



# Optimized Expression and Purification of Humbug in *Pichia pastoris* and Its Monoclonal Antibody Preparation

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## Abstract

**Background:** The humbug gene is a truncated isoform of Aspartyl  $\beta$ -hydroxylase (ASPH) gene that is overexpressed in many human malignancies. In recent years, since humbug has received increasing attention, it is considered as a potential therapeutic molecular target. Therefore, it is necessary for preparing humbug protein and its monoclonal antibody to investigate its structure and function.

**Method:** The optimized humbug gene, synthesized by Genscript in Nanjing, China on December 21st 2013, was expressed in *Pichia pastoris* cells that were cultured in a 10-L bioreactor. The recombinant protein was further obtained and purified by using ion exchange chromatography and Sephadex G75. The humbug protein was used to immunize Balb/c mice to generate the monoclonal antibodies. The specificity and sensitivity of the monoclonal antibodies were assessed by indirect enzyme-linked immunosorbent assay. Finally, the humbug monoclonal antibodies were used to detect the expression of humbug in several tumor cell lines via indirect immunofluorescence.

**Results:** Firstly, the recombinant humbug was expressed in *P. pastoris* successfully and efficiently by using a gene-optimized strategy. Secondly, the purification process of humbug was established via multiple chromatography methods. In addition, four monoclonal antibodies against humbug were obtained from the immunized Balb/c mice, and the result of indirect immunofluorescence was indicated that the humbug monoclonal antibody showed the high affinity with humbug protein, which expressed in several tumor cell lines.

**Conclusion:** The over-expression of recombinant humbug provides adequate sources for its structural study and the preparation of the humbug-specific monoclonal antibody can potentially be used in tumor initial diagnosis and immunotherapy.

**Keywords:** Humbug, Fermentation, *Pichia pastoris*, Monoclonal antibody, Tumor diagnosis

## Introduction

Humbug is a novel  $\text{Ca}^{2+}$ -binding protein of the endoplasmic reticulum (ER) membrane (1). It has drawn a great deal of attention over the past several years. As a type II membrane protein, humbug belongs to the aspartyl  $\beta$ -hydroxylase (ASPH) protein family (2). By alternative splicing, the ASPH gene can be translated into four functional proteins: aspartyl  $\beta$ -hydroxylase, the structural protein of sarcoplasmic reticulum junctin, humbug (ASPH-type junctate) and junctin-type junctate (3).

Humbug, a truncated aspartyl  $\beta$ -hydroxylase construct missing the catalytic domain, is shown to be a  $\text{Ca}^{2+}$ -binding protein at ER membranes (4) and to be overexpressed in a variety of malignant neoplasms (5-9). Furthermore, the aspartyl  $\beta$ -hydroxylase/humbug gene is frequently overexpressed in a variety of carcinomas from Chinese patients (10-12). Despite of these discoveries, there is still lack of fully understanding of humbug gene. For example, few studies have been performed on its structure and function. Previous research mainly

focuses on the distribution, and overexpression of humbug in tumor tissues and cells (13-15). It is essential to establish an effective recombinant expression system for the further study to examine the structure and function of human membrane proteins such as humbug, which is medically significant but not easily accessible.

Humbug has been successfully expressed in *Escherichia coli* (16) with biological activity. However, it is necessary to obtain ample amounts of functional humbug by using a eukaryotic expression system in order to explore further its structure and function. In the past decades, due to high cell densities, strong AOX promoters and effective post-translational modifications, the *P. pastoris* expression system has been widely used (17). Furthermore, the system can stably integrate the exogenous gene into expression vector at specific sites and secrete exogenous protein into culture medium, which simplifies the subsequent purification (18).

With the unlimited quantities and the permitted standardization of reagent and technique, monoclonal antibodies generated in vitro are commonly used in the biological and medical science. In addition, monoclonal antibodies have high specificity and homogeneity, which shows greater values, especially in the diagnosis and the therapy of human diseases.

In this study, a method of the expression and purification of large quantities of humbug was established by using a gene-optimized strategy in *P. pastoris* expression system. Moreover, the purified humbug can be used as an immunogen to produce monoclonal antibodies (mAbs), which are potential tools in tumor diagnosis. The efficient expression of r-humbug will provide adequate sources to study its structure and the humbug-specific monoclonal antibody can potentially be used in tumor initial diagnosis and immunotherapy for human health.

