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# CLONING AND EXPRESSION OF RECEPTOR OF EGG JELLY PROTEIN OF POLYCYSTIC KIDNEY DISEASE 1 GENE IN HUMAN RECEPTOR OF EGG JELLY PROTEIN

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**ABSTRACT**

Autosomal dominant polycystic kidney disease (ADPKD) is caused by a mutation in the polycystic kidney disease1 (PKD1) gene, which is responsible for 85% of ADPKD cases. The PKD1 gene encodes a polycystin-1 (PC1) protein that has a large extracellular area containing many polypeptide motifs. The extracellular region of PC1 includes several well-defined peptide domains that show that it has been involved in cell-cell and/or cell-matrix interactions. One of the regions that we focused on in this study is the receptor of the egg jelly (REJ) domain. This study conveys novel findings, in which we utilized a successful methodology to clone and express the REJ protein. The REJ gene is located in many exons separated by introns, which made it impossible to clone the whole REJ region. Therefore, synthetic DNA technology was used to clone a fragment located on exon 15 of the REJ gene. The PCR technology was used to amplify the REJ region by using a universal primer to express the domain located on exon 15 in humans. The REJ from synthetic DNA was cloned by using the phosphoramidite synthetic method. Sequencing technology and bioinformatic tools were applied to confirm the validation of the coding region. The results indicated that we successfully cloned and expressed the REJ protein using the DNA synthesis method instead of the conventional methodology.



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## Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a genetic disorder that is signified by the growth of numerous cysts in the kidneys. According to global statistics, ADPKD affects approximately 4 to 7 million people in the world. In Europe and North America, ADPKD is responsible for approximately 6 to 10% of end-stage kidney disease patients [1]. In Saudi Arabia, men are affected more than women by 95% [2].

In 1995, the International Polycystic Kidney Disease Consortium reported the final structure of the polycystic kidney disease (PKD1) gene and its protein [3]. ADPKD is caused by mutations in the PKD1 gene on chromosome 16p13.3 or the PKD2 gene on chromosome 4 and is responsible for 85% of cases [3]. The PKD1 gene encodes the polycystin-1(PC1) protein which regulates the signaling pathways that preserve the function and structure of the renal tubules. Nigro and Boletta, 2021 [4] demonstrated the functions of PC1 as a mechanosensory. Their results highlight the potential cystogenesis events that the mutation in PC1 causes. They suggested that the stiffness of the extracellular environment's mechanical stimuli could be sensed by PC1. These findings have hypothetically important implications for understanding ADPKD pathophysiology and the opportunity of designing novel treatments [4].

PC1 is an integral transmembrane protein and a member of a novel family of proteins with a multidomain structure [5]. It has a predicted mass of approximately 460 kDa and a glycosylated mass of 520 kDa. It also consists of transmembrane domains, a large N-terminal extracellular domain mediating cell-cell and cell-extracellular matrix binding, and a carboxy-terminal region [6]. The extracellular domain contains a novel combination of motifs that are predicted to be involved in cell-cell and cell-

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matrix interactions [7]. This region contains two leucine-rich repeats flanked by cysteine-rich domains (LRR), a C-type lectin domain (CLD), a cell wall integrity and stress response component (WSC) domain, 16 immunoglobin-like domains (PKD repeats), a low-density lipoprotein-like domain (LDL-A domain), a GPS domain, and a receptor of egg jelly protein (REJ) domain. The domains in the PC1 protein could be capable of binding ligands in the extracellular matrix (ECM) [7].

In this study, we cloned and expressed the receptor of the egg jelly (REJ) domain, which is the largest domain located in the extracellular region of PC1 (GenBank: AAC37576.1) (residues 2146 to 3109). We chose the REJ gene from 2151 to 2451 for two reasons: First, the PC1 protein has a single domain of REJ, which is the largest domain in the N-terminal of PC-1 protein [8]. Second, few copies that are expressed in the tissues of the kidneys and testicles exist, and these proteins are characterized by similar biological functions.

