (pRSET–ORFplac1) was grown overnight in a small culture volume (3ml of LB broth with amp), in an orbit-rotating incubator at 37 °C. The next day, 1.5 ml from this pre-culture was transferred into 10-50 ml of LB broth with amp (for small scale expression) or into 400-1000 ml of LB broth with amp (for large scale expression). Then, cultures were incubated at 37 °C until obtaining $O.D_{600nm}$ in the range 0.3-0.9. The expression was induced using a gradient of IPTG concentrations (Thermo scientific, USA), at gradient induction temperatures (37 °C, 22 °C, 16 °C) for gradient periods of expression induction time (3-4 h, 7-8 h, and overnight 16-20 h), respectively. After that, the culture was harvested by centrifuging at 4000 rpm for 15 min. The expression of sfGFP was induced as positive control by induction BL21 transformed with (pRSET-sfGFP) with 0.5 m IPTG at 16 °Covernight as mentioned in (Al-jaghasi et al., 2021). Later, pallets were manually purified by re-suspending with phosphate-buffered saline (PBS) and cells were lysed by repeated freezing and thawing on ice with a vortex. Then, solutions were centrifuged at 4000 rpm at 4 °C for 2 min, the supernatant was diluted with binding buffer to the final concentration; 20 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, and 200 µl Nickle charged Nitrilotiace acid (Ni-NTA) agarose beads 50 % slurry (Qiagen, Germany); 1 ml binding buffer /200 µl Ni-NTA /1 ml supernatant with rolling for 1h. Later, rPLAC1 was purified from the cytoplasmic extract by IMAC using a PD-10 column of NTA super sepharose (Qiagen, Germany), and washed with 10 volumes of binding buffer (after incubating for 1h). After that, bound protein (rPLAC1) was eluted by 300 µl elution buffer consisting of 20 mM sodium phosphate, 300 mM NaCl, and 250 mM imidazole after incubating for 1 h. Then, the eluted fraction was detected by SDS-PAGE followed by WB. The concentration of the purified protein was determined according tothe Bradford method (Bradford, 1976) using the Bradford reagent (Sigma-Aldrich, USA). In addition, the percentage of the purifiedrPLAC1 was estimated by ImageJ software.

2.4. SDS- PAGE of PLAC1 Protein.

The presence of rPLAC1 whole-cell lysate and purity of rPLAC1 was evaluated by Comassie-stained SDS-PAGE. Protein samples were diluted with 5X sample buffer consisting of 0.01 % bromophenol blue, 25 % glycerol, 10 % SDS, 5 % β-mercaptoethanol, and 16 mM Tris-HCl PH 6.8), and incubated at 95 °Cwater bath for 5 min. Then, they were separated by SDS-PAGE using a Bio-Rad Mini-Protean Tetra Cell system following the manufacturer's instruction in a gel that was prepared using stacking gel 4.5% and resolving gel 15%. Then, electrophoresis was applied using 100 V in electrophoresis buffer (25 mM Tris-base, 192 mM Glycine, 0.1 % SDSfor 1L d H_2O). The gel was stained in Comassie brilliant blue buffer (0.25 % Comassie R250, 10 % acetic acid, 40 % methanol) for 45-60 min and then washed several times in distaining buffer (30 % methanol, 10 % acetic acid).

2.5. Immunoblotting of PLAC1 Protein

Firstly, protein samples were separated in acrylamide gel, blotted onto 0.2 µm nitrocellulose membrane (Whatman, Germany) using 1X transfer buffer (25 mM Tris-base, 192 mM Glycine, 20 % Methanol for 1L d H_2O). Then, the membrane was blocked overnight at 4 °Cin 8 % skimmed milk diluted in T-BST buffer (10 mM Tris-HCl, 154 mM NaCl, 0.1 % Tween 20, pH=7.5). Then, incubated with Rabbit anti-HIS antibodies; 1/500 dilution (Bethyl laboratories USA) for 1 h with shaking at room temperature, washed again with T-BST buffer, and incubated with secondary antibody antibodies (Goat anti-Rabbit antibodies conjugated with alkaline phosphatase (AP); 1/1000 dilution), (Bethyl laboratories) for 1 h with shaking at room temperature. Finally, this membrane was incubated in darkness with chromogen substrate 33 µl Nitro blue tetrazolium (NBT) and 33 µl 5-Bromo-4-chloro-3-Indolyl phosphate (BCIP) (Sigma-Aldrich); diluted in 10 ml of substrate buffer (100 mM Tris-base, 100 mM NaCl, and 5 mM $MgCl_2$, pH=9.5) for 2-3 min.