## Materials and Methods

### *Strains, vectors, reagents, and cell lines*

As a cloning host *E. coli* strain, Top10 was purchased from Invitrogen (Carlsbad, USA) on December 10th, 2014. This strain was used for DNA

subcloning and the propagation of the expression plasmids. The *Pichia* expression kit, containing the *P. pastoris* strain GS115 and the pPIC9k vector was also purchased from Invitrogen. The Quick-Change site-directed mutagenesis kit was obtained from Stratagene Inc. (La Jolla, CA, USA). Oligonucleotide primers were synthesized by Genscript (Nanjing, China). Restriction endonucleases and T4 DNA ligase were purchased from Takara Bio Inc. (Dalian, China).

According to the instructions of *Pichia* expression kit manufacturer, Luria-Bertani (LB) medium, minimal dextrose (MD) medium, buffered complex glycerol (BMGY) medium, yeast extract peptone dextrose (YPD) medium, buffered complex methanol (BMMY) medium, and fermentation basal salts (BSM) medium supplemented with trace elements solution PTM1 were all prepared. The origins of other chemical were indicated in the study.

The tumor cell lines of human renal adenocarcinoma (ACHN), bladder cancer (BIU-87), breast carcinoma (MCF-7), hepatic carcinoma (SMMC-7721), laryngeal cancer (Hep-2), cervix cancer (HeLa), and ovary cancer (SKOV) were purchased from the Wuhan Cell Institute of Chinese Academy of Sciences (Wuhan, China). They were maintained in DMEM or RPMI-1640 cell culture mediums (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (heat-inactivated at 56 °C for 30 min), 10 mM nonessential amino acids, 100 IU/ml of penicillin, and 100 mg/ml of streptomycin in a humidified 5% CO<sub>2</sub> atmosphere at 37 °C.

### *Humbug gene synthesis and construction of the expression vector*

The DNA codons of humbug (GenBank, accession number KC009577) with a low usage percentage (<15%) were replaced by those with a higher use frequency. The designed gene was synthesized by a Nanjing bioscience company of Genscript in China and optimally analyzed by OptimumGene™ based on the preferred codons in *P. pastoris*. The DNA fragment of humbug was amplified by using the synthetic gene as a template to yield a product of 897 bp. The primer pairs' sequences, containing added sites for the restriction

enzymes *Xho*I and *Not*I (underlined), a cleavage sequence (in bold) and terminal sequences (in bold), were designed as follows: humbug-F, 5'-CTCTCGAGAAAAGAGAGGCTGAAGCTA-TGGTGATTGCATTGCTG-3'; humbug-R, 5'-TAG-CGGCCGCTTAAGTTTCTGGTGGTACTTC-3'. PCR amplification was performed at 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 45 sec and 72 °C for 30 sec, with a final extension at 72 °C for 10 min. The *Xho*I<sub>5710</sub> site in the pPIC9k vector was mutated to use the *Xho*I<sub>1193</sub> site for the desired gene cloning. The single-site mutation (in bold) was generated by PCR-based quick-change mutagenesis by using the following primers: 5'-TTAATCGCGGCCTAGAGCAAG-AC-GTTTC-3'; 5'-GAAACGTCTTGCTCTAGGCCGCG-AT-TAA-3'. The thermocycling program was optimized as follows: one cycle of 95 °C for 30 sec; 12 cycles of 95 °C for 30 sec, 52 °C for 1 min and 68 °C for 7 min. The PCR product was ligated into the pPIC9k vector between the *Xho*I and *Not*I sites in-frame with the  $\alpha$ -factor signal sequence to generate the pPIC9k-humbug plasmid. The recombinant plasmid was transformed into *E. coli* Top10, and all the constructs were verified by DNA sequencing.