This REJ domain of PC1 includes several well-defined peptide domains, which are predicted to be involved in cell-cell and/or cell-matrix interactions. It is also one of the major components in the PC1 ectodomain, which extends to approximately 1000 amino acids. Approximately 230 mutations exist, which include 80 missense mutations of the REJ region, and among these missense mutations, the authors of [9] predicted that 65 are the disease-causing ones. the REJ region is encoded by PKD1 exons15 to 23 [9].

At the GPSG-protein-coupled proteolytic site, PC1 undergoes cleavage, and this process involves the entire REJ region (10). GPS cleavage is an essential process for both kidney function and structure, and the number of mutations at the REJ region affects GPS cleavage by disruption [10].

We aimed to produce a recombinant form of REJ-His fusion protein in a bacterial host and evaluate its biophysical properties. We also aimed to investigate the molecular expression mechanisms and predict the molecular structure of the extracellular domains of PC1 that could assess its potential importance in ADPKD etiology.

## Materials and Methods

The PCR reagents and universal primers were obtained all from Macrogen (Korea) and the work was performed at King Fahad medical research center. The kits and general reagents were obtained to construct the recombinant plasmid, digestion enzymes, and CutSmart Buffer from New England BioLabs (UK). The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were purchased from Merck (Germany) together with the expression solution, Luria–Bertani, from SolarBio (China).

### *REJ Gene Cloning*

Based on the sequence obtained from GenBank, we synthesized residues of the 2151 to 2451 genes with the accession number AAC37576.1 using the phosphoramidite synthetic method [11]. In this experiment, the DNA synthesis technique was used instead of the traditional amplification method due to the difficulty of amplifying the REJ gene with standard PCR methods. Therefore, the REJ gene sequence was designed and manufactured at Synbio (USA) and cloned with a pUC57-Amp cloning vector. The digested vector was added to the loading dye and then separated on a 1.2 % agarose gel electrophoresis. In this current study, all plasmids were digested using Snap Gene Viewer software [12].

### *REJ Gene Amplification*

A PCR reaction was performed, followed by restriction enzyme digestion to amplify the REJ constructs. The PCR reaction included the pUC57-REJ constructs (1µl) and forward and reverse M13 primers (2µL of M13 F, GTAAAACGACGGCCAGT, and M13R, GTCATAGCTGTTTCCTG). A master mix (25µL) (Sigma Aldrich, USA) was used, and deionized water was added to a total volume of 50µL. The forward and reverse primers were used to generate a 300 bp DNA fragment representing the coding region of the REJ gene. The PCR products were electrophoresed using 0.5 % (w/v) agarose gel in a 1X TPE buffer. Ethidium bromide (0.5 µg/L) was used to stain the DNA in agarose gel. Electrophoresis was performed at 80V. The PCR products were visualized using an alpha imager (UVP, UK).

### *Sub-Cloning of REJ Gene into PET-21a (+) Expression Vector*

The REJ gene fragment that had been confirmed via the DNA sequencing was subcloned into the pET-21a (+) expression vector to make plasmid pET-21a (+)-REJ. First, double digestion reactions were performed using XhoI and BamHI restriction endonuclease. The reaction included a pET-21a (+) expression vector (2µl) and a BamHI and XhoI restriction enzyme (1µL). Then, a 10x (5µL) NEB buffer r3.1 (restriction enzyme buffer) was added to the mixture. Finally, nuclease-free water was added up to a total volume of 50µL. The mixture was incubated for 1 hour at 37°C. The restriction map of the pET-21a (+) expression vector is shown in **Figure 1**, indicating the production of the fusion protein as His-tagged [13].

