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High-efficiency Expression and Application of Anti-p53 Recombinant Antibodies in CHO Cells

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Abstract

p53 is an important tumor suppressor gene that plays a crucial role in cell cycle regulation and DNA repair, with mutations associated with over 50% of tumors. Clinically, p53 antibodies are used to detect abnormal p53 proteins, aiding in tumor diagnosis and prognosis analysis. However, existing p53 antibodies are expensive and rely on imports, limiting their application in pathological testing and research. To address this issue, this study achieved high-efficiency expression of anti-p53 recombinant antibodies in Chinese hamster ovary (CHO) cells to reduce costs. Research began by constructing the eukaryotic expression vector pEE12.4-p53-GS, transfecting CHO cells, and screening stable cell lines. A hydrophilic and immunogenic p53 antigen was selected, and monoclonal antibodies were generated using BALB/c mice. The 4H12E8 cell line was identified through Western blot and immunohistochemistry (IHC) with an antibody titer of 1:1024 k, comparable to commercially available p53 antibodies. The antibody gene was cloned from hybridoma cells by RT-PCR, and the pEE12.4-p53-GS vector was constructed and validated for stable expression in CHO-GS KO cells. After optimizing the fermentation process, the antibody expression level in the 3 L fermentation tank reached 1 g/L, reducing the cost by 4-5 times compared to imported products. Ultimately, the anti-p53 recombinant antibody demonstrated good specificity and applicability, showing strong performance across 65 cancer

samples. This study not only addresses the ethical issues associated with monoclonal antibody production but also significantly reduces production costs, providing important support for pathological testing and p53 protein research, and laying the foundation for the development of high-affinity humanized p53 antibodies.

Keywords: p53; CHO-GS; recombinant antibody; monoclonal antibody;

1. Introduction

Recombinant antibodies, also known as genetically engineered antibodies, refer to antibodies obtained by modifying and recombining antibody gene sequences as needed through recombinant DNA and protein engineering technologies, followed by transfection or transformation of the constructed plasmid into suitable host cells for expression (Ayyar et al. 2017). Compared to traditional antibodies, recombinant antibodies offer several advantages:

1. A significant advantage of recombinant antibodies is their ease of engineering. Using molecular biology and synthetic biology techniques, recombinant antibodies can be humanized, effectively addressing the human immune response triggered by animal-derived antibodies, thereby improving antibody efficacy. Alternatively, the heavy chain, light chain, or specific fragment regions of recombinant antibodies can be rearranged or replaced to design novel antibodies with desired properties. Furthermore, technologies like phage display enable high-throughput screening of recombinant antibodies (França et al. 2023), allowing for the rapid identification of candidates with high specificity for therapeutically relevant targets and potential for drug development.

2. The quality and function of monoclonal and polyclonal antibodies produced by traditional methods often vary significantly between batches. In contrast, recombinant antibodies are produced using molecular cloning and protein expression techniques, which greatly reduce batch-to-batch variability (Groff et al. 2015).

3. Recombinant antibody production represents a standardized process. It offers benefits such as known antibody sequences, the possibility of long-term storage of antibody genes, stable properties, and good experimental reproducibility (Chu et al. 2021). This approach mitigates risks associated with monoclonal antibody production and storage, such as the loss of antibody genes during storage or the failure of cells to grow or their death upon revival during production.

CHO cells were originally epithelial adherent cells but have been adapted for suspension culture in serum-free media. They are widely used as host cells for large-scale recombinant protein production (Chu et al. 2001). CHO cells offer numerous advantages for recombinant antibody production:

1. CHO cells are easy to culture and adapt to suspension growth, making them suitable for large-scale industrial suspension fermentation. They can also thrive in various serum-free and chemically defined media (Kim et al. 2017;Lai et al 2013).

2. CHO cells perform accurate post-translational modifications (e.g., glycosylation). Consequently, recombinant antibodies expressed in CHO cells exhibit physicochemical properties and biological activities closer to natural antibody molecules (Kim et al. 2017;Lai et al 2013).

3. CHO cells secrete recombinant proteins extracellularly. Since CHO cells secrete relatively low levels of endogenous proteins (Xu et al. 2011), this facilitates the separation and simplifies the downstream purification of the target protein.

4. CHO cells are not susceptible to infection by many human viruses, significantly reducing biosafety risks in industrial production processes (Kastenhuber et al. 2017) . This lower susceptibility may be because many viral genes cannot be expressed after entering CHO cells (Kastenhuber et al. 2017).

5. Various gene amplification systems have been developed for CHO cells, enabling high-efficiency gene amplification and protein expression.

The p53 gene was discovered in the early 1970s during research on tumor-related genes. As a critical tumor suppressor gene, it plays a key regulatory role in the cell cycle. p53 is a master regulator of cellular stress responses and is one of the most frequently mutated genes in human cancers, with over 50% of tumors exhibiting abnormalities in the p53 signaling pathway involving p53 mutations (Lamb et al. 1986). The human p53 gene is located on chromosome 17p13.1, spanning 16-20 kb (Liu et al. 2019). It encodes a protein with a molecular weight of 43.7 kDa. However, due to a proline-rich region that slows its migration in gels, the protein band appears at the 53 kDa marker position during electrophoresis, hence the name p53 (Chen et al. 1990). The p53 gene structure includes coding and non-coding regions. The coding region consists of 11 exons. Exons 2, 4, 5, 7, and 8 encode five evolutionarily highly conserved domains (Liu et al. 2019), and it contains a start codon and a stop codon. These exons are spliced to form mature mRNA, which is then translated into the p53 protein. The non-coding regions include promoters, enhancers, and transcription factor binding sites, which are crucial for the transcriptional regulation of the p53 gene (Liebl et al. 2021).