#### ***Transformation of P. pastoris and selection of the multicopy clones***

The pPIC9k<sup>+</sup>-humbug and pPIC9k (as a negative control) plasmids were both digested by *Sac*I for linearization and transformed into the *P. pastoris* wild-type strain GS115 (Invitrogen, Carlsbad, USA) through electroporation at 25  $\mu$ F and 2000 V by using a micropulser (Bio-Rad, Hercules, USA). After pulsing, 1 ml cold 1 M sorbitol was immediately added to the *P. pastoris* cells at 30 °C for 1 h. After cultivation, all transformants were spread and incubated on MD and MM plates to select for Mut<sup>+</sup> or Mut<sup>S</sup> recombinants at 30 °C for 72 h. Multicopy clones were selected on a YPD agar plate containing 2-4 mg/ml G418 at 30 °C for 72 h. Genomic DNA was extracted from the different transformants to confirm that the humbug gene was integrated into the *P. pastoris* genome. Integration was analyzed through PCR by using the cloning primers.

#### ***Production of recombinant humbug using high cell-density fermentation***

According to the protocol of a 10-L bioreactor and the *P. pastoris* fermentation guidelines (Invitrogen, Carlsbad, USA), the fermentation procedure of methanol feeding of Mut<sup>+</sup> strains was performed. The fermentation was executed by using a 10-L bioreactor (Biostat B plus, Gottingen, Germany) equipped with four additional feed pumps, a gas mixer, an oxygen supply, a water cooler and a computer for automatic control and data acquisition. The 3 L starting basal salt medium supplemented with PTM1 trace salts was inoculated with 500 ml culture grown in BMGY medium at 30 °C for 24 h. The gas mixture (air and oxygen), airflow, glycerol and methanol feed, and stirring parameters were adjusted to ensure that the dissolved oxygen (DO) levels were maintained at 20-40% during the fermentation process. Temperature and pH were automatically held at 30 °C and pH 5.0 by water-cooling and the addition of 50% ammonia, respectively. Briefly, the fermentation procedure consisted of three steps. Initially, the DO levels were maintained above 20%. When the initial glycerol was depleted, fed-batch fermentation was initiated with a glycerol fed-batch medium at a rate of 20 ml/(h·L). After a 24 h fed-batch phase, the methanol induction phase was started with a feed containing methanol. The fed rate of methanol was used to keep the DO level stable above 20%. Samples were withdrawn at 6 h time intervals during the induction phase, and the DO level, the cell wet weight and the protein concentration were analyzed.

#### ***Purification of recombinant humbug***

The supernatant was separated from the culture by centrifugation at 12,000 rpm for 20 min and was filtered with a 0.22  $\mu$ m hydrophilic multilayer Durapore membrane (Millipore, Boston, USA). The filtrate was transferred to the 1,000 ml vessel of a tangential flow filtration (TFF) ultrafiltration device (Labscale TFF System, Millipore, Boston, USA) equipped with a regenerated cellulose membrane (MW cut-off: 5 kDa) to concentrate the supernatant to approximately 50 ml.

The concentrated samples were purified using a DEAE Sepharose FF column. The sample was eluted in a solution of 3 mM NaCl and 10 mM sodium phosphate (pH 7.4) at a flow rate of 5 ml/min. The peak fraction containing humbug was collected, and 50  $\mu$ L of each eluted sample was analyzed following separation on a 12% polyacrylamide gel. The samples containing recombinant humbug were pooled and concentrated by using an AmiconUltra 15 (Millipore, Boston, USA) with a 5 kDa MW cut-off filter. The concentrated samples were further purified by using a Sephadex G25 gel-filtration column. The concentration of the protein at each step was determined by using a BCA protein assay kit (Pierce, Rockford, USA), and bovine serum albumin was used as the standard.

#### ***Generation, purification and characterization of a monoclonal antibody against recombinant humbug***

The desalted and lyophilized humbug protein was weighted and diluted with PBS to a concentration of 1 mg/ml, which was used as an immunogen. For the initial immunization, five female BALB/c mice were subcutaneously vaccinated with 100- $\mu$ g immunogen emulsified with an equal volume of complete Freund's adjuvant. Two and four weeks after the initial injection, booster injections were given intraperitoneally with the same quantity of immunogen. The antiserum of each mouse was collected from the retrobulbar plexus, and each antiserum titer was determined by indirect ELISA. The best-performing mouse was selected for hybridoma production and boosted with 100- $\mu$ g immunogen 2 days before fusion.