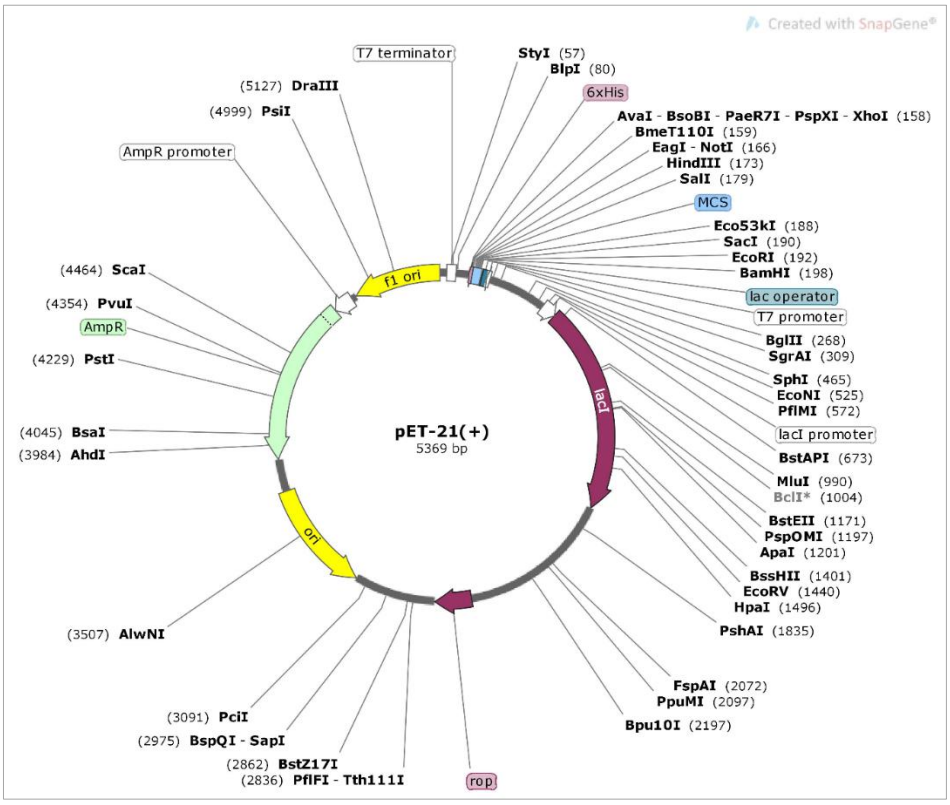


**Figure 1.** Restriction map of pET-21a (+) expression vectors (5369bp)

A ligation reaction was provoked by using a 1:3 insert-to-vector ratio. The amount of the pET-21a (+) expression vector was calculated to estimate the inserted REJ gene needed using NEB tool software. The ligation reaction was performed using a T4 DNA ligase enzyme (1µL) and incubated overnight at 4ºC. In 1 hour, the transformation process was performed using BL21-DE3 competent cells (Sigma-Aldrich, US). Competent *E. coli* cells that contained the pET-21a (+)-REJ were spread onto Luria–Bertani (LB) plates containing 100 µg/ml ampicillin and were left to grow overnight at 37°C [14].

*Identification of Positive Clones*

In order to select a positive colony with a plasmid containing the REJ gene (pET21a (+)-REJ, a colony PCR test was performed. The colony PCR procedure had been done by selecting 27 colonies on a culture plate and picking each colony with a 10-ul pipette tip and mixing each of them with 10 uL PCR super mix, that contains 5µL master mix, the T7 promoter right primer (1µ, TAATACGACTCACTATAGGG), T7 terminator left primer (1µL, GCTAG-TTATTGCTCAGCGG), and 3µL of $dH_2O$.PCR reaction conditions were programmed at 94 ºC for 4 min, cycling for 32 times (94 ºC for 30 Sec, 48 ºC for 30 Sec, and 72 ºC for 1 min), and finally the end of cycling at 72 ºC for 10 min. The annealing temperature for used primers was 48 ºC [15].

*Plasmid DNA Purification and Extraction*

The positive plasmid with a colony PCR was confirmed. The selected positive colony was left for an overnight culture, followed by DNA isolation for a subsequent sequencing reaction. The selected bacterial clone was picked up from the plate with a sterile toothpick, suspended in 5 ml of LB media containing 1% (w/v) ampicillin (5µl), and left overnight in a 190-rpm orbital shaker at 37 °C. The pET-21a (+)-REJ was isolated with the ZymoPURE™ II Plasmid Maxiprep kit according to the manufacturer's protocol [16].