During tumorigenesis and development, mutations in the p53 gene and alterations in its expression product are common. High p53 expression in patients is often associated with poor prognosis, indicating its important role in the formation of various tumors. Therefore, detecting p53 gene mutations and expression products is significant for the early diagnosis of cancer. Mutations in the p53 gene can alter the physicochemical properties of the protein, significantly increasing its stability. These changes can be detected and analyzed using molecular biology, immunohistochemistry (IHC), and serological methods. IHC is widely used clinically for tumor detection and diagnosis, especially for challenging cases. It allows for the analysis of mutant p53 protein expression in tumor tissues for both clinical diagnosis and biological research. Positive immunostaining for p53 gene products in tumor tissue typically indicates abnormality. This change can be used to evaluate tumor proliferation and malignancy grade, serving as an auxiliary tool for assessing patient prognosis. IHC detection of p53 protein primarily relies on p53 antibodies. However, currently available p53 antibodies, predominantly monoclonal antibodies produced in animals, are expensive and largely dependent on imports, which limits their application.

The significance of this study lies in utilizing hybridoma technology to produce a p53 monoclonal antibody, followed by employing genetic engineering techniques to retrieve the antibody genes from the hybridoma cells to generate a recombinant antibody. High-yielding monoclonal cell lines will be selected, and large-scale fermentation conditions will be explored to enhance antibody expression levels, thereby reducing production costs and making the product more price-competitive. This approach offers advantages such as low cost, evident effectiveness, and the avoidance of animal ethics issues. It aims to provide beneficial support for tumor detection and in-depth research on the p53 protein, while also obtaining a high-affinity antibody sequence as a foundation for subsequent humanized antibody development.

2. Materials and Methods

2.1 Strains and Plasmids

Cell lines: A549 (human non-small cell lung carcinoma cells), HEK 293T (human embryonic kidney cells), HEK 293FT (human embryonic kidney cells), HT-29 (human colorectal adenocarcinoma cells), SW480 (human colorectal adenocarcinoma cells), U251 (human glioma cells), T-47D (human ductal breast carcinoma cells), and CHO-GS KO (Chinese hamster ovary cells) were preserved in our laboratory.

Bacterial strains: *E. coli* DH5 α and BL21(DE3) were preserved in our laboratory.

Plasmids: pET32a, pEE6.4-mIgG, and pEE12.4-mCk-GS were constructed and maintained in our laboratory.

2.2 Primers

The primers are listed in Table 1 and Table 2.

Table 1 Primers used in this study

Primers	Sequences(5'-3')
D1 V _K -F1	GACAWTGTCTCTACCCAGTC
D2 V _K -F2	GACATCCAGATGACACAGWC
D3 V _K -F3	GATRTTGTGATGACCCAGWC
D4 V _K -F4	GACATTSTGMTGACCCAGTC
D5 V _K -F5	GATGTTGTGVTGACCCAAAC
D6 V _K -F6	GACACAACGTGACCCAGTC
D7.V _K -F7	GAYATTKTGCTCACTCAGTC
D8 V _K -F8	GATATTGTGATRACCCAGGM
D9 V _K -F9	GACATTGTAATGACCCAATC
D10 V _K -F10	GACATTGTGATGWCACAGTC
D11 V _K -F11	GATRTCCAGATGAMCCAGTC
D12 V _K -F12	GATGGAGAAACAACACAGGC
D13 V _K -R1	GCGTTTBATTTCCAGCTTGG
D14 V _K -R2	GCGTTTTATTTCCAATTTTG
D15 V _H -F1	GAGGTTCDSTGCAACAGTY
D16 V _H -F2	CAGGTGCAAMTGMAGSAGTC
D17 V _H -F3	GAVGTGMWGCTGGTGGAGTC
D18 V _H -F4	CAGGTTAYTCTGAAAGAGTC
D19 V _H -F5	GAKGTGCAGCTTCAGSAGTC
D20 V _H -F6	CAGATCCAGTTSYGCGAGTC

D21 V _H -F7	CAGRTCCAACTGCAGCAGYC
D22 V _H -F8	GAGGTGMAGCTASTTGAGWC
D23 V _H -F9	GAAGTGAAGMTTGAGGAGTC
D24 V _H -F10	GATGTGAACCTGGAAGTGTC
D25 V _H -F11	CAGATKCAGCTTMAGGAGTC

Table 2 Primers used in this study

Primers	Sequences(5'-3')
D26 V _H -F12	CAGGCTTATCTGCAGCAGTC
D27 V _H -F13	CAGGTTCACCTACAACAGTC
D28 V _H -F14	CAGGTGCAGCTTGTAGAGAC
D29 V _H -F15	GARGTGMAGCTGKTGGAGAC
D30 V _H -R1	CGAGGAGACGGTGACMGTGG
D31 V _H -R2	CGCAGAGACAGTGACCAGAG
D32 V _H -R3	CGAGGAGACTGTGAGASTGG
p53-F	CCATGGCTGATATCGGATCCATGGAGGAGCCGCAGTCAG
p53-R	TCGAGTGCGGCCGCAAGCTTGTTTCAGGGCAGGGGAGTAC
T7-F	TAATACGACTCACTATAGGG
T7-R	GCTAGTTATTGCTCAGCGG
M13-F	TGTAAAACGACGGCCAGT
M13-R	CAGGAAACAGCTATGACC
p53-V _H -F	CACAGGAGCCCACAGCCAGATCCAGCTGGCCC
p53-V _H -R	GGGGTGTCGTTTTAGCGGCGCTCACTGTCACC
p53-V _κ -F	TCCAGCTAGCCGCGGCGATATCGTGATGACAC
p53-V _κ -R	TTGGTGCAGCATCTGCCCTCTTCATCTCCAGC
mIgG-F	GCTGACAGACTAACAGACTG
mIgG-R	TTATGTTTCAGGTTTCAGGGG
mC _κ -F	GAGATCATGAGGGTGTCTCT
mC _κ -R	CTACTGTCCTCATCTTCCCCG

2.3 Design and Expression of the p53 Antigen

The basic information of the human p53 protein (UniProt ID: P04637), including the number of amino acids, molecular size, amino acid sequence, and subcellular localization, was retrieved from the UniProt database. Subsequently, the secondary structure was predicted using DNASTAR software to identify the position, length, and sequence of α -helices and β -sheets, as well as their spatial arrangement and relative positioning. Further analysis was conducted on the distribution of hydrophobic and polar residues within the secondary structure, along with interactions such as hydrogen bonds and ion pairs, to evaluate the protein's antigenicity and stability. Finally, B-cell epitopes were predicted from the amino acid sequence using the BepiPred-3.0 server. Based on the prediction results, the optimal antigenic sequence was selected for full gene synthesis. The successfully sequenced target gene was cloned into the pET32a vector. The recombinant plasmid was transformed into *E. coli* BL21(DE3) cells, and protein expression was induced at 18°C. The antigen was purified using a nickel-nitrilotriacetic acid (Ni-NTA) affinity column, analyzed by SDS-PAGE, and quantified by the Bradford method.