Mouse myeloma cells Sp2/0 cell lines were cultivated in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin under a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C, and cells in the exponential growth phase were grown to concentrations of 4 $\times$ 10<sup>5</sup> cells/ml before cell fusion. The harvested spleen cells were fused with Sp2/0 cells at a ratio of 10:1. Hybridomas were selectively cultured for approximately 2 weeks, and the supernatants were screened by indirect ELISA. Finally, the hybridoma-

mas, produced antibodies with good reactivity against humbug, were cloned twice by limiting dilution followed by expansion for the large-scale production of the mAb.

After injection of the hybridoma cells (5 $\times$ 10<sup>5</sup>), ascites fluid was produced in BALB/c mice in 7-14 days. The fluids were purified by using a Protein G Sepharose 4 Fast Flow column, and then the purity was analyzed by SDS-PAGE. According to the manufacturer's recommendations, isotyping of the mAbs against humbug was determined by a gel gold test strip mouse mAb isotyping kit (Pierce, Rockford, USA).

#### ***SDS-PAGE and Western blot analysis***

As for SDS-PAGE analysis, proteins in the culture supernatants were mixed with a 2x loading buffer (pH 6.8) containing 1 M Tris, 20% glycerol, 10% SDS, 0.1% bromophenol blue and 5%  $\beta$ -mercaptoethanol. A low molecular weight range ladder (TakaRa, Otsu, Japan) was used as a standard to evaluate molecular mass. Electrophoresis was carried out on 12% polyacrylamide gel under denaturing conditions for approximately 90 min with a constant voltage of 120 V. Humbug protein samples were visualized with Coomassie brilliant blue R-250 staining.

As for Western blot analysis, the fractionated proteins were transferred onto nitrocellulose membranes (Bio-Rad, USA) by electroblotting and probed with diluted (1:1,000) primary antibody for 1 h, and then nitrocellulose membranes were incubated with goat anti-mouse IgG/HRP (diluted 1:2000) (Caltag Laboratories) as the secondary antibody. The Western blots were blocked, washed, and probed at room temperature in 10 mM sodium phosphate (pH 7.4) containing 150 mM NaCl, 0.1% bovine serum albumin and 0.1% Tween 20. The detection of humbug was performed by using the ECL Western Blotting Substrate kit (Pierce, Rockford, USA).

#### ***Immunofluorescence cell staining***

Cells (4 $\times$ 10<sup>5</sup>) in 3 ml culture medium were seeded onto 6-well cell culture plates overnight at 37 °C. After washing 3 times with ice-cold PBS, the cells were permeabilized for 30 min with 2% Triton X-

100 at room temperature and then blocked with 3% bovine serum albumin (BSA) at 37 °C for 30 min. The monoclonal antibody against humbug at a dilution of 1:100 (9 mg/ml) was applied for 1 h and then was washed by PBS for 3 min. The cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:200) and Hoechst 33258 at 37 °C for 1 h in the dark. After washing with PBS, the cells were mounted with 50% glycerol/PBS mounting medium. Images were immediately observed and captured by using fluorescent microscopy.

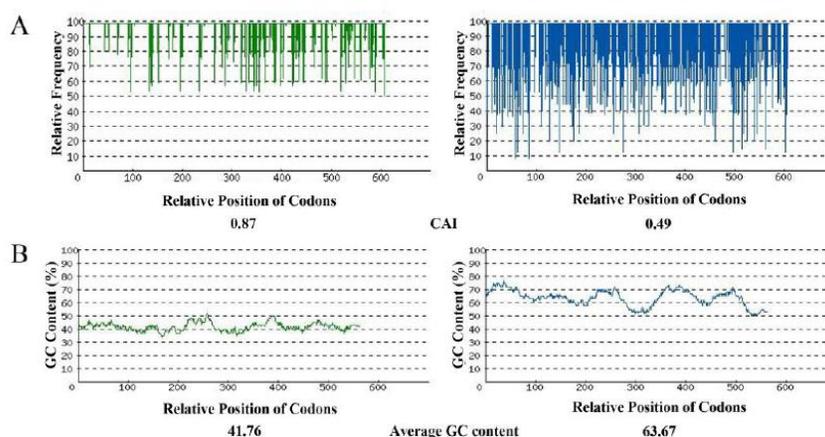
## Results

### Optimized humbug gene design and construction of expression vector

Compared to the wild type, a codon-optimized humbug gene of 897 bp was synthesized by the OptimumGene™ algorithm to obtain humbug gene with enhanced translation efficiency. A BLAST search showed that the nucleotide sequence of the synthesized gene was 77% identical to the wild-type humbug gene. Considering the codon usage bias in *P. pastoris*, the CAI value was increased from 0.49 to 0.87. The GC content of

the sequence was decreased from 63.67 to 41.76% (Fig. 1).

To use the *Xho*I<sub>1193</sub> site for the desired gene cloning, the *Xho*I<sub>5710</sub> site in the expression vector pPIC9k was mutated by using single site-directed mutagenesis. The humbug fragment was cloned in frame with the  $\alpha$ -factor signal sequence from *Saccharomyces cerevisiae*. The KEX2 cleavage site between the *Xho*I and *Not*I restriction sites of the mutated pPIC9k expression vector, generating the pPIC9k-humbug construct. The expression vector was linearized by using *Sac*I to generate an integrative fragment containing humbug and the His4 selectable marker. Approximately 280 His<sup>+</sup> transformants of the *P. pastoris* GS115 competent cells were obtained by electroporation for the linearized plasmid. Transformants were then selected on YPD plates with increasing concentrations of G418 for the rapid screening of the multicopy clones. Most of the transformants (150-200) appeared on plates with 1 mg/ml G418 after 3-4 days of incubation at 30 °C, whereas 14 transformants appeared on plates with 3 mg/ml G418. Only 5 transformants could be observed on plates with 4 mg/ml G418 after 5 days of incubation.



**Fig. 1:** Codon Adaptation Index (CAI) and GC Content Adjustment after OptimumGene™ Optimization.

- (A) The distribution of codon usage frequency along the length of the gene sequence. A CAI of 1.0 is considered perfect in the desired expression organism, and a CAI of > 0.8 is regarded as good, in terms of high gene expression level; (B) The percentage distribution of codons in computed codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism.

All clones selected on YPD plates with 4 mg/ml G418 were tested by PCR to confirm the integration of the humbug-coding region into the *P. pastoris* genome, and three positive clones were selected. PCR and electrophoresis confirmed the expected size of the constructs (Fig. 2).

### Expression and purification of recombinant humbug in 10-L bioreactor

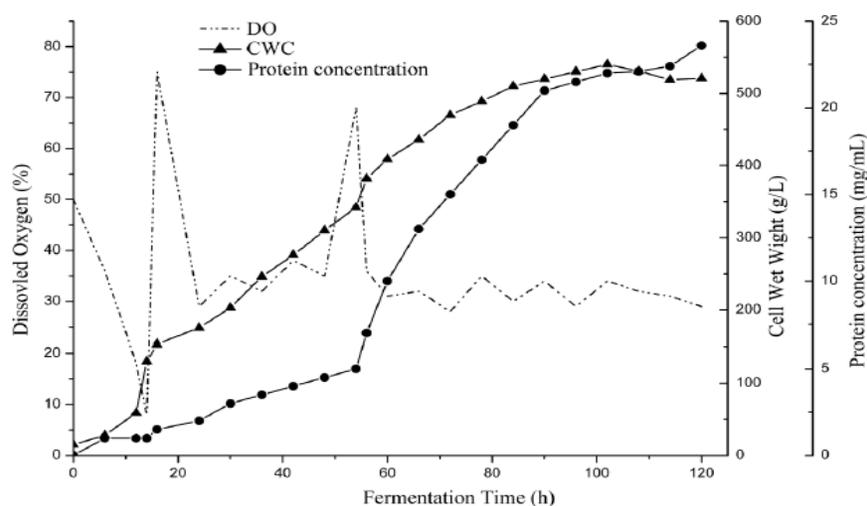
High-expressed fermentation of humbug was achieved by using a 10-L bioreactor. The wet weight of cell and the concentration of protein in supernatant were monitored throughout each

stage of the fermentation. The initial batch phase began with a cell-wet weight of 153 g/l. The culture was switched to a glycerol fed-batch phase for 24 h with a cell-wet weight of 342 g/l. The methanol fed-batch phase is made up of the methanol-adoption and methanol-utilization phases. The fermentation was terminated at 118 h with a cell-wet weight of 521 g/l. The cultivation profile was shown in Fig. 3, and the SDS-PAGE and Western-blot analysis of humbug protein expression at different hours was shown in Fig. 4A, B.



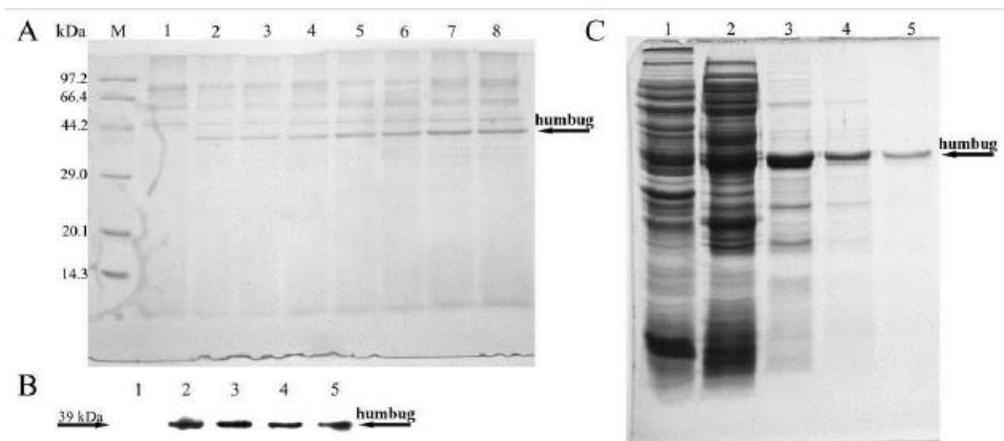
**Fig. 2:** Construction and transformation of pPIC9K/Humbug in *P. pastoris*

- (A) Restriction endonuclease profiles of pPIC9K/Humbug digested with *Xho*I and *Not*I. Lane M: DNA marker; Lane A, B, C: pPIC9K (9.3 kb) Humbug gene (765 bp); (B) Screening the multicopy clones on YPD plates with G418. (C) Agarose gel electrophoresis of positive clone.



**Fig. 3:** Time course of recombinant humbug expression in high cell-density fermentation

DO (---), cell wet weight (▲) and protein concentration (●) were monitored at various time intervals during the fermentation.



**Fig. 4:** SDS-PAGE, Western blot analysis and purification of recombinant humbug during fermentation

**(A)** SDS-PAGE analysis. Lane M, molecular weight standard; Lanes 1-8, recombinant humbug (fermentation time: 0 h for lane 1, 12 h for lane 2, 24 h for lane 3, 36 h for lane 4, 48 h for lane 5, 72 h for lane 6, 96 h for lane 7 and 120 h for lane 8). The main recombinant protein band was denoted with an arrow; **(B)** Western blot analysis. Lane 1, samples of the whole culture supernatant before induction (negative control); Lanes 2-5, samples of the whole culture supernatant after 120 h induction. The samples were probed with an anti-HAAH polyclonal antibody; **(C)** Purification of recombinant humbug expressed in *P. pastoris*. Lane 1, negative control for the secreted protein; Lane 2, crude secreted protein; Lane 3, protein was purified by using Ultrafiltration; Lane 4, protein was purified by using DEAE ion-exchange chromatography; Lane 5, protein was purified by using Sephadex G25 gel-filtration chromatography. The gel was stained with Coomassie brilliant blue R-250.

All steps of the purification were monitored by SDS-PAGE (Fig. 4C). The cell-free supernatant was concentrated by ultrafiltration and purified on a DEAE Sepharose FF column. Fractions of some protein impurities were pooled, concentrated and further purified by using a Sephadex

G25 gel-filtration column. The purification procedure resulted in the recovery of 96.9 mg/l recombinant humbug from the culture supernatant, accounting for 41.2% of the total protein content (Table 1).

**Table 1:** Purification of recombinant humbug protein

Fraction	Protein (mg/l)	Yield (%)
Cell-free supernatant	235.1	100
Ultrafiltration	192.3	81.8
DEAE Sepharose FF column	131.6	55.9
Sephadex G25 gel-filtration column	96.9	41.2

#### **Generation, purification and characterization of a monoclonal antibody against recombinant humbug**

After an initial injection and three booster immunizations, the maximum antibody titer in mice sera against the purified humbug was estimated to be

$5 \times 10^3$  by an indirect ELISA (data not shown). The mouse was boosted again and selected for hybridoma fusion, which increased the titer to  $1 \times 10^4$  (data not shown).

The fusion cells were seeded in a 96-well culture plate. After 2 weeks culturing, hybridoma cell

clones formed in 85 wells, giving a fusion ratio of approximately 88.5%. Seven hybridoma clones in the 85 wells were initially selected based on their strong ELISA reactivities with humbug protein and were subsequently subjected to cloning procedures. Four clones (B7, C2, E5, and F3) with the best titers, affinities, and cell growth statuses were finally selected for further limiting dilution. An indirect ELISA assay showed that the titers of

mAb specific against humbug in the fluids reached  $5 \times 10^4$ - $1 \times 10^5$ . These mAbs could specifically react with humbug, determined by Western blot analysis. The Ig subclasses of mAbs that were secreted by the four cell strains were all IgG1 kappa by using a mouse mAb isotyping test kit. The affinity constant ( $K_{aff}$ ) of the four mAbs ranged between  $1.6 \times 10^8$  and  $6.2 \times 10^8$  were measured by noncompetitive ELISA (Table 2).

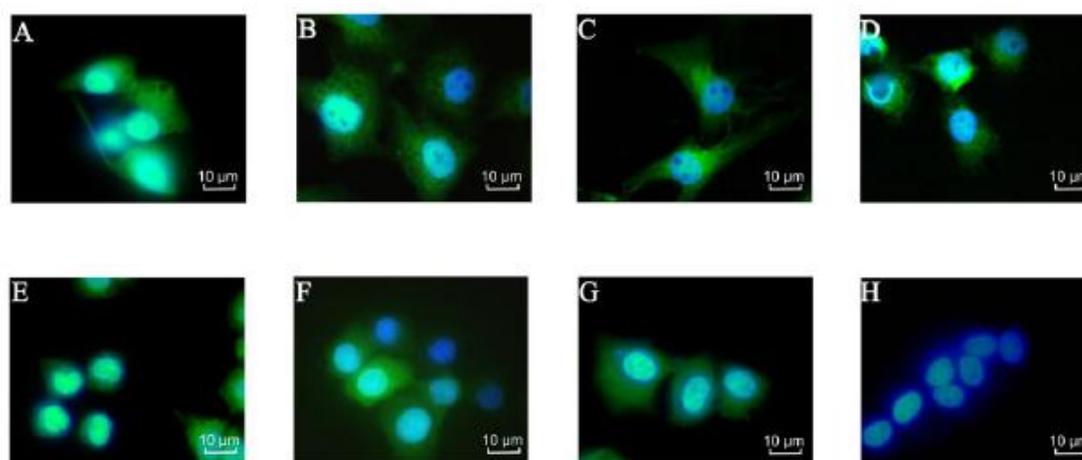
**Table 2:** Identification and characterization of anti-humbug mAbs

Hybridoma	Class and subclass	Type	Titer of supernatant of ascites	Affinity constant ( $M^{-1}$ )
B7	IgG <sub>1</sub>	ê	1:5000	$1.6 \times 10^8$
C2	IgG <sub>1</sub>	ê	1:7000	$2.3 \times 10^8$
E5	IgG <sub>1</sub>	ê	1:12000	$6.2 \times 10^9$
F3	IgG <sub>1</sub>	ê	1:9000	$3.7 \times 10^8$

**Immunofluorescence cell staining in carcinoma cell lines**

To identify the specificity of the humbug mAbs, the humbug protein expression levels and its distribution in several carcinoma cell lines were evaluated with the E5 mAb by immunofluorescence. As shown in Fig. 5, the humbug mAb exhibited high levels of binding to the seven human tumor

cell lines (ACHN, BIU-87, MCF-7, SMMC-7721, Hep-2, HeLa and SKOV). As a negative control, human liver cells L02 were found to be unreactive. The distribution pattern of humbug in the tumor cell lines was mainly cytoplasmic with unambiguous perinuclear. Plasmalemmal accentuation was shown by fluorescent staining, whereas the normal human liver cells exhibited no staining.



**Fig. 5:** Results of indirect cellular immunofluorescence (A)ACHN; (B)BIU-87; (C)MCF-7; (D)SMMC-7721; (E)Hep-2; (F)HeLa; (G)SKOV; (H)L02.

## Discussion

ASPH, junctin, junctate, and humbug are four functionally different isoforms formed by alternative splicing of a 6 kb mRNA transcript (6). As a truncated homolog of ASPH, humbug has been identified in the peripheral coupling of the ER and the plasma membrane in eukaryotic cells. It also has been shown to contribute to  $Ca^{2+}$  homeostasis by forming a multi-molecular  $Ca^{2+}$  entry channel (1,2,20-23). Humbug has also been implicated in regulation of cell motility in malignant neoplasm metastasis (3,4). Recent evidences demonstrate that the overexpression of humbug promote the malignant progression and potentially metastasis of human gastric cancer cells (24-26).

Several hosts such as *E. coli* and mammalian cell cultures are used for the expression of recombinant humbug fusion proteins (7, 14). In earlier attempt, pProEX expression system is used to express humbug in *E. coli* (16). One of the major drawbacks of the expression system in *E. coli* was that the formation of inclusion bodies and its re-folding purification could reduce the recovery yields (27). A great quantity of high purity, crystalline protein is necessary for three-dimensional structural studies. Hence, some optimized methods need to be developed in this study.

Based on prior studies, gene expression levels are regulated and influenced by several factors such as codon usage bias (19), GC content and repeated sequences. Utilizing the OptimumGene™ algorithm and gathering as many of these factors as possible, the optimum gene sequence for high levels of expression was produced. In addition, the unfavorable regions in the original sequence were eliminated to prolong the half-life of the mRNA, and the stem-loop structures, which affect ribosomal binding and stability of mRNA, were broken. The optimum construction strategy of the expression vector was based on the sequence of the pPIC9k expression cassette. On the one hand, the processing of the factor signal cleavage in *P. pastoris* was performed in two steps: KEX2 cleavage (28), which recognized the sequence Glu-Lys-Arg\*Glu-Ala-Glu-Ala (\* denotes the site of cleavage) and STE13 cleavage, which cleaved the Glu-

Ala repeats subsequently. The first restriction site *SnaB* I was not a conventional enzyme cut site and generated blunt ends. If *SnaB* I were not chosen, at least two amino acids would be left on the N-terminus of the expressed protein of interest. On the other hand, there were two *Xho*I restriction enzyme sites (1193, 5710) in the original expression vector pPIC9k. The former site was in the reading frame of the  $\alpha$ -factor signal sequence, and the latter was in the kanamycin resistance gene region. As the *Xho*I<sub>5710</sub> was mutated by using site-directed mutagenesis, the humbug gene with *Xho*I and *Not*I sites at the two ends was successfully cloned into the  $\alpha$ -factor signal expression cassette. The procedure for protein expression and purification was involved in three steps. Firstly, a eukaryotic *P. pastoris* expression system being compatible with the insertion of and selection for multiple gene copies was chosen for expression. Secondly, high cell-density fermentation in a 10-L bioreactor facilitated the effective production of recombinant protein under controlled induction conditions such as pH, temperature and DO concentration. Finally, a two-step purification procedure was utilized to maintain functionality and yield of recombinant humbug.

In the previous study, a monoclonal antibody against ASPH/humbug was obtained by co-immunizing mice with naked plasmid DNA containing the N-terminal domain of the ASPH gene and recombinant humbug that have been expressed in *E. coli* (16). The affinity constant of the present anti-humbug antibody was higher and stronger than the former one. Immunofluorescence cell staining results from the present study illustrated that humbug was overexpressed in many tumor cell lines. Moreover, the normal human liver cell line L02 was utilized as a negative control. Furthermore, more functional investigations such as signal transduction should be performed to determine the role of humbug in proliferation, invasion and metastasis of tumors.

## Conclusion

The successful production of stable, functional recombinant humbug in *P. pastoris* can help pro-

mote research on the structural features of this protein. The development of a monoclonal antibody against humbug with high antigen-binding activity also can facilitate further research into the function of the protein. Further study will obtain crystalline humbug for X-ray diffraction analysis and explore the relationship between ASPH/humbug and human malignancies. As a result, this study can potentially be used in further structural research of humbug and can be applied in tumor initial diagnosis and immunotherapy for human health.

## Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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