After extraction and purification, the pET-21a (+)-REJ was sent to the King Fahd medical research center, Jeddah, KSA, for sequencing to prove the identity of the plasmid sequence. The resulting pET-21a (+)-REJ sequence was analyzed using the Thermo Hitachi ABI Applied Biosystems 3500XL Genetic DNA Analyzer, Japan. The results were displayed using Finch TV [17]. and then multiple sequence alignment software was used (European Bioinformatics Institute), to align the REJ sequence (GenBank: AAC37576.1) with the sequencing result [18].

*REJ-His Fusion Protein Expression*

*E. coli* BL-21 was transformed as the expression system with the pET-21a (+) positive clones harboring the plasmid were picked and left to grow overnight in liquid LB containing 100 µg/mL of ampicillin at 37°C to control the expression of the desired protein. After reaching 0.6 OD 600nm, the expression was induced using isopropyl-1-thio-β-D-galactopyranoside (IPTG). The expression was optimized using different IPTG concentrations (0.5,1,1.5 mM), different times (4, 5, 6 hours), and temperatures (20°C,25°C,37°C). After optimization, E. coli cells were cultivated in larger volumes and centrifuged in the final

suspension at 6000rpm at 4 °C for 10 min to remove the cellular debris. Then, the cells were re-suspended in a lysis buffer containing 50 mM $NaH_2PO_4$, 300 mM NaCl, and 10 mM imidazole. The culture mixture was disrupted with sonication, and the lysate was centrifuged at 6000 rpm for 10 min at 4 °C to pellet the supernatant aliquots to determine the recombinant protein expression via SDS-PAGE and Coomassie brilliant blue staining. The inclusion bodies were parted and solubilized by a denaturing lysis buffer containing 100 mM $NaH_2PO_4$, 10 mM Tris-HCl, and 6 M urea, then aliquots from the supernatant and pellet were used to determine the recombinant protein expression. Twenty percent SDS-PAGE was used to detect the REJ-His fusion protein solubility in the supernatant and pellet [19, 20].

**Results and Discussion**

REJ proteins support $Ca^{2+}$ influx, and the localization of the REJ protein domains in the extracellular region of the PC1 protein allows it to interact with the ECM proteins and provides an excellent example model to investigate its function in ADPKD [7].

*REJ Gene Cloning*

The bands representing the vector and the digested vector were visualized in an agarose gel electrophoresis obtained from Synbio. Two segments of the digested pUC57 plasmid with a size of 500 and 1200 bp were visualized in lane 1, while in lane 2 and the uncut pUC57 plasmid was 5000 bp **(Figure 2)**.

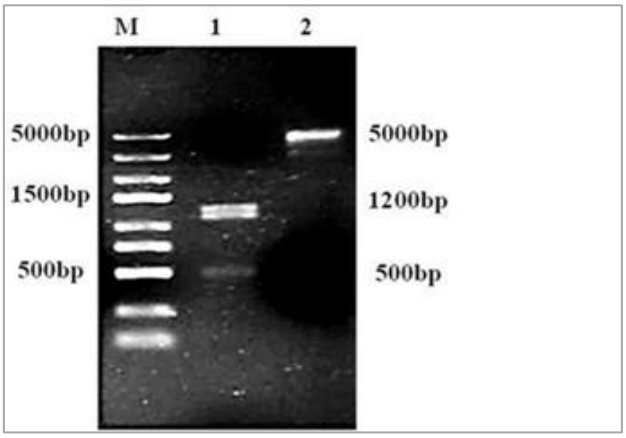


**Figure 2.** Gel electrophoreses analysis of the digested and undigested pUC57 plasmid in 1.2% (w/v) agarose gel electrophoresis. Lane M is a DNA marker. Lane 1 contains pUC57 plasmid digested by ApaL-I, presenting 500bp and 1200bp. Lane 2 contains the uncut pUC57 plasmid containing 5000bp.