2.4 Preparation and Characterization of Monoclonal Antibodies

Healthy female BALB/c mice (6-8 weeks old) were acclimatized for one week prior to immunization. The antigen (1 mg/mL) was emulsified with Freund's adjuvant at a 1:1 (v/v) ratio for 120 seconds and loaded into a medical syringe. After disinfecting the mouse backs with 75% ethanol, each mouse was subcutaneously injected with 100 μ g of the antigen at multiple sites. The immunization schedule consisted of a prime immunization, followed by a boost three weeks later, and subsequent boosts at two-week intervals. Typically, 4-5 immunizations were performed, up to a maximum of seven. Serum antibody titers were determined by indirect ELISA. Briefly, plates were coated with antigen diluted to 1 μ g/mL and incubated overnight at 4°C, followed by blocking with skim milk. PBS served as the negative control. Sera were initially diluted at 1:500 and subjected to two-fold serial dilution up to 1:512,000, then incubated at 37°C for 1 hour. After washing five times with PBST, HRP-conjugated secondary antibody was added and incubated at 37°C for 30 minutes. Plates were washed again, TMB substrate was added for 3-5 minutes, the reaction was stopped with 0.1 M H₂SO₄, and the absorbance was measured at 450 nm.

HAT medium, RPMI-1640, and PEG 1500 were pre-warmed to 37°C. SP2/0 cells (1×10^7) were harvested and resuspended in RPMI-1640. Splenocytes were aseptically prepared from the immunized mouse and mixed with the SP2/0 cells at an appropriate ratio. Cell fusion was induced by adding PEG 1500 over 80 seconds, followed by dropwise addition of RPMI-1640 to a total volume of 30 mL within 3 minutes to terminate fusion. After centrifugation, the cell pellet was gently resuspended in pre-warmed HAT selection medium supplemented with 20% FBS and 1% penicillin-streptomycin, and plated for hybridoma selection.

Supernatants from hybridoma cultures were screened by indirect ELISA. The 30 wells showing the highest OD values were selected, and their supernatants were further analyzed by IHC. Positive clones were subsequently subcloned to ensure monoclonality.

For Western blot analysis, samples were mixed with 5 $\times$ SDS-PAGE loading buffer and heated. Electrophoresis was performed at 90 V for 30 minutes, followed by 140 V for 80 minutes. Proteins were then transferred to a PVDF membrane (activated with absolute ethanol) at 300 mA for 30 minutes. After blocking, the membrane was washed with PBST and incubated with diluted primary and secondary antibodies. Signals were detected using ECL substrate and visualized with a chemiluminescence imaging system.

For IHC, tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval using a heated citrate buffer. Endogenous peroxidases were quenched with 3% H₂O₂, and non-specific binding was blocked with 3% BSA. Sections were incubated with primary antibody, followed by HRP-conjugated secondary antibody. Color development was performed using DAB, and nuclei were

counterstained with hematoxylin. Staining was observed under a bright-field microscope. The protocol for immunofluorescence (IF) was performed according to the instructions provided by Servicebio.

2.5 Cloning of Antibody Genes and Construction of Eukaryotic Expression Vectors

Total RNA was extracted from the hybridoma cells and reverse-transcribed into cDNA. The variable region genes of the heavy chain (VH) and light chain (VL) were amplified by PCR using degenerate primer pools (Table 1), with separate reactions for heavy and light chains. The PCR protocol was as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 7 sec; followed by a final extension at 72°C for 5 min. The PCR products were analyzed via agarose gel electrophoresis, and target bands of the expected size were excised and purified.

The purified VH and VL fragments were subsequently used as templates for a second round of PCR with specific primers (p53-VH-F/p53-VH-R and p53-Vκ-F/p53-Vκ-R, Table 2) to introduce homologous recombination arms. The resulting products were purified via agarose gel electrophoresis.

The pEE6.4-mIgG vector was digested with HindIII and EcoRI, and the pEE12.4-mCκ-GS vector was digested with XbaI and EcoRI. Digestion was carried out at 37°C for 20 minutes, and the linearized vectors were purified. The variable region genes were then ligated into the corresponding linearized vectors using an In-Fusion Cloning Mix for homologous recombination. The reaction mixture contained the linearized vector, the insert fragment, and 2× In-Fusion Cloning Mix Plus in a total volume of 20 µL. The recombinant products were transformed into competent cells, plated, and incubated overnight at 37°C. Positive colonies were identified by colony PCR using M13 forward and reverse primers and further verified by restriction enzyme digestion. Colonies with inserts of the correct size and orientation were selected for large-scale culture and plasmid extraction.

2.6 Establishment of Stable Cell Lines and Antibody Expression

HEK 293FT cells were thawed and cultured to a volume of 100 mL, reaching a density of 3×10^6 cells/mL. For transfection, Solution A (300 µg of plasmid DNA in 5 mL of Transpro CD01 medium) and Solution B (600 µL of PEI in 5 mL of Transpro CD01) were prepared. The solutions were mixed gently and incubated at room temperature before being added to the cells. The day after transfection, a feed supplement and a transient transfection additive were introduced into the culture. The culture was maintained with regular monitoring of cell viability and glucose concentration. When cell viability dropped below 70%, the supernatant was harvested by centrifugation at $8,000 \times g$ for 10 minutes.