3. Results

3.1. Cloning of ORF plac1 into pRSET vector

The amplified ORFplac1 fragment (about 593 bp in length) was identified by agarose gel-electrophoresis following PCR (Figure 2A), digested (Figure 2B), and ligated with the digested pRSET vector (Figure 2C). Transformed *E. coli* top10 with ligated products were screened by colony-PCR approach, which enabled comparing between empty pRSET containing colonies (negative colonies), which gave a fragment of 276 bp and pRSET–ORFplac1 containing colonies (positive colonies), which gave a longer fragment of 860 bp due to the existence of the inserted gene within them (Figure 2D). Plasmid constructs were extracted from positive PCR colonies and digested to ensure these positive colonies, where the pRSET–ORFplac1 containing colonies gave two fragments of 584 bp and 2877 bp after digestion due to the existence of the inserted gene within them (Figure 2E). Finally, this construction pRSET–ORFplac1was sequenced and compared with sequences in the Gene Bank, whereas results confirmed that no alteration or base substitution in the ORFplac1 sequence which was 100 % identical with that of *Homo sapience* (Genbank accession number:10761) and has been sent to NCBI and provided with (GenBank accession number:OK880267).

3.2. Expression and Purification of rPLAC1 Protein

Production of rPLAC1 was obtained after the transformation of BL21cells with the confirmed pRSET–ORFplac1 plasmid construction (Figure 1C). The protein expression was induced at decreased-gradient induction temperatures for increased-gradient induction periods (37 °C/3-4h, 22 °C/7-8h, 16 °C/16-20) in the first log phase ($O.D_{600nm}$=0.3-0.5) with 0.5 mM IPTG (phase-1) (Figure 3A). Then, we tested the optimum condition from phase-1 using gradient O.D at the early-log phase of the bacterial growth curve ($O.D_{600nm}$=0.3-0.5, and $O.D_{600nm}$=0.9) (Figure 3B), followed by testing it using gradient concentrations of inducer (0.5-1 mM IPTG) (Figure 3C), and gradient time course expression (Figure 3D). Then, protein expression was induced on a large scale (Figure 4A). The optimum condition for rPLAC1 production in soluble form was to induce bacteria at the first log phase ($O.D_{600nm}$=0.3-0.5), using 0.5 mM IPTG at 22°C for an incubation period of about 7-8 h and rPLAC1 was obtained as ~29 kDa band on SDS-PAGE (Figures 3A, B, C &D). The expression on a large scale was not different from a small scale since rPLAC1 was obtained as ~29 kDa band on SDS-PAGE within the same optimum expression conditions (Figure 4A). rPLAC1 was purified from the cytoplasmic extract by IMAC and the purification product was separated in SDS-PAGE, which has obtained a clear main band of about ~29 kDa presenting the pure rPLAC1 in the soluble fraction (Figure 4B). However, there was a remaining part of rPLAC1 in the insoluble fraction (Figure 4B). Although rPLAC1 was partially purified from bacteria cytoplasmic extract, the purification has been done without using any reducing /denaturing agents like urea, and the yield of pure rPLAC1was about half the total amount of rPLAC1 in the extract as mentioned in the purification recovery table (Table 1). The purity of the soluble form of rPLAC1was estimated at 37.76% (Table 2) as there were some non-specific bands besides rPLAC1 (Figure 4B).

Table 1. Purification recovery table, S; soluble form, I; insoluble form.