The REJ gene theoretical sequence obtained from the REJ domain of polycystin-1 from the NCBCI-BLAST (GenBank: AAC37576.1) was compared with the sequence obtained from our research. The gel electrophoresis results in the lab presented identical matches between these sequences. The REJ gene sequence showed 300 bp, as shown in **Figure 5**. The sequence alignment between the theoretical REJ gene sequence was compared with the REJ sequence produced from the sequencing reaction in the lab. The result obtained from the King Fahad medical research center lab using a Sanger Sequencing Analyzer is shown in **Figures 3 and 4** which showed the Sequencing Chromatograms of the REJ gene.

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CLUSTAL O(1.2.4) multiple sequence alignment


Sequencing      GGGGCATCAGCGCTTTCCAGTCACGACGTTGTAAACGACGGCCAGTCTCGAGCGCGTCGT    60
REJ             ------------------------------------------------CTCGAGCGCGTCGT    14
                                                                **************

Sequencing      GCTGCAGACCAACCAGGTCACCCGGCAGCATCAGAACGTCCTGACCGTGCAGGGTAACAG    120
REJ             GCTGCAGACCAACCAGGTCACCCGGCAGCATCAGAACGTCCTGACCGTGCAGGGTAACAG    74
                ************************************************************

Sequencing      AGTACTGCGCCGGCGGACCCGCCGCAACAGAGAACAGGAATTCACGCCACAGCGCGTACG    180
REJ             AGTACTGCGCCGGCGGACCCGCCGCAACAGAGAACAGGAATTCACGCCACAGCGCGTACG    134
                ************************************************************

Sequencing      GCGCACCCGGCAGACCCGGACCAGAGGTGCAACCGTTCGCGCACGCCTGCGGGTGGCAAG    240
REJ             GCGCACCCGGCAGACCCGGACCAGAGGTGCAACCGTTCGCGCACGCCTGCGGGTGGCAAG    194
                ************************************************************

Sequencing      ACGCGTCCAGCGGCAGGCAGATGTTCGCACCCGGGGATCCGGCGTAATCATGGTCATAGC    300
REJ             ACGCGTCCAGCGGCAGGCAGATGTTCGCACCCGGGGATCC----------------------    234
                ************************************
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**Figure 3.** The alignment between the pUC 57-REJ obtained from sequencing and the theoretic (GenBank: AAC37576.1) REJ DNA sequence. Differences are marked by a dot (.) and matches are marked with a star (*).

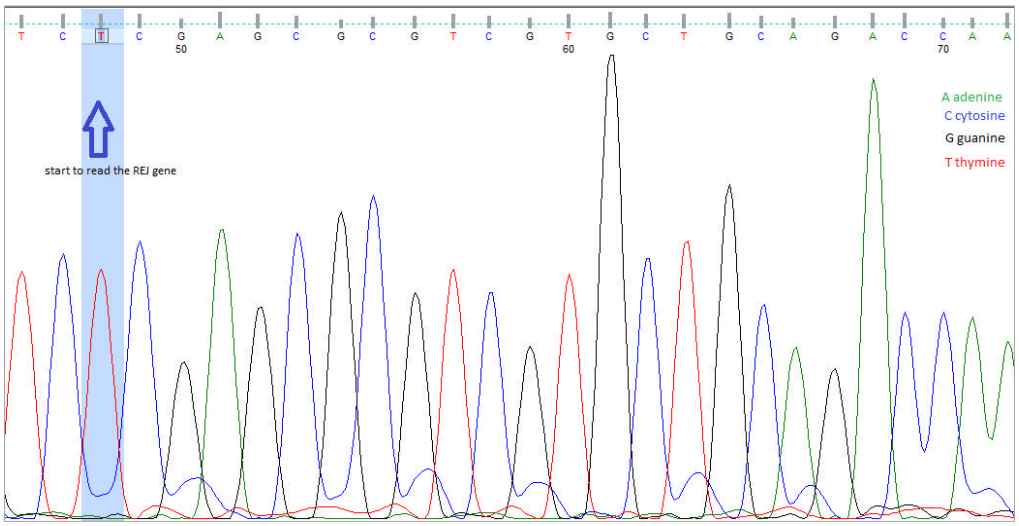



**Figure 4.** Sequencing Chromatograms of REJ gene. A single color is presented in each of the spaced-out peaks and the baseline noise is quite low.

*REJ Gene Amplification*

The REJ gene was visualized with 0.5 % (w/v) agarose gel electrophoresis. The cloned gene size presented a 300 bp band that is shown in **Figure 5**.

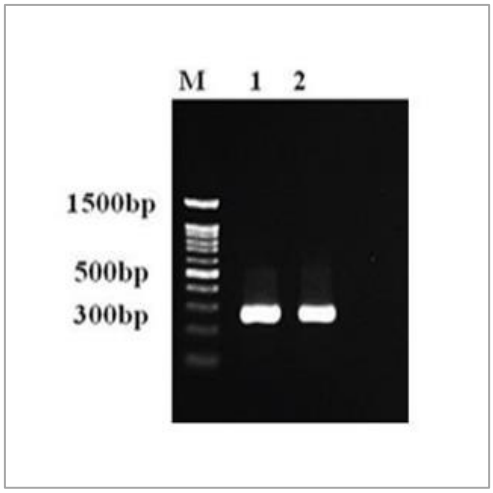



**Figure 5.** A 0.5% (w/v) agarose gel electrophoresis presents the insert size of 300bp band of REJ PCR product lane 2. Lane 3 presents REJ gene-positive control sample. Lane M is the DNA ladder (100bp).

The PCR result appears to be migrating in the same position as the 300 pb DNA marker in **Figure 5**, precisely measuring 300 bp. The REJ gene encodes a 13 kDa protein, which is what is shown in **Figure 5**. The inserted gene is next ligated into the pET21a (+) expression vector using that REJ fragment as the target gene.

*Identification of Positive Clones*

The recombinant plasmid was confirmed with gene sequencing by a colony PCR, which estimated the recombinant REJ with 500bp, which was the correct size for the desired colony **(Figure 6)**. Subcloning was performed to obtain REJ genes in suitable quantities. In this study, we used the pET21a (+) expression vector because it has the T7 terminator, and T7 promoter primers the region between these primers contains the recombinant plasmid according to colony PCR and the calculation of Snap Gene Viewer software that region presents 500bp. **Figure 6** shows evidence of a successful ligation into the pET21a (+) expression vector [21].

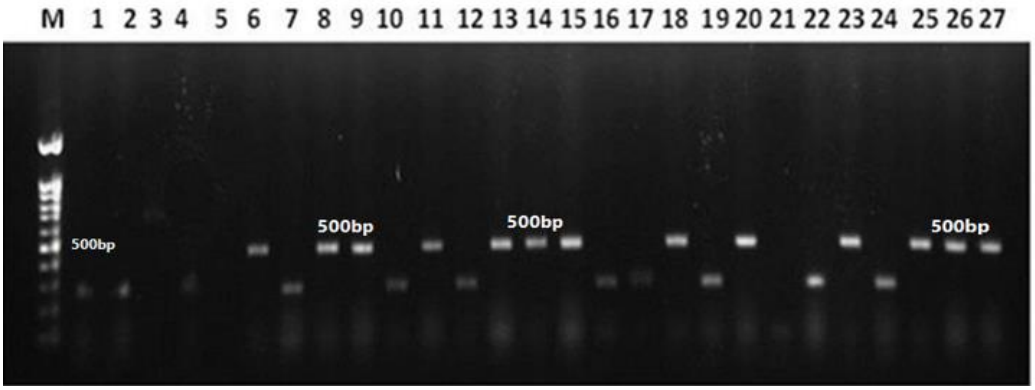
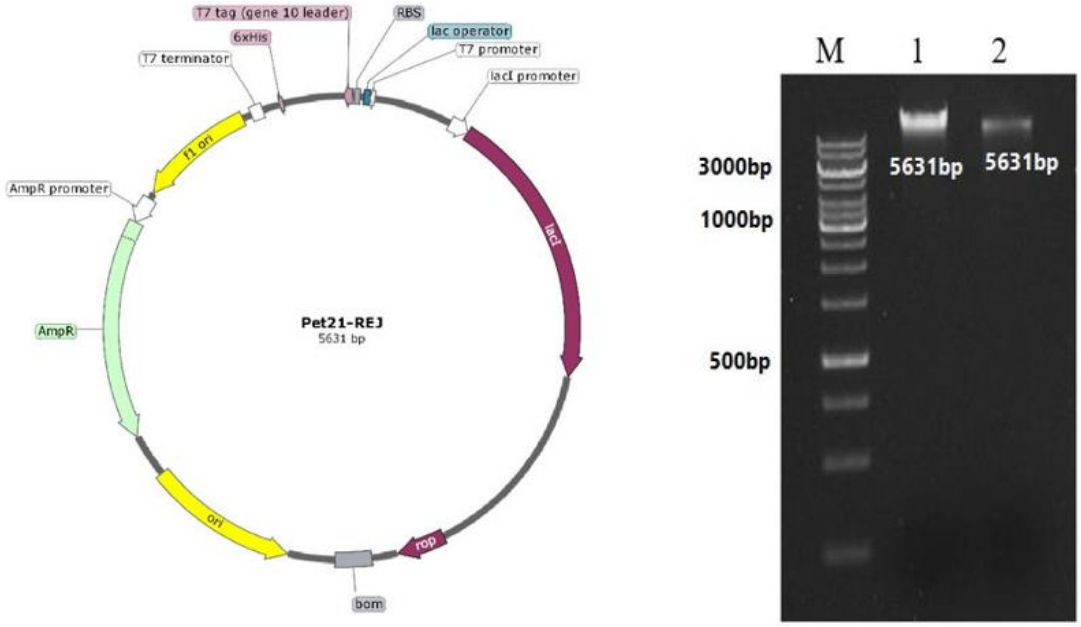