CHO cells were thawed and counted. A portion of the cells was centrifuged at 1,000 rpm for 5 minutes, resuspended in serum-free RPMI-1640 medium, and adjusted to a density of 4×10^6 cells/mL in a final volume of 4 mL. This cell suspension was aliquoted into two wells of a 6-well plate. The transfection mixture was prepared as follows: Solution A (12 µg of plasmid DNA in 200 µL RPMI-1640) and Solution B (36 µL of PEI in 200 µL RPMI-1640) were mixed separately and allowed to stand for 5 minutes. Solution B was then added to Solution A, mixed gently, and incubated for 15 minutes at room temperature. This DNA-PEI complex was added dropwise to the cells. The cells were placed in a 37°C shaking incubator. Six hours post-transfection, the cells were centrifuged at 800 rpm for 5 minutes and resuspended in 4 mL of Growth A medium supplemented with 6 mM glutamine. After 24 hours, the cells were centrifuged again and resuspended in 4 mL of glutamine-free Growth A medium to begin selection. The medium was replaced with fresh glutamine-free Growth A for the selection process. Cells were monitored daily, and cell density and viability were measured every other day. When the cell density reached 2×10^6 cells/mL, the culture was transferred to a 250 mL flask.

Following successful transfection and initial adaptation, the cells were maintained under selection in glutamine-free medium. After selection was complete, the culture was scaled up to 100 mL. When the cell density reached 4×10^6 cells/mL, 4% BalanCD CHO Feed 4 was added to enhance production. Cell density, viability, and glucose levels were monitored daily, ensuring glucose concentration

remained above 3 g/L. When the cell density reached 2×10^7 cells/mL or after 4 days of culture, the temperature was shifted to 32°C. The culture was harvested by centrifugation at $8,000 \times g$ for 10 minutes when the cell number decreased significantly or viability fell below 90%, typically on day 13 or 14.

The harvested supernatant was filtered through a 0.45 µm membrane. The antibody was purified using a Protein A gravity column. The purified sample was analyzed by SDS-PAGE, concentrated via ultrafiltration, and quantified using the Bradford method to determine the antibody concentration and the expression level of the mixed pool. Finally, the functionality of the purified antibody was validated by Western Blot, IHC, and IF assays.

2.7 Screening of High-Producing Monoclonal Cell Lines

High-expressing cells were thawed and the cell density was adjusted to 1×10^6 cells/mL. After serial dilution, the cell suspension was dispensed into 96-well plates. Following seven days of culture, wells were examined under a microscope to identify and mark monoclonal colonies. After an additional seven days of culture, the supernatant was transferred to a 48-well plate for further expansion, and the antibody expression level was assessed via dot blot assay. The ten monoclonal clones exhibiting the highest expression levels were selected for scale-up culture. The final chosen cell line was cryopreserved. Subsequently, the cryopreserved cells were thawed and expanded to a 50 mL culture volume. Antibody expression was induced when the cell density reached 4×10^6 cells/mL with a viability exceeding 95%. The antibody expression titer was measured, and the monoclonal clone with the highest yield was selected for subsequent functional characterization and titer determination.

2.8 Scale-up Production of the Antibody

An orthogonal experimental design was employed to determine the optimal conditions for antibody expression. Three factors— inoculation density, temperature shift time, and temperature shift setpoint—were investigated using a three-factor, three-level orthogonal array. Each condition was tested with at least three replicates. Samples were taken every other day, centrifuged, and the concentrations of glutamine, antibody, ammonium, and lactate in the supernatant were measured using a biochemical analyzer. CHO-p53-GS cells were thawed to generate the seed train. A bioreactor was assembled, sterilized, and inoculated. The aeration rate was set to 15 mL/min, and 1.3 L of medium was added. The conditions were set to 37°C, pH 7.0, and an agitation speed of 180 rpm. After 4 hours of gassing, the dissolved oxygen (DO) was set to 40%. The bioreactor was inoculated with seed cells at a density of 0.3×10^6 cells/mL, and the working volume was adjusted to 1.5 L. An initial sample was taken for baseline measurements, and the pH probe was calibrated.

Samples were taken daily to monitor cell density and the concentrations of glutamine, antibody, ammonium, and lactate in the supernatant. On day 3, a feed consisting of 2% DN .Feed 1 and 0.2% DN Feed B was added, with subsequent feeding every other day thereafter. On day 6, the culture temperature was shifted down to 33°C. The culture was terminated after 17-18 days when a significant drop in cell viability was observed. The cell broth was harvested from the bioreactor and centrifuged at $8,000 \times g$ for 10 minutes to collect the supernatant for subsequent purification

3. Results

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3.1. Selection and Expression of the p53 Antigen

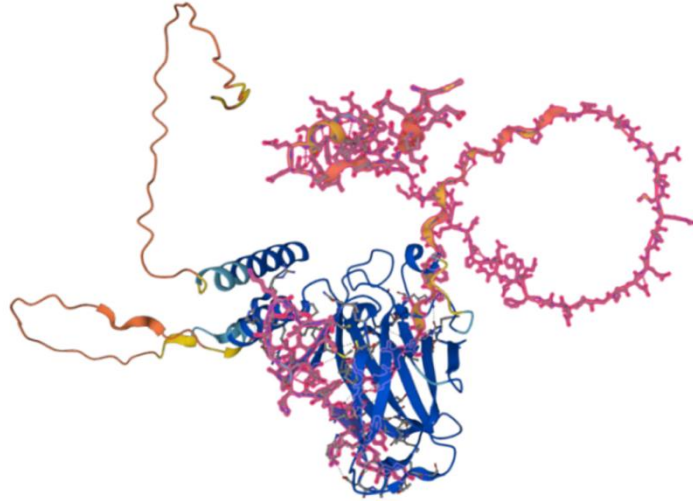


Fig 1 Prediction of three-dimensional structure of p53 antigen.

The antigen was selected based on comprehensive analyses, including protein tertiary structure prediction and hydrophilicity analysis performed using DNASTAR and PyMOL software(Figure 1). A region encompassing amino acids 1-131 of the p53 protein was chosen as the recombinant antigen for expression. This region was selected for its strong antigenicity, good hydrophilicity, and surface accessibility on the native p53 protein. The corresponding gene fragment was expressed in *E. coli*. Since p53 is a eukaryotic protein and may be difficult to express solubly in a prokaryotic system, the p53 antigen was fused with a TrxA solubility-enhancing tag to form a recombinant fusion protein for expression. The recombinant expression plasmid, pET32a-p53, was constructed. This plasmid was then transformed into *E. coli* BL21 (DE3) cells. Protein expression was induced at 18°C for 18 hours. Bacterial cells were harvested by centrifugation, resuspended, and lysed. The expression of the recombinant protein was analyzed by SDS-PAGE (Figure 2A). The theoretical molecular weight of the fusion protein is 33.3 kDa. However, the SDS-PAGE result showed a band at approximately 42 kDa (Figure 2A). This aberrant migration is likely due to the high proline content in the p53 antigen, which can retard electrophoretic mobility. The analysis indicated that the fusion protein was expressed predominantly in the soluble fraction, with a smaller portion forming inclusion bodies. The expression result was further confirmed by Western Blot analysis (Figure 2B).

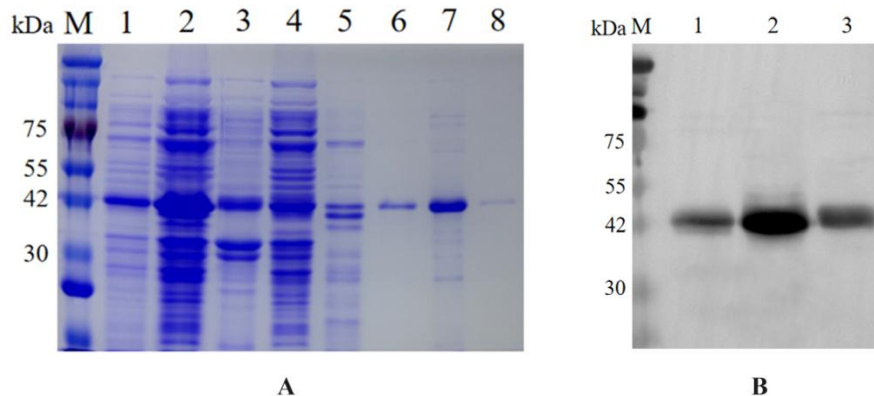


Fig 2 (A) SDS-PAGE analysis of antigen purification. M: Protein marker; Lane 1: Whole-cell lysate; Lane 2: Supernatant after cell lysis; Lane 3: Pellet after cell lysis; Lane 4: Flow-through; Lanes 5-8: Elution fractions with 50 mM, 100 mM, 200 mM, and 500 mM imidazole, respectively. (B) Western blot analysis. M: Protein marker; Lane 1: Whole-cell lysate; Lane 2: Supernatant after cell lysis; Lane 3: Pellet after cell lysis.

3.2. Preparation of Mouse Monoclonal Antibodies

Mice were subcutaneously immunized with the antigen emulsified in adjuvant (complete Freund's adjuvant was used for the primary immunization). After the third immunization, blood was collected from the mice, and the serum antibody titer was measured by indirect ELISA. Immunizations continued until the fifth immunization, after which blood was drawn again to determine the antibody titer and for analysis by Western blot. The results showed that after the third immunization, the titer for mouse #1 was 1:128,000, while mice #2-#4 had titers of 1:64,000. After the fifth immunization, the titer for mouse #1 reached 1:1,024,000; mice #2 and #4 had titers of 1:256,000, and mouse #3 had a titer of 1:128,000. Western blot results (not shown) indicated that the serum from mouse #1 yielded the best results, with clear and bright bands. Consequently, mouse #1 was selected for cell fusion. Splenic B cells from mouse #1 were fused with SP2/0 myeloma cells using polyethylene glycol (PEG) induction. As antibodies are secretory proteins, they can be detected directly in the supernatant of the cultured hybridoma cells. The following day, the supernatant was harvested and subjected to ELISA. The supernatants from 15 wells showing higher optical density (OD) values were selected for further analysis by Western blot.

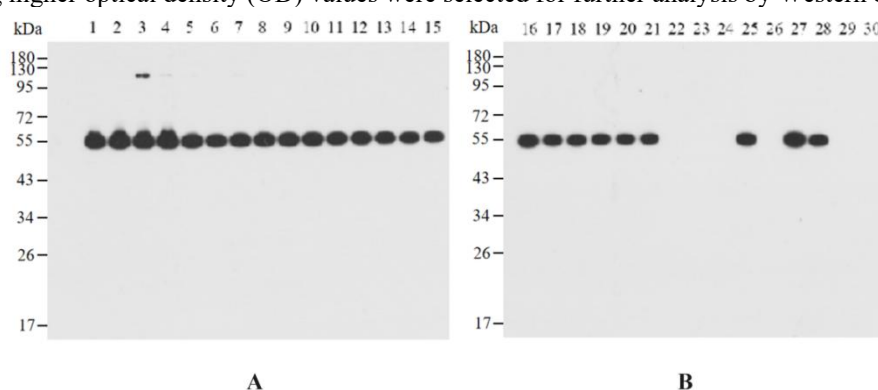


Fig 3 Western blot analysis of fusion supernatant. Lanes 1-15 correspond to the supernatants from 15 positive wells, respectively. The detection antigen was HT-29 cell protein.

From the fused cells, six clones with the best detection results were selected for subcloning. After one week of culture, the supernatant was collected for ELISA. The supernatants from 30 wells exhibiting higher OD values were analyzed by Western blot (Figure 3). Based on these results, five stable hybridoma cell lines were selected: 4 (designated 4H12E8), 13 (6C10G7), 15 (7F10G9), 18 (7G3A5), and 21 (7G10F11). These cell lines, which stably secreted the p53 antibody, were expanded in culture and then injected intraperitoneally into BALB/c mice.

Ascites were collected from five mice five days post-injection, selecting those in good condition. The ascitic fluid was centrifuged, and the supernatant was collected for antibody purification. Mouse ascites contains a significant amount of host proteins; thus, purification was performed using Protein A affinity chromatography to obtain high-purity antibodies. The eluted fractions were concentrated via ultrafiltration and buffer-exchanged into PBS. Protein concentration was determined using the Bradford method. The highest antibody yield (2 mg after purification) was obtained from the ascites of mice inoculated with the 4H12E8 hybridoma cell line. The purified antibodies were characterized by Western blot (Figure 4), immunohistochemistry (IHC), and immunofluorescence (IF) assays (Figure 5). For IHC, a goat anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (HRP) was used. For IF, a goat anti-mouse IgG secondary antibody conjugated to Cy3 was used.

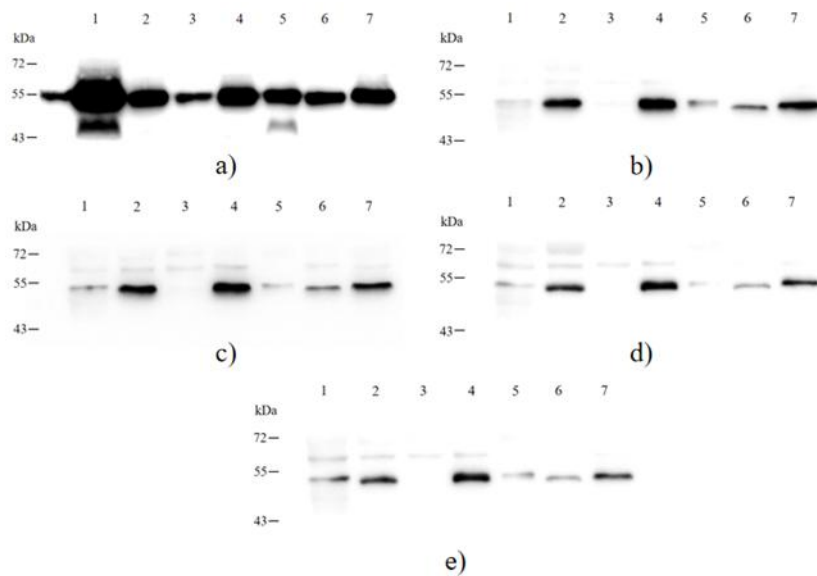


Fig 4 Western blot analysis of ascites antibodies. a) 4H12E8; b) 6C10G7; c) 7F10G9; d) 7G3A5; e) 7G10F11. Lanes 1-7: Protein lysates from HT-29, SW480, A549, 293, 293T, U251, and T-47D cell lines.

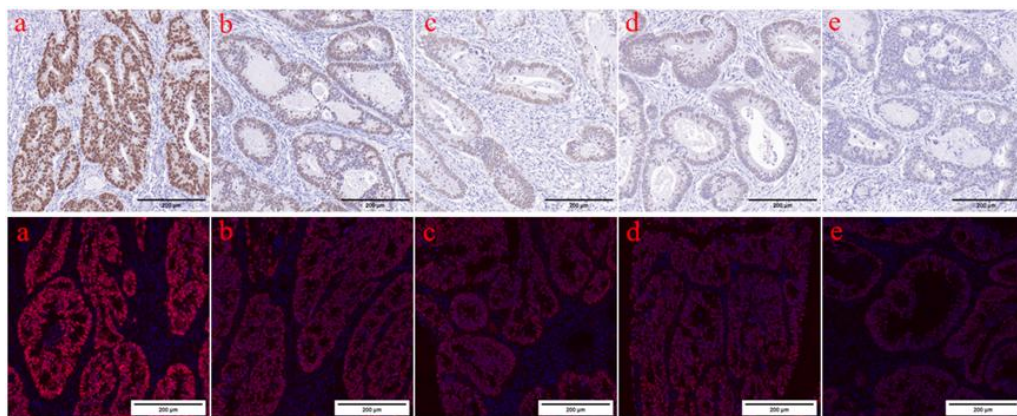


Fig 5 IHC and IF test results. a:4h12e8; b: 6c10g7; c: 7f10g9; d: 7g3a5; e: 7g10f11

The results demonstrated that the antibody produced by the 4H12E8 clone performed the best in the assays. In Western blot analysis, it exhibited minimal non-specific bands, with the target band appearing clear and bright. In esophageal cancer tissue samples, both IHC and IF staining showed no background or non-specific staining. The positive signals (brown in IHC, red in IF) were the most pronounced, indicating high specificity and titer of the antibody. Isotype testing confirmed that the antibody secreted by the 4H12E8 clone is of the IgG1 subclass. The 4H12E8 cell line was subsequently expanded, cryopreserved, and designated as the master cell line for subsequent antibody production and as a template for antibody gene retrieval.

3.3. Cloning and Transient Expression of Antibody Genes

Total RNA was extracted from the hybridoma cells and reverse-transcribed into cDNA. Primers were designed based on the nucleotide composition of the framework regions (FRs), mixed in equal proportions to create a primer pool, and used to amplify the antibody variable region genes. After amplification, agarose gel electrophoresis was performed. Upon successful amplification of the antibody heavy chain variable region (VH) and light chain variable region (VL), the target DNA bands were excised and subjected to gel extraction. The purified DNA fragments were then cloned using a T-A cloning strategy and sent for sequencing verification. Based on the sequencing results, specific primers