Procedure	Yield (mg)	Percentage (%)	Recovery
Total protein in extract (S, I)	18.1	100	
Nickle column	10	55.24	55.24% of total

Supplementary Data:

Table 2. Percentage of purified protein (Soluble form of rPLAC1), which was estimated depending on intensity rPLAC1 band in SDS-PAGE analysis of purification stage, by ImageJ software

	Percentage of purified protein
The soluble form of rPLAC1	37.76%

3.3. Immunoblotting of rPLAC1 Protein.

Detection of rPLAC1 was done after migration of total sample extract and pure rPLAC1 in SDS-PAGE (acrylamide 15%), blotting on nitrocellulose membrane and incubating with primary antibodies followed by secondary antibodies, and substrate. The location of rPLAC1 on nitrocellulose membrane was detected and defined as ~29 kDa compared to the protein molecular weight ladder (Figure 4C).

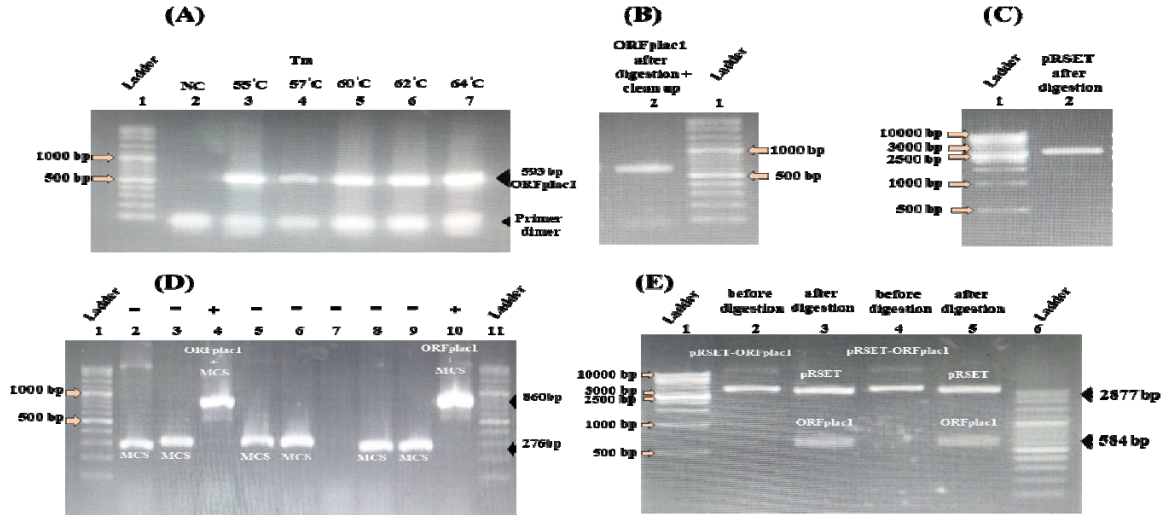

Figure 2. Electrophoresis of multiple stages of the Cloning (ORF PLAC1 into pRSET vector) products in 1 % agarose gel. **(A)** PCR product using gradient Tm; from the left; lane 1 DNA Ladder 100pb, lane 2 negative control (NC) (without genomic DNA), lane 3 Tm=55°C, lane 4 Tm=57°C, lane 5 Tm=60°C, lane 6 Tm=62°C & lane 7 Tm=64°C, Tm; Temperature of melting (annealing). **(B)**ORFplac1 after digestion and clean up; from the right; lane1 DNA Ladder 100pb, lane 2 ORFplac1 after digestion with *XhoI* and *Kpn1* enzymes and clean up. **(C).** pRSET plasmid extracted from *E. coli* top10; from the left; lane 1 DNA Ladder1kb, lane 2 extracted pRSET after digestion with *XhoI* and *Kpn1* enzymes. **(D)** Colony-PCR products of transformed *E. coli* top10 colonies with pRSET-ORFplac1; from the left; lanes 1&11 DNA Ladder 100pb, lanes 4&10 positive colonies (+), lanes 2, 3, 5, 6, 7, 8&9 negative colonies (-). **(E)** Extracted plasmid from the two positive colonies; from the left; lane 1 DNA Ladder 1 kb, lanes 2&4 extracted pRSET–ORFplac1 constructions before digestion, lanes 3&5 extracted pRSET–ORFplac1 constructions after digestion with *XhoI*, and *Kpn1* enzymes, &lane 6 DNA Ladder100bp.

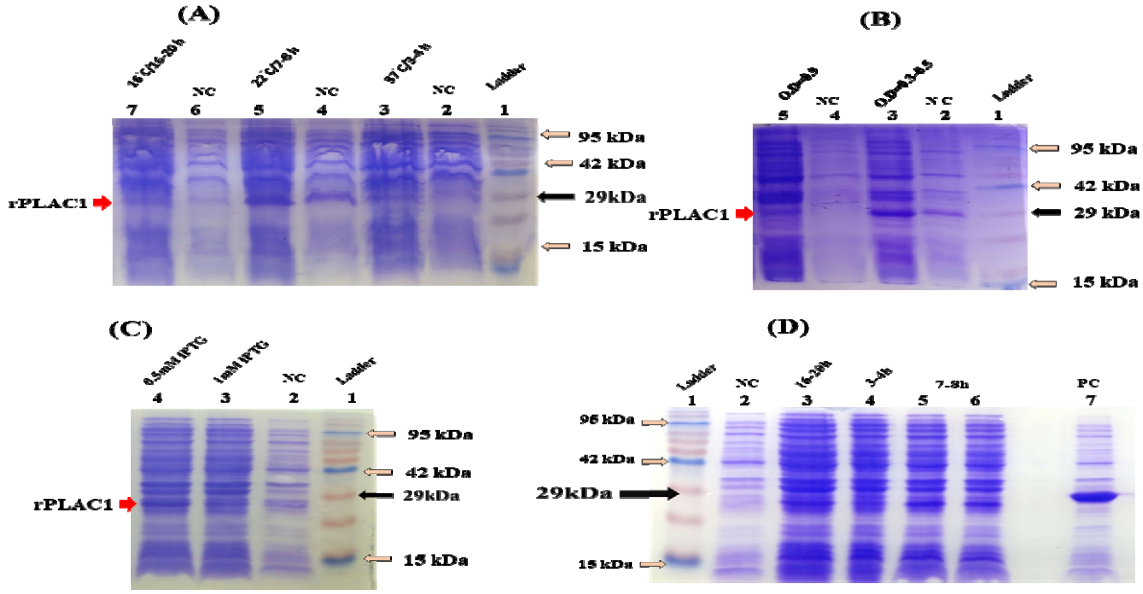

Figure 3.SDS-PAGE of *E. coli* BL21 transformed with (pRSET-ORFplac1) extracts before and after induction in optimized culture cultivation conditions and processing with 5X sample processing buffer and 1X PBS. **(A)** Optimization of induction temperature/period. From the right; lane1 protein molecular weight ladder, lanes 2, 4 &6 extracts before induction NC. Lane 3 extract after induction with 0.5 mM IPTG at O. D_{600nm}= 0.3-0.5 of bacterial growth phase in **37 °C** for **3-4h**. Lane5 extract after induction with 0.5 mM IPTG at $O.D_{600nm}$=0.3-0.5 of the bacterial growth phase at**22 °C** for **7-8h** (*). Lane 7 extract after induction with 0.5mM IPTG at $O.D_{600nm}$= 0.3-0.5 of bacterial growth phase in **16 °C** for **16-20h**. **(B)** Optimization of O.D in induction time. From the right; lane1 protein molecular weight ladder, lanes 2&4 extracts before induction NC, lane 3 extract after induction with same conditions (*), lane 5 extracts after induction with same conditions (*), except $O.D_{600nm}$, thus the used was $O.D_{600nm}$=0.9. **(C)** Optimization of IPTG concentration. From the right; lane1 protein molecular weight ladder, lane 2 extract before induction NC, lane 3 extract after induction with same conditions (*), except IPTG concentration, thus the used was 1mM IPTG, lane 4 extract after induction with same conditions (*). **(D)** Optimization of time course induction. From the left; lane1 protein molecular weight ladder, lane 2 extract before induction NC, lane 3 extract after induction with same conditions (*), except the period of induction, thus the used was 16-20h, lane 4 extract after induction with same conditions (*), except the period of induction, thus the used was 3-4, lanes 5&6 extracts after induction with same conditions (*). &Lane 7 PC (*E. coli* BL21 transformed with (pRSET-sfGFP) after induction at 16 °C overnight).

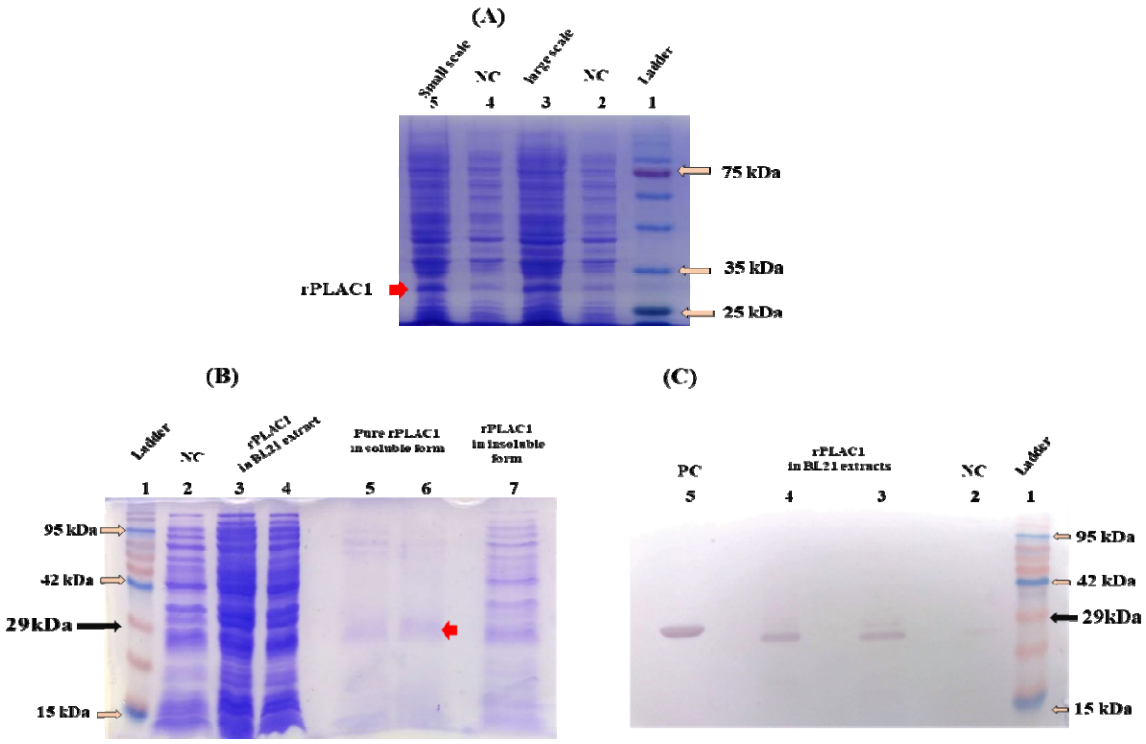

Figure 4.SDS-PAGE &WB analysis of *E. coli* BL21 transformed with (pRSET-ORFplac1) extracts in small and large scales before and after induction with optimum conditions (0.5mM IPTG in O.D= 0.3-0.5 at 22°C for 7-8h) and purification**. (A)** SDS-PAGE analysis for large &small scales extracts. From the right; lane 1 protein molecular weight ladder, lanes 2 &4 NC (extract before induction), lane 3 extract after expression on a large scale, &lane 5 extract after expression on a small scale. **(B)**SDS-PAGE analysis for extracts before and after induction with Optimum condition (*) and purification. From the left; lane1 protein molecular weight Ladder, lane 2 NC (extract before expression), lanes 3 &4 extract after expression and before purification, lanes 5 &6 extracts after expression and purification (soluble form) without using urea, &lane 7 extract after expression (insoluble form). **(C)** WB analysis; From the right; lane 1 protein molecular weight ladder, lane 2 NC (extract before induction), lanes 3 &4 extracts after expression in optimum conditions (0.5mM IPTG in O.D= 0.3-0.5 at 22°C for 7-8h), &lane 5 PC (*E. coli* BL21 transformed with (pRSET-sfGFP) after induction in 16 °C for overnight).

4. Discussion

PLAC1 is an important member of the CTAs family, serves as a prognostic and diagnostic cancer biomarker, and is a promising therapeutic target for many cancers (Cocchia et al., 2000; Nejadmoghaddam et al., 2017; Mahmoudian et al., 2019). *E.coli* BL21 is a strong expression system that presents a high productivity yield in a short time (Gopal and Kumar, 2013; Kaur et al., 2018). However, producing rPLAC1 in *E.coli* BL21 strain has faced many obstacles, especially no expression at all or accumulation of rPLAC1 in IBs needing urea or other denaturing agents for purification, which denatures its structure and alters its function, despite employing many strategies (Ghods et al., 2014b; Nazari et al., 2017; Nejadmoghaddam et al., 2017; Mahmoudian et al., 2019). Since there are no universal strategies for protein production as each specific protein needs its specific strategies for proper production (Gopal and Kumar, 2013; Kaur et al., 2018), hence it is suggested to employ efficient strategies to produce rPLAC1 in soluble form without compromising the correct form. In our study, we have amplified ORFplac1 without ss. Because it is a cleavable domain and its only function is to direct protein through secretory pathway in eukaryotes (Roldán, 2012). Besides, recent studies in prokaryotes proved that removing of ss increased the stability of RPs without changing its biochemical characteristics (Gopal and Kumar, 2013). We also concentrated on optimizing the conditions that affect rPLAC1 expression in a soluble form depending on recent studies (Gopal and Kumar, 2013; Kaur et al., 2018). First, we induced the *E. coli* BL21 strain at different induction temperatures and for different induction periods (37 °C/3-4h, 22 °C/7-8h, 16 °C/16-20) in the first log phase ($O.D_{600nm}$=0.3-0.5) with 0.5 mM IPTG (phase-1). Our results showed that low induction temperature, about 22 °C, with an induction period of about 7-8h are optimal conditions for soluble expression. However, hydrophobic interactions are the main cause of IBs formation and they decreased when the temperature is lowered. So the lower temperature, the less amount of IBs, and the more soluble form of protein (Gopal and Kumar, 2013; Kaur et al., 2018). Moreover, both short and too long induction periods are inefficient for the expression of RPs as bacteria need a period to adapt to culture conditions and their metabolic activity is decreased, respectively (Gopal and Kumar, 2013; Kaur et al., 2018). Second, we induced BL21 in gradient O.Ds at the early-log phase (about $O.D_{600nm}$=0.3-0.9) (phase-2). We found that the optimum phase for induction bacteria is the first log phase ($O.D_{600nm}$=0.3 -0.5) as it is reported that bacterial cultures

that are induced at the early-log phase presented a low endogenous protein production of about 30%, and high exogenous RPs, which leads to efficient protein purification (Gopal and Kumar, 2013; Kaur et al., 2018). whereas the induction at the late-log phase decreased the RPs due to the high density of bacteria that reduced its metabolic efficacy (Gopal and Kumar, 2013; Kaur et al., 2018). Our results also revealed that using a low concentration of IPTG (about 0.5 mM IPTG) is the optimum. The high amount of inducer leads to the accumulation of RPs in IBs because the rate of protein synthesis overwhelms the folding machinery whereas lower concentration leads to inefficient induction (Gopal and Kumar, 2013; Kaur et al., 2018). In this study, we obtained pRSET–ORFplac1 construction without any mutation or frame-shift, and achieved the expression of the full length of rPLAC1 in soluble form in *E. coli* BL21 strain, as ~29 kDa molecular weight after separating and detecting rPLAC1 using 15% SDS-PAGE and WB, respectively. Our results agree with different studies reporting the molecular weights of rPLAC1 around 27 kDa (Nejadmoghaddam et al., 2017), 27.2 kDa (Nazari et al., 2017), and 28-30 kDa (Fant et al., 2007). Nevertheless, some reports obtained different molecular weights such as 25kDa (Mahmoudian et al., 2020), 26kDa (Koslowski et al., 2007; Dong et al., 2008), and 35kDa (Silva et al., 2007), which can be attributed to several factors like PTM and existence of different isoforms (Mahmoudian et al., 2019). Besides, the length of PLAC1 (truncated, full length, or fused with tags) (Silva et al., 2007; Nazari et al., 2017; Mahmoudian et al., 2020). Some studies used the His tag (Fant et al., 2007; Dong et al., 2008; Nazari et al., 2017; Nejadmoghaddam et al., 2017; Mahmoudian et al., 2020), and others used Enhanced-GFP (EGFP) (Mahmoudian et al., 2020) and Glutathione S-transferase (GST) (Silva et al., 2007). In addition, some of them produced PLAC1 without ss (Fant et al., 2007; Nazari et al., 2017; Nejadmoghaddam et al., 2017), and others expressed it with ss (Dong et al., 2008; Mahmoudian et al., 2020). Even some reporters used other *E. coli* strains like *E. coli* M13 (Dong et al., 2008), or eukaryotic expression systems like CHO-K1 cells (Mahmoudian et al., 2020)and baculovirus expression systems with Sf9 cells (Fant et al., 2007) (Table3). Here, we produced a full length rPLAC1 in soluble form as half amount of produced protein with 12.44 µg of purePLAC1/ml of bacteria culture, and ~10mg in 1L of bacteria culture, after purifying with IMAC and without using denaturing agents. However, we have not overcome the IBs completely as the half amount of protein in extracts was insoluble form, which maybe attributed to a lack of chaperons, codon usage, and a reducing state of bacterial cytoplasm (Baneyx, 1999; Nazari et al., 2017). In comparison with other studies, neither the soluble truncated nor the full length rPLAC1 was produced using BL21 as an expression system, except in one study, which produced rPLAC1 fused with thioredoxin (Trx) tag (Nazari et al., 2017) (Table3). The full length of rPLAC1 was expressed as the insoluble form as IBs in all strains, and the soluble form was only obtained after PLAC1 had been expressed with a large size fusion tag like Trx (Nazari et al., 2017). Our rPLAC1 had good purity; nevertheless, some of the non-specific bands were still obtained, which may be attributed to the tendency of some *E. coli* endogenous proteins to strongly bind to metal ions like Nickel or the presence of superficial groups of His residues (Bolanos-Garcia and Davies, 2006). Purification of full length rPLAC1 in our study, and using 5% of deoxycholic acid for purification of fused rPLAC1 with Trx tag in recent studies. We successfully optimized expression conditions for the production of the full length of rPLAC1 in soluble form, using *E. coli* BL21.

Table 3. Comparison between different expression systems that have been used for the production of Human rPLAC1. HCC; Human hepatocellular carcinoma, GST; Glutathione-s-transferase, SA of TFR1; signal anchor of transferrin receptor 1, NI; no information has been told in the article.

Reference studies	Expression in an insoluble form	Expression in soluble form	Urea Or any denaturing agent	Purification technique	ss	tags	Cultivation conditions	Protein concentration	Protein KDa	Protein aa	Inserted gene Bp	Expression vector	Host strain	Host systems
(Nazari et al., 2017)	-	Expression only in Origami TM and Shuffle T7 with no expression in BL21 at all	2M urea & 12pH for denaturing for purification of truncated or full lengthPLAC1 Or 5% of deoxycholic (DOC) acid for purification of Trx-PLAC1	IMAC & gel filtration chromatography with 95% purity technical estimated	-	6X HIS tag and Trx tag at N-terminal in protein level and 5´end in gene level	The optimum condition for T-plac1 is Induction at $O.D_{600nm}$=0.6-0.9 with 0.1, 1 mM IPTG and induction temperatur15 °C for 24 hours	10mg/L Using Far-UV CD spectrum	22KDa & 18KDa	117-212aa	Truncated-plac1 (T-plac1) 351-636pb	p- cold vector	OrigamiTM (DE3)	Prokaryotes
													Shuffle T7 (DE3)	
	All strains express as IBs with the high amount in OrigamiTM and Rosetta2with no observed expression in BL21 using WB	No expression at all strains including BL21						35mg/L Using Far-UV CD spectrum	27.2KDa	23-212aa	Full length-plac1 (F-plac1) 69-636bp		Rosetta2 (DE3)	
	Little amount	The main part in all strains with the high amount in Rosetta2 with noobserved expression in BL21 in WB					Induction at $O.D_{600nm}$=0.6-0.9 Witno observedM IPTG and induction temperature 22 °C for 12 hours	25mg/L Using Far-UV CD spectrum	45KDa	23-212aa Fused with trx tag	Fused plac1 with trx (Trx-plac1)	pET32-a	Rosettagami (DE3) pLysS	E.coli
													BL12 (DE3)	
Our study	half of the total amount	There is Obvious expression in BL21 with an amount of about half of total rPLAC1 in the lysate	urea or any denaturing agent hasn't been used at all	Manually IMAC using Nickel-charged NTA column with about 37.76% purity	-	6X HIS tag at N-terminal in protein level and 5´end in gene level	Induction at $O.D_{600nm}$=**0.3-0.5** with **0.5** mM IPTG and induction temperature 22 °C for **7-8** hours	10mg/L Using spectrophotometer with Bradford method	~29KDa	21-212aa	ORFplac1 63-636bp 573bp from Human genomic DNA	pRSET	BL12DE3)	
(Nejadmoghaddam et al., 2017)	+	-	2M urea & 12pH for denaturing for purification	Same(Nazari et al., 2017)					~27 kDa	23-212aa	Full length-plac1 (F-plac1) 69-636bp	Same (Nazari et al., 2017)	Same(Nazari et al., 2017)	
(Dong et al., 2008)		NI		IMAC using Nickel-charged NTA agarose risen	+	6X HIS tag	Induction at temperature 37 °C for **5** hours	NI	26KDa	212aa	196bp from Human HCC cDNA	pQE30 vector	M13	
(Silva et al., 2007)		NI		IMAC with Glutathione-sepharose Beads	-	GST tag in C-terminal	NI		35KDa	125-212aa Fused with GST tag	The coding sequence of PLAC1 125-212aa	pGEX-4T	E. coli strain	
(Fant et al., 2007)		NI		IMAC using Nickel-charged NTA	-	6X HIS tag at N-terminal	Induction for **7-8** hours	NI	28-30KDa	23-212aa	PLAC1 Coding sequence	pAcGP67 transfer vector	Baculovirus with Sf9 cells	Eukaryotes
(Mahmoudian et al., 2020)	Cytoplasmic protein			NI	+	6X HIS tag at C-terminal	Induction at temperature 37 °C for **24-48** hours	NI	25KDa	1-212aa	mRNA 291bp converted to cDNA	LeGo-iG2 vector	CHO-K1	
									39KDa	50-212aa With SA of TFR1	The coding sequence of PLAC1 50-212aa With SA of TFR1	pIRES2-EGFP vector		

5. Conclusion

PLAC1 is one of the important CTAs, which serve as prognostic, diagnostic, and immunotherapeutic targets for many cancers. In our study, we have partially optimized the production conditions of rPLAC1 in a simple and cost effectively method. We found that the optimum conditions to produce the full length of rPLAC1 are induction of *E.coli* BL21 at the early-log phase of growth ($O.D_{600nm}$=0.3-0.5) with 0.5 mM IPTG and induction temperature of 22 °C for 7-8 hours. We obtained a soluble form of rPLAC1 in a concentration equal to 12.44 µg of purePLAC1/ml bacteria culture. Our team tries to produce ScFv antibodies-library against this rPLAC1 for diagnosing prostate cancer later.

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