**Figure 6.** A 0.5% (w/v) agarose gel electrophoresis of the colony PCR. The desired bands with a 500 bp can be seen in lanes 6,8,9,11,13,14.15,18,20,23,25,26, and 27. The rest of the lanes (300bp) represented a weak positive band and thus did not consider for further analysis. The DNA ladder was 100bp.

The colonies that contained positive recombinant REJ were selected for plasmid extraction and purification by miniprep; then, the REJ recombinant plasmid was transformed to a BL21 *E. coli* to be expressed. As a result, the REJ recombinant plasmid was successfully cloned, cloned the REJ gene into pUC57, and subcloned into the pET-21a (+) expression vector. Furthermore, the REJ nucleotide sequence of the cloned fragment displayed 100% identity with the REJ nucleotide sequence in GenBank: AAC37576.1.

*Plasmid DNA Purification and Extraction*

The subcloning of pET21a (+)-REJ was successful as seen in **Figure 7**. The agarose gel electrophoresis showed two bands at 5631bp of pET21a (+)-REJ. This plasmid was further used in the expression of REJ fusion protein. These results were the same as the Snap Gene Viewer reading. As mentioned before the length of pET-21a (+) was 5369bp.




a) b)

**Figure 7.** The schematic presentation of pET21a (+)-REJ vector. Lane M is the DNA ladder (1kb). Lane 1, 2 is pET21a (+)-REJ vector at (5631 bp). a) Snap Gene Viewer software and b) is a 0.5% (w/v) of agarose gel electrophoresis lane M was the DNA ladder, and lanes 1,2 were the pET21a (+)-REJ.

*REJ-His Fusion Protein Expression*

The results of the valuation of the protein expression in the cell lysate (soluble and insoluble) fractions revealed that a considerable amount of REJ-His fusion protein was distributed into the insoluble fraction, as indicated in the SDS-PAGE in **Figure 8**.

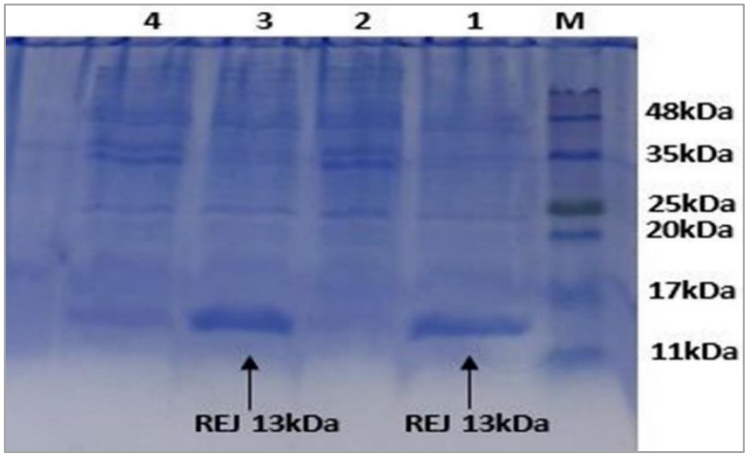



**Figure 8.** A 20% (w/v) SDS-PAGE of the expressed REJ proteins. Lanes 1 and 3 present the insoluble REJ protein. Lane 2 shows the noninduced fraction. Lane 4 shows the native protein fraction and M presents the protein marker.

This study found that the optimized condition for REJ-His fusion protein expression was at 25 °C for 6 hours with 1 mM IPTG. His REJ protein was expressed in the insoluble fraction in the pellet. This is consistent with the study of Hasegawa *et al*., (2002) which showed that proteins with a molecular weight less than 30kDa require the fusion with solubility enhancers to be expressed in a soluble fraction [22]. Our study is the first to explore REJ proteins with previously unknown functions and mechanisms, and the use of synthetic nucleotides to produce REJ-His fusion protein is novel.

Many REJ protein functions are unidentified and, in some cases, debated. To understand REJ's function and structure, researchers have produced enough pure protein for detailed biophysical and biochemical studies [23]. A straightforward process to produce a recombinant protein with a high yield would be appreciated. Therefore, we attempted to simplify the human REJ and expression process by cloning the gene in the pET-21 system. Though, because the structure of many human proteins is comparatively complicated, the expression of recombinant forms might be enhanced upon the use of eukaryotic hosts. Our study was parallel to Gholami *et al*., 2018 [24].

Based on the proteins database, the localization of REJ in different exons separated by many introns led us to clone the REJ gene via the phosphoramidite synthetic method. Therefore, we designed REJ nucleotide sequences and sent them to Synbio, US, to create a product that was identical to the product that was theoretically proposed [25]. Similarly, Schröder *et al.*, 2011, studied the segments of small molecular weight of an expressed REJ protein. They found that the REJ domain contains a considerable number of β-sheets, which E. coli could express, and is possibly similar to fibronectin type III; therefore, the REJ module is not a single domain [26].

Using BL21-DE3 competent cells to transform the recombinant REJ guarantees the efficiency of more than 60% of the other competent cells [27, 28]. However, the REJ domain has 1000 amino acids, which indicates 75kDa of translated protein, meaning this significant molecular weight cannot be produced by BL21-DE3 competent cells. Thus, researchers have expressed proteins in insects as these proteins have a greater molecular weight than the proteins expressed by bacteria, they can also go through post-translational modifications such as glycosylation, and phosphorylation is avoided. In addition, the expression in insects facilitates protein folding [29, 30].

## Conclusion

In conclusion, we successfully cloned and expressed the human REJ synthesized gene. The production procedure was easy to implement and allowed us to produce large quantities of recombinant protein with no need for special equipment or media. Furthermore, the REJ DNA synthesis saved time and material consumption and allowed us to avoid the occurrence of a post-transcription modification. With the intent of enhancing the expression conditions to produce soluble protein, this is novel research that remarkably contributes to the understanding of the molecular mechanisms of inherited ADPKD diseases. Protein solubility is necessary for protein expression, function, and folding; therefore, in future works, our team intends to use His 6-Tag and a maltose-binding-protein double-affinity fusion system to produce more soluble REJ proteins. Our results demonstrated a powerful experimental approach to further studying the solubility, function, and REJ- ECM interactions of proteins and should pave the way to systematically characterizing the effects of disease-causing mutations in the REJ module of human PC1.

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**Conflict of interest:** None

**Financial support:** None

**Ethics statement:** None

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