were designed: p53-VH-F and p53-VH-R for the heavy chain, and p53-V κ -F and p53-V κ -R for the light chain (kappa chain). PCR was performed using these specific primers to amplify the respective variable region genes. The plasmid pEE6.4-mIgG was digested with HindIII and EcoRI, and the plasmid pEE12.4-mC κ -GS was digested with XbaI and EcoRI. The purified antibody gene fragments (inserts) were then ligated into the corresponding linearized vectors via homologous recombination: the heavy chain gene (VH) was inserted into the pEE6.4-mIgG vector, and the light chain gene (V κ) was inserted into the pEE12.4-mC κ -GS vector. The resulting recombinant plasmids were designated as pEE6.4-mIgG-VH and pEE12.4-mC κ -V κ -GS, respectively. The plasmids pEE6.4-mIgG-VH and pEE12.4-mC κ -V κ -GS were co-transfected into HEK 293FT cells for transient expression. The cells were cultured in a fed-batch mode with a final volume of 100 mL. Approximately 7 days post-transfection, when cell viability dropped below 70%, the culture supernatant was harvested. The antibody was purified from the supernatant using a Protein A gravity column and analyzed by SDS-PAGE. The results (Figure 6A) showed that under reducing conditions, the heavy chain and light chain had apparent molecular weights of approximately 50 kDa and 25 kDa, respectively. Under non-reducing conditions, where disulfide bonds remain intact, the antibody migrated at approximately 150 kDa, consistent with the expected size of the full-length immunoglobulin. The antibody expression yield, as determined by biochemical analysis, was 36 mg/L.

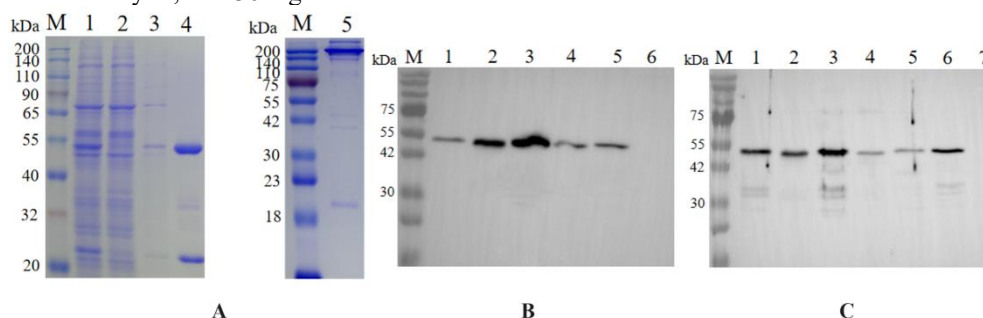


Fig 6 (A) SDS-PAGE analysis of purified transiently expressed antibody. M: Protein marker; Lane 1: Supernatant; Lane 2: Flow-through; Lane 3: Wash; Lane 4: Elution fraction; Lane 5: Elution fraction under non-reducing conditions. (B) Western blot analysis of antibody functionality. Detection was performed using the transiently expressed p53 antibody. (C) Western blot analysis of antibody functionality. Detection was performed using a commercial p53 antibody. M: Protein marker; Lanes 1-5: Protein lysates from HT-29, SW480, 293T, U251, and T-47D cell lines, respectively; Lane 6 (a): Protein lysate from H1299 (-) cells; Lane 6 (b): Protein lysate from A431 cells; Lane 7: Protein lysate from H1299 (-) cells.

The obtained p53 antibody was used as the primary antibody for immunohistochemistry (IHC) and immunofluorescence (IF) assays (Figure 6B). The antibody was diluted at ratios of 1:1000 (0.82 μ g/mL) for IHC and 1:300 (2.73 μ g/mL) for IF. An HRP-conjugated goat anti-mouse secondary antibody was used for IHC detection, while a Cy3-conjugated goat anti-mouse secondary antibody was employed for IF. Negative controls were performed by replacing the primary antibody with PBS.

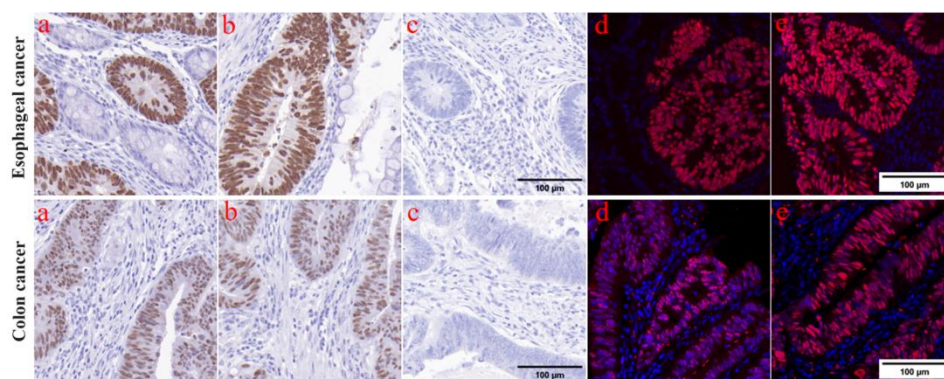


Fig 7 Immunohistochemistry (IHC) and immunofluorescence (IF) staining results in esophageal and colon cancer tissues. (a, d) Staining with the transiently expressed p53 antibody. (b, e) Staining with a commercial p53 monoclonal antibody. (c) Negative control.

The results (Figure 7) demonstrated that the transiently expressed p53 antibody exhibited high specificity with no background staining in both IHC and IF assays. In esophageal cancer tissue samples, the positive signals (brown in IHC, red in IF) were strong, and the staining effect was consistent with that observed using the commercial p53 monoclonal antibody.

3.4. Construction and Expression of Stable Transfectants

Following plasmid transfection and selection, a mixed pool of transfected cells was obtained. Although all cells in the pool contained the same target gene, the integration site within the chromosome varied, leading to a positional effect. This effect causes variations in antibody expression levels among individual cells. Therefore, the expression level was first evaluated using the mixed cell pool to preliminarily determine the efficiency of stable transfection. Concurrently, the functionality of the antibody expressed by the stable pool was assessed. The purification and functional characterization of the antibody from the stable transfectant pool are shown in Figure 8. The results indicated that the antibody was purified effectively, with no significant non-specific bands observed. Under reducing conditions on SDS-PAGE, the heavy and light chains migrated at approximately 50 kDa and 25 kDa, respectively (Figure 8A). Under non-reducing conditions, the antibody exhibited an apparent molecular weight of approximately 150 kDa, which is consistent with the expected size of an intact immunoglobulin (Figure 8A). The antibody expression level from the mixed pool, as measured by biochemical analysis, was 112.30 mg/L.

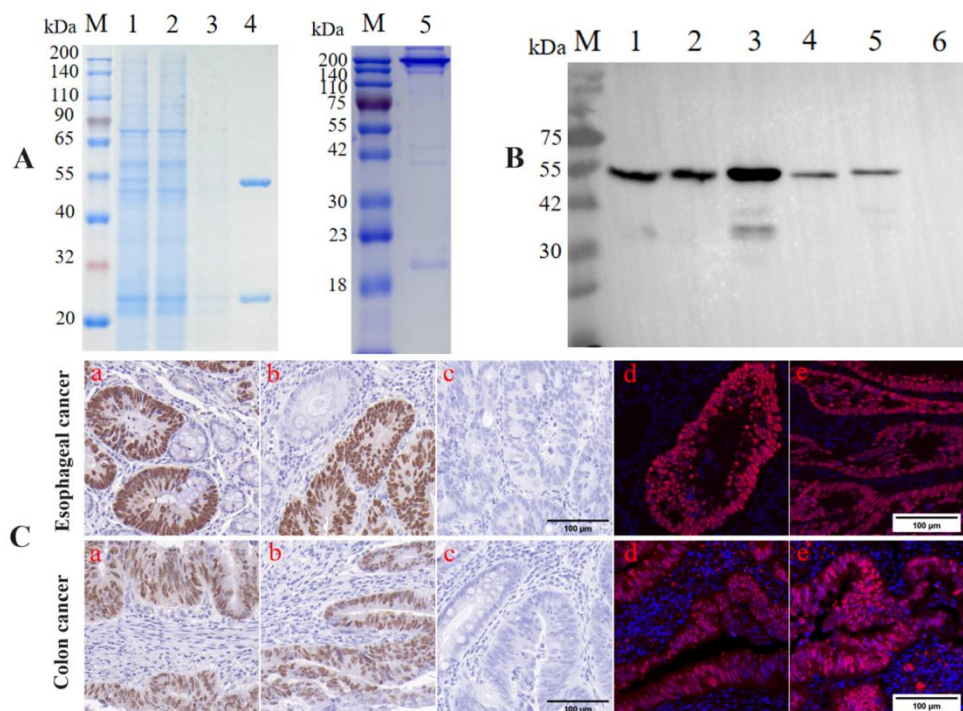


Fig 8 A: SDS-PAGE analysis of the purification results of stabilized antibody; B: Western Blot test for antibody function; C: IHC and IF detection junction for esophageal and colon cancer.

Western blot analysis was performed using the p53 antibody obtained from the stable transfectants (final concentration: 1 $\mu\text{g/mL}$). The results demonstrated that the antibody exhibited high specificity, with no noticeable non-specific bands observed (Figure 8B). The same antibody was also used for ICH and IF assays at a dilution of 1:6000 (working concentration: 0.61 $\mu\text{g/mL}$). The results showed strong specific staining with no background, distinct positive signals, and a performance consistent with that of the commercial p53 monoclonal antibody (Figure 8C).

3.5. Selection of High-Expression Clones

Following the functional characterization of the antibody, high-producing monoclonal cell lines were selected using the limiting dilution method. Monoclonal colonies were observed under a microscope on day 7. On day 14, the contents of viable wells were transferred to a 48-well plate. Dot blot assays were performed the next day to analyze the antibody concentration in the supernatants. Based on the dot blot results, the ten clones exhibiting the highest signal intensity were selected for small-scale expression in 50 mL shake flasks to measure antibody production. Among these, the monoclonal clone 3C2 achieved the highest expression level, reaching 512.40 mg/L (Figure 9B).

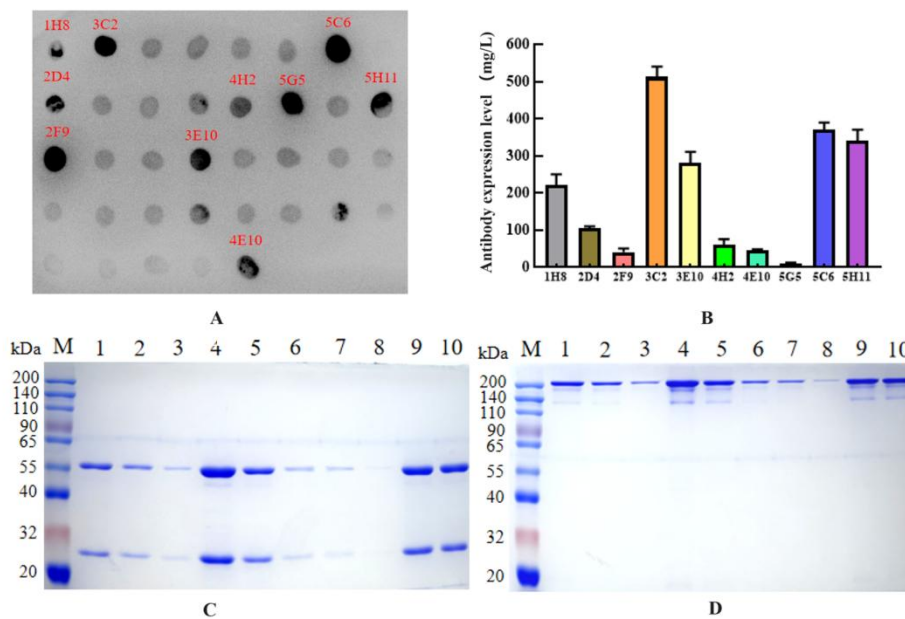


Fig 9 (A) Dot blot analysis of culture supernatants. (B) Antibody expression levels of the ten selected monoclonal cell lines. (C) Reducing SDS-PAGE analysis of purified antibodies from the ten monoclonal cