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CLONING OPTIMIZATION OF USP47 IN *E. COLI*

Blnd Ibrahim Mohammed*, Abdulkarim Yasin Karim and Bushra Karim Amin

Department of Biology, College of Science, Salahaddin University-Erbil, Erbil, Iraq.

Abstract

Ubiquitin is an important protein, which has a great role in protein turnover in eukaryotic cells, However, ubiquitin can be removed from target protein by the action of deubiquitylating enzymes (DUBs), thus delaying the protein degradation, thus, ubiquitin and deubiquitylating enzymes plays a key role in the regulation cell proteins in many cases including cancer, muscle wasting, and inûammation. The present study aimed to determine amplicon size, optimized condition for the amplification of target USP47 gene and cloning of USP47 in prokaryotic cells like *E. coli*. By using pGEX-6P-1 vector. Result of present study concluded that best annealing temperature for USP47 amplification by conventional PCR was (55°C, 1 minute), amplicon size was 942bp and USP47 gene inserted in pGEX-6P-1 by using two restriction enzymes which were (BamHI and XhoI).

Key words : Polymerase Chain Reaction, optimization, *E. coli*, ubiquitin.

Introduction

Many protein-encoding genes present in eukaryotic cells and these proteins are in dynamic state in which synthesis and degrade continuously (Bradshaw, 1989). Ubiquitin, a highly conserved 76-residue protein which present in eukaryotic cells either free or covalently joined to a great variety of proteins, Ubiquitin attachment regulate the degradation of proteins via the proteasome and lysosome (Mukhopadhyay and Riezman, 2007). Initially, single ubiquitin conjugate to target protein and change three dimensional structure of target protein which stimulate other ubiquitin subunit to conjugate and once Polyubiquitylated proteins formed degradation take place by 26S proteasome (Liu *et al*., 2016). However, ubiquitin can be removed from target protein by the action of deubiquitylating enzymes (DUBs), thus delaying the protein degradation, thus, ubiquitin and deubiquitylating enzymes plays a key role in the regulation cell proteins in many cases including cancer, muscle wasting, and inûammation (Harrigan *et al*., 2018). The human genome encodes approximately 95 putative DUBs which belong to the superfamily of proteases, uniquitin specific protease consider as the most characterized from of DUBs family (Clague *et al*., 2012).

Author for correspondence

Uniquitin specific protease 47 also named Ubiquitin-specific-processing protease 47 or Ubiquitin thioesterase 47, in human this protein encoded by USP47 gene which is located on chromosome 11p15.3 and this enzyme plays a direct role in regulating cell cycle, and its overexpression has been reported to be involved in tumor progression (Weinstock *et al*., 2012 and Yu *et al*., 2019).

The present study aimed to determine amplicon size, optimized condition for the amplification of target USP47 gene and cloning of USP47 in prokaryotic cells like *E. coli* by using pGEX-6P-1 vector.

Materials and Methods

The short sequences of USP47 gene was obtained from the National Centre for Biotechnology Information (NCBI) database and one set of specific primer has been designed by SnapGene software with BamHI/XhoI overhangs as shown in table 1. Target gene has been amplified by PCR and recombinant plasmid constructed from target gene with special vector pGEX-6P-1 by using of restriction enzymes (BamHI/XhoI) with ligation kit, constructed recombinant plasmid transferred to chemically prepared competent cells *E. coli* BL21(DE3), finally antibiotic resistance done for the selection of bacterial cell with constructed plasmid (USP47-pGEX-6P-1).

Table 1 : Primers used for PCR amplification of USP47 gene, designed by SnapGene software.

Primer name	Sequence	Amplicon length
Forward-USP47	GAGAACCAACTGGTCCCGAA	942bp
Reverse-USP47	TGCTTGGCTGGACCCATAAG	942bp

Polymerase Chain Reaction

PCR was performed with GoTaq Green PCR master mix (Promega) according to the manufacturer's instructions. The 50 µl reaction mixtures were composed of 25 µl master mix, 1 µl (100 ng) DNA templates, 1 µl for each forward and reverse specific primer (10 pmol), and the volume was completed by adding 22 µl of nuclease free water.

Agarose Gel Electrophoresis

Amplicons were separated using agarose gel electrophoresis in order to confirm the size, location and quality of the PCR specific product for specific primers. The electrophoresis tank was prepared using a standard method (De Gregoris *et al*., 2011).

Recombinant plasmid construction

PCR product (USP47) and vector (pGEX-6P-1) individually digested with the appropriate restriction endonucleases, reactions were set up each in a total volume of 20 which consisted of 5µl PCR product or vector, 1µl BamHI and 1µl XhoI, 2µl tango buffer and the volume was completed using ddH_2O, then incubated at 37°C for 16 hours, finally enzymes inactivated in 65ºC for 10 min and the mixture used for ligation. Additionally, ligation of target gene and vector was carried out in the smallest volume possible, typically 10 µL containing 2 µL vector, 1 µL insert (at a 6:1 insert: vector molar ratio), 1 µL T4 DNA ligase, 2 µL 10X reaction buffer, and 4 µL ddH2O, reaction was allowed to incubate at room temperature (approximately 21°C) for 4h.

Transformation and selection of bacterial cells

Vectors were transformed into competent *E. coli* DH5á using a heat shock approach according to the previous study (Froger and Hall, 2007). For the purpose of bacterial selection with recombinant plasmid antibiotic resistance has been used, 100µl of the cell suspension were plated onto LB agar supplemented with 100 µg/µl ampicillin, incubated at 37°C overnight.

Results and Discussion

PCR optimization

Gradient PCR has been used for selection of PCR condition according annealing temperature, result showed that following condition was the best for the amplification of target gene (USP47), PCR cycling profile for USP47 amplification was composed of an initial 4-minute denaturation step at 95°C followed by 35 cycles of denaturation (94°C, 1 minute), annealing (55°C, 1 minute) and extension (70°C, 1 minutes) and a final extension at 70°C for 8 minutes. Amplicons were separated by agarose gel electrophoresis (l%) as shown in fig. 1.

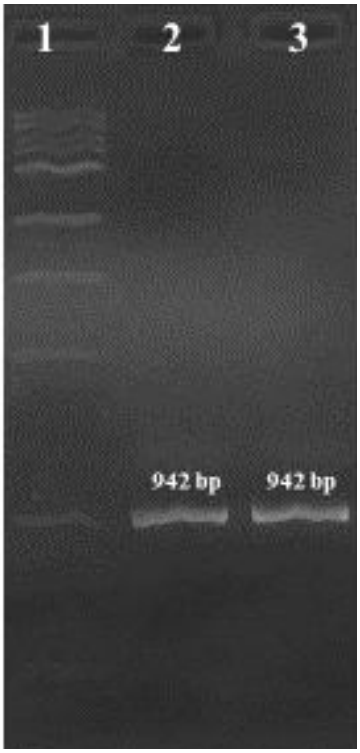

Fig. 1 : Amplification of USP47 gene which examined through Agarose gel electrophoresis (1%) under UV light. Lane 1: Ladder, Lane2 and 3: positive sample.

Constructed Plasmid (USP47-pGEX-6P-1)

Restriction enzymes (BamHI and XhoI) and ligation kit have been used for the construction of recombinant plasmid (USP47-pGEX-6P-1) as mentioned in methodology section, after increasing recombinant plasmid checked by using Agarose gel electrophoresis, result showed that recombinant plasmid was about 5760 bp, as shown in fig. 2. Many studies used pGEX-6P-1 vector for the cloning and synthesis of many recombinant human proteins (Huang *et al*., 2004 and Liu *et al*., 2014).

Antibiotic Resistance test

E. coli BL21(DE3) selected depending antibiotic resistance, 100µl of the cell suspension were plated onto LB agar supplemented with 100 µg/ µl ampicillin, incubated at 37°C overnight.

Fig. 2 : USP47-pGEX-6P-1 examined through Agarose gel electrophoresis (1%) under UV light. Lane 1: Ladder, Lane 2: recombinant USP47-pGEX-6P-1, which was 4860bp.

The advantages of *E. coli* include its relatively rapid growth, its utilization of inexpensive cultural techniques, its ease of transformation and its ease of maintenance. *E. coli* BL21 (DE3) contain T7 lysozyme gene in its genomic DNA and T7 lysozyme increases the tolerance of *E. coli* cells against the toxicity of the protein, additionally, this bacterium lacks both Ion protease and the outer membrane protease ompT which degrade expressed protein, thus, present study chosen *E. coli* BL21 (DE3) as an expression host. Previous studies reported that *E. coli* successfully utilized to produce many functional human proteins such as human growth hormone, pro-insulin, interferon-gamma and antibody fragments (Santala and Lamminmaki, 2004).

Conclusion

Present study concluded that best annealing temperature for USP47 amplification by PCR was (55°C, 1 minute), amplicon size was 942bp and this gene inserted in pGEX-6P-1 by using two restriction enzymes which were (BamHI and XhoI).

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