# cDNA identification, cloning and expression of the carp Ubc9 gene

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## Abstract

**The Ubc9 gene encodes the only ubiquitin-like binding enzyme in the SUMO process, which plays an important role in the ubiquitination modification process. In order to explore the sequence and expression of carp Ubc9 gene, a cDNA fragment of EP28bc9 with a length of 1228 bp was amplified by PCR. The fragment sequence consisted of a 12 bp 5' untranslated region (5'UTR) and a 715 bp 3' untranslated region (5 'UTR) and a 474 bp coding sequence encoding 158 amino acids in length. The prokaryotic expression vector (pET22b-Ubc9) of EPCUbc9 gene was constructed and transformed into rosetta, which was induced by IPTG. The expression of fusion protein was identified by SDS-PAGE. The target protein was purified by nickel column. The expression of protein was detected by Western blot. The results showed that the expression was successful. The Ubc9 protein was purified and laid the foundation for the subsequent functional study of Ubc9.**

## Keywords

**Carp Ubc9, clone expression, EPC cells, protein purification.**

## 1. Introduction

The Ubc9 gene encodes the only E2-binding enzyme in the Small Ubiquitin-like Modifiers (SUMO) process, which plays an important role in SUMOylation. It binds directly to the SUMO molecule and binds it to the substrate protein, which is SUMOylation. The key node in the modification. The amount of Ubc9 expressed in the cells affects the change in the overall SUMOylation level of the cells. In addition, Ubc9 is closely related to the occurrence and development of tumors [1]. Ubc9 gene is overexpressed in many malignant tumors such as breast cancer and ovarian cancer. Altering the expression of Ubc9 gene in cancer cells can lead to cell proliferation and cell cycle changes. Studies have shown that Ubc9-mediated SUMOylation is closely related to cell and viral replication [2-7]. Chan-I Sua et al [8] found that interference with Ubc9 expression in 293T cells attenuated RNA replication by dengue virus. Lee, H. S. et al. [9] found that Ubc9 silencing impaired HCV replication in JC1-infected cells. Zhan Wenbin et al [10] found that RNA interference Ubc9 can inhibit white spot Syndrome Virus (WSSV) copy number and viral gene expression, and reduce WSSV shrimp mortality. Yu Fei et al [11] found that overexpression of Ubc9 leads to an increase in the replication efficiency of Reovirus. These all suggest that Ubc9 plays an important regulatory role in the development of viral infection replication. However, the expression of Ubc9 gene and the mechanism of viral replication during the infection of iridescent virus are still unclear. Moreover, the carp Ubc9 gene has not been cloned for expression and functional studies. Therefore, the prokaryotic expression of Ubc9 cDNA clone was established in this paper, which laid a foundation for further study of the function of EPCUbc9.

## 2. Materials and Methods

### 2.1 Materials

The squid epithelial cell line, Escherichia coli DH5α strain was preserved in the laboratory and the pET22b vector was preserved in the laboratory; the restriction enzymes Hind III, EcoRI and reverse

transcription kit were purchased from TaKaRa; agarose gel recovery reagent The kit and the small plasmid kit were purchased from OMEGA; the protein Marker was purchased from Tiangen Biochemical Technology Co., Ltd.; the highly efficient eukaryotic transfection reagent was purchased from Beijing Kangrun Chengye Biotechnology Co., Ltd.; the protein RP lysate was purchased from KGI; rabbit source HA primary antibody was purchased from Sigma; sheep anti-rabbit source IgG and ECL chemiluminescence were purchased from Thermo Fisher.

### 2.2 Primer Information

Table 1 Cloning and expression primer sequence of Ubc9

| Primer name | Primer sequence |
|---|---|
| All-Ubc9-F | CGTCGTACCGCGGGTCCTGATTAGA |
| All-Ubc9-R | CGCATTTTGTAGAAAGAGCTTTATT |
| pro-Ubc9-F | GGAATTCCATATGATGTCTGGCATTGCTCTGAG |
| pro-Ubc9-R | CGGAATTCAACGACGGGGAGAATTTTTTG |

### 2.3 Identification of Ubc9 gene and construction and acquisition of recombinant vector

The Ubc9 gene sequence of grass carp (KU760729.1) was searched in NCBI, and the primers were designed with Primer Premier 5 software using this sequence as a template. Primer synthesis and sequencing were performed by Huada Company. The EPC total RNA was extracted by Trizol method, reverse-transcribed into cDNA by reverse transcription kit, and the cDNA was used as a template for PCR to carry out PCR reaction, and the EPC Ubc9 ORF fragment was amplified. PCR reaction loading system: 2 μL cDNA template, 1.8 μL of upstream and downstream primers (10 μmol•L-1), 20 μL of 2× PCR Master Mix, and sterile deionized water to a total volume of 40 μL. The PCR reaction conditions were set: pre-denaturation at 94 °C for 3 min; denaturation at 94 °C for 30 s, annealing at 54.5 °C for 30 s, extension at 72 °C for 29 s, 34 cycles, and extension at 72 °C for 10 min. The PCR product was detected by 1.2% agarose gel electrophoresis, and the gel was recovered and sequenced. Then, the pET22b plasmid and Ubc9 were digested with NdeI and EcoRI, respectively, and the digested product was recovered by PCR purification kit, and the purified plasmid and fragment were ligated with T4 DNA ligase to obtain a prokaryotic expression recombinant plasmid of pET22b-Ubc9-His.

### 2.4 Prokaryotic expression of recombinant plasmid pET22b-Ubc9-His

The correctly sequenced recombinant plasmid was transformed into Rosetta competent state and plated on LB solid medium of ampicillin and chloramphenicol to finally obtain monoclonal pET22b-Ubc9-His. This monoclonal was transferred to 4 ml of LB liquid medium containing ampicillin and chloramphenicol at 37 ° C, 225 rpm. Induction: When the OD value reached 0.6, the inducer IPTG (0.5 μmol•L-1) was added and the culture was continued. The cells were cultured at 20 ° C for 16 h to collect the cells: centrifugation, the supernatant was discarded, and the cells were collected. Expression detection: buffer A suspension was added to the collected cells, dissolved thoroughly by sonication, centrifuged, and the precipitate after centrifugation was dissolved using buffer B. Separate supernatant and sedimentation, prepare samples, prepare SDS. -PAGE detection.

### 2.5 Ubc9 protein purification, detection and validation

Equilibration: Take 5 ml Ni-IDA and wash the equilibration column with a 10 column bed of Binding buffer at a flow rate of 5 ml/min. Incubation: The crude protein was incubated with the equilibrated column packing for 1 h. Upper column: The product after incubation is applied to the column and collected and discharged. Washing: Wash the column with Washing buffer and collect it. Elution: Elution with Elution buffer, collection and efflux. Purification test: separate processing of crude protein, washing and effluent, elution and effluent, sample preparation, and preparation for SDS-PAGE. Verification of the target protein SDS-PAGE detection: processing the purified protein, preparing the sample, running the gel, and detecting the molecular weight.Western Blot verification: The purified protein was processed and sampled. After 12% SDS-PAGE, the gel was transferred to a PVDF membrane by a semi-dry film transfer machine at 28 V for 31 min, and 5% degreased. The

milk was blocked at 4 ℃ for 2 h; rabbit anti-his tag antibody (1:2000 dilution) was added, and incubated at 4 °C for 14 h; washed 3 times with TBST, and added with HPR-labeled goat anti-rabbit secondary antibody (1:3000 dilution) ), incubated at 4 ° C for 2 h; washed 4 times with TBST, ECL developed color.

## 3. Results

### 3.1 Identification results of the carp Ubc9 cDNA sequence

The Ubc9 gene sequence of grass carp (KU760729.1) was searched in NCBI, and the amplification primers were designed using this sequence as a template. The total RNA of EPC cells was obtained, and the Ubc9 gene cDNA fragment was amplified by PCR using this RNA as a template, and the PCR product was purified and sequenced. Primer synthesis and sequencing were performed by Huada Company. The sequence is as follows (Fig. 1):

GAACCGATGTGTTTTCGGGAGGGAAACTTTCACAGCTTTGGTACCGAGTCAAACACGGTTCCAGTGAGCGGGCCCAGAAG

ACGAATAACACTGGGATTCACAAGGATCAGTGGTC

ATGTCTGGCATTGCTCTGAGTCGACTTGCACAAGAGCGCAAAGCATGGAGGAAAGACCATCCATTCGGCTTTGTTGCTGTG

M S G I A L S R L A Q E R K A W R K D H P F G F V A V

CCGACGAAAAACCCCGATGGCACCATGAATCTCATGAACTGGGAATGTGCTATTCCAGGAAAGAAGGGGACTCCATGGGAA

P T K N P D G T M N L M N W E C A I P G K K G T P W E

GGAGGGCTGTTTAAGTTAAGGATGCTGTTTAAAGATGACTACCCCTCATCACCCCCAAAGTGTAAATTTGAGCCTCCGCTCTT

G G L F K L R M L F K D D Y P S S P P K C K F E P P L

CCACCCGAATGTGTATCCGTCAGGAACAGTGTGTCTTTCCATCCTGGAGGAGGATAAAGACTGGAGACCTGCCATCACCATT

F H P N V Y P S G T V C L S I L E E D K D W R P A I T

AAACAGATCCTCTTAGGGATTCAGGAGCTCCTAAATGAACCGAACATACAGGATCCAGCTCAGGCAGAGGCGTACACCATA

I K Q I L L G I Q E L L N E P N I Q D P A Q A E A Y T

TACTGTCAGAATAGAGTGGAGTACGAGAAGAGAGTTCGTGCACAGGCCAAAAAATTCTCCCCGTCGTAA

I Y C Q N R V E Y E K R V R A Q A K K F S P S *

GCGAGACCGAGGCGAATGGAAAGAGGGATTATGTGAATACTCGTGCTATCCTCTCACCGGGACTAAAAGGACTTAAAATATG

ATGTCTGTGCCATCAACCCCAAACCCTTCCCCTGCACCACAAAATTTACACTCTTCTTCCACCCTTTTAATTTCATTTGCTCTC

CGTTTCCCTTAGAACGCAGTCGCCAATTCAGTATCCACAGGTTTTGTTGCGTTTCAAACCAGCTTGGATCAGAATGGACTGTT

GGGATTTTAGCTCCTGGTGTCTGTCTGTGTGCTTACTTGCACTCGCCATTTTATTATGTTCGTGAAGCTTTGTTTCCTCTTGAA

AAACATGCGAGTGTGTGTGTGTTGCTCTGGTTTAGGTTGGTTTATAGCGTCTTATATGTGGGAAAGTGCCTCATGGTGACGTT

TCCCCTCAGAATGCTGATCTAGGACCAGTTTCCTCTCCATTGGGCTCGTTTGTACTTGACCAGCCACGTGGTCTTAGATCTGT

CTTGGATTGTGTAATGCTGATTCGTTAAATAATGTCCAGAGTTATTGCTCCTGTGAGCAGCAGAACCAGAGTCCAATTCTCTT

AAACATGCTGTACATGGTTTCTTGTTTGTTTGATGGGAACATGTGTCCTGCGATTGCT

Fig. 1 Nucleotide and deduced amino acid sequences of EPC Ubc9

### 3.2 Construction of prokaryotic expression vector

According to the method 2.3, the EPC Ubc9 cDNA was isolated, and the cDNA was cloned into the prokaryotic expression vector pET22b to construct the prokaryotic expression vector pET22b-Ubc9. The plasmid was identified: the prokaryotic expression plasmid pET-22b-Ubc9-His was digested with XbaI and EcoRI, and a 480 bp target fragment was observed, which was as expected (Fig. 2). The results of plasmid sequencing were compared with the original sequence, and the DNA bases were completely identical, indicating that the recombinant plasmid was successfully constructed.

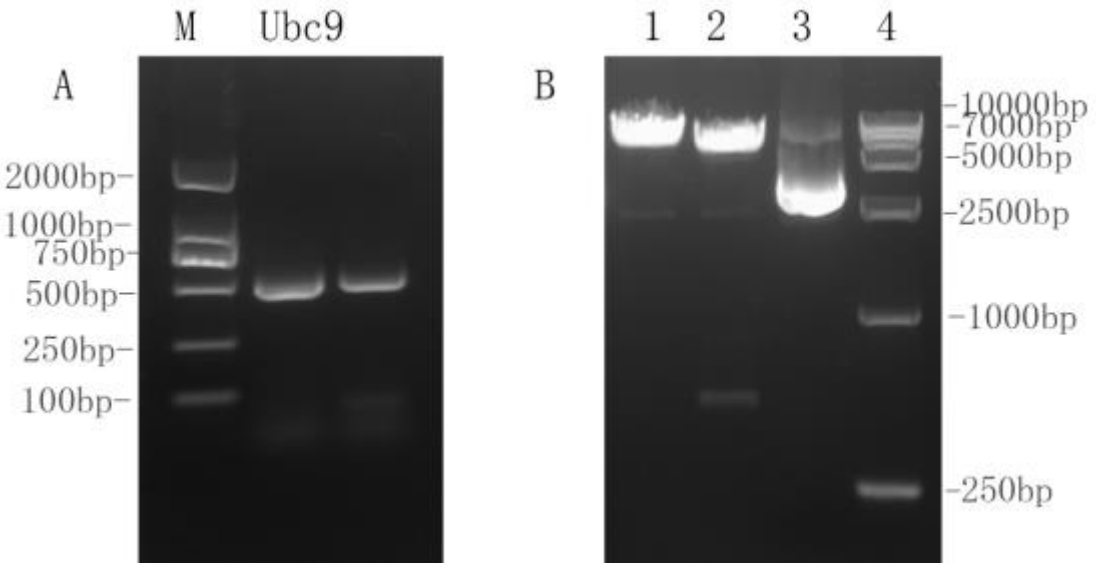


Fig.2 Identification of pET22b-Ubc9-His Recombinant Plasmid
A: pET22b-Ubc9-His transformant colony PCR identification; B: pET22b-Ubc9-His recombinant plasmid (1) Xba I single digestion, (2) XbaI and EcoRI double digestion, (3) uncut, ( 4) DNA molecular weight marker.

### 3.3 Purification of Ubc9 protein

The constructed pET22b-Ubc9 recombinant plasmid was transformed into E. coli Rosetta expressing bacteria for Ubc9 protein expression experiments. The expressed product was identified by 12% SDS-PAGE. Analysis showed that the His-tagged recombinant Ubc9 protein was correctly expressed in the precipitated sample with a size of 19 kDa, which was consistent with the expected results (Fig. 3).

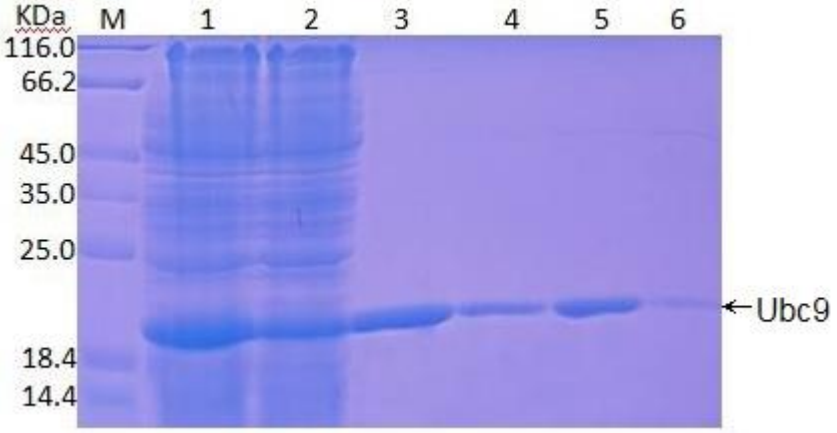


Figure 3 Nickel Sepharose affinity chromatography purification SDS-PAGE analysis
M: Protein marker; 1: loading; 2: efflux; 3-4: 20 mM Imidazole elution fraction; 5: 50 mM Imidazole elution fraction; 6: 500 mM Imidazole elution fraction

### 3.4 Detection and identification of Ubc9 protein

The obtained protein was mixed with the corresponding loading buffer to prepare a Western Blot sample. Western Blot was used to detect Ubc9 protein expression. Analysis showed that the His-tagged recombinant Ubc9 protein was correctly expressed in the precipitated sample with a size of 19 kDa (Fig. 4), which was consistent with the expected results. Moreover, Western Blot results showed that the expressed recombinant Ubc9 protein specifically binds to the Anti-His tag antibody (Fig. 5). This indicates that the experiment successfully purified and identified Ubc9 protein.

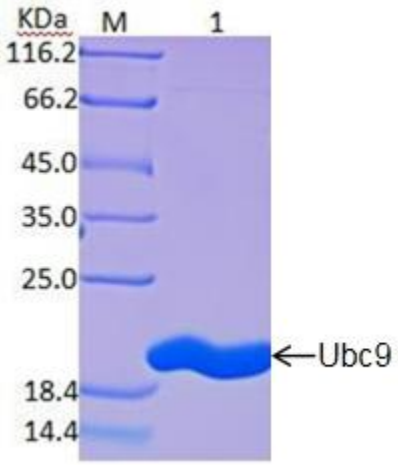


Figure 4 Protein final purification SDS-PAGE analysis
M: Protein marker; 1: Target protein

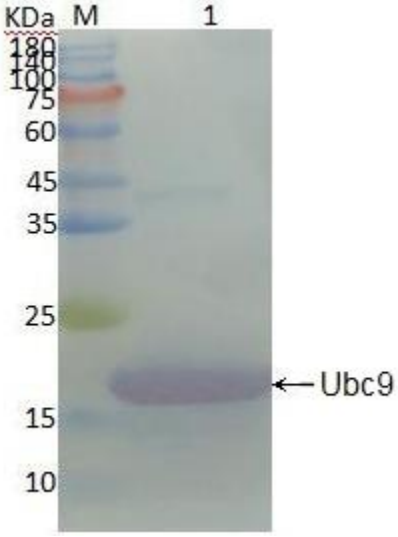


Figure 5 Protein final purification Western Blot analysis
M: Protein marker; 1: Target protein

## 4. Conclusion

In order to study the biological sequence and structural information of the Ubc9 gene, a cDNA fragment of the Ubc9 gene of the squid epithelial cell line was obtained by PCR amplification. The EPC Ubc9 gene sequence was obtained by sequencing. The BLASTP homology search results showed that it has high similarity with the SUMO-binding enzyme-Ubc9 of other species in the NCBI protein database. Zhou Peng et al also found this phenomenon in the cloning study of the large yellow croaker Ubc9 gene. The largest open reading frame of EPC Ubc9 cDNA encodes a protein of 158 amino acids in length, which is consistent with the results of Zhou Peng et al. on the Ubc9 gene of large yellow croaker, and sequence analysis of the ubiquitinated E2 binding enzyme Ubc9 of grass carp. It contains 159 amino acids differing by only one amino acid. The biological information predicts that the EPC Ubc9 protein domain contains both a characteristic conserved domain with a ubiquitinated ligase family (amino acids 7-146) and SUMO ligase (amino acids 1-156). It is suggested that ubiquitinated ligase and SUMO ligase may have the same competitive substrate. The Residue annotation of the protein residue was predicted to show five E3 interaction residues (7S, 69P, 70S, 105P, 106A) and one cysteine active site (93C). The multi-sequence alignment revealed that the five interaction sites and cysteine active sites of Ubc9 and E3 ligase in three animals (grass, ginseng, zebrafish) were completely conserved. The role of the interaction site is to link the SUMO molecule to the lysine residue of the substrate protein through Ubc9 and E3 to complete the SUMOylation

modification of the specific protein. The high degree of conservation of this key interaction site in Ubc9 also further suggests that the protein performs similar or identical biological functions in these three classes of animals. Therefore, it is speculated that the cysteine active site (93C) of EPC Ubc9 may be linked to the SUMO molecule. The prokaryotic expression vector of EPC Ubc9 gene was constructed and the expression of Ubc9 protein was successfully expressed by selecting 0.5 mM IPTG and expressing at 16 °C for 16 h. Prokaryotic expressed Ubc9 protein can provide materials for antibody preparation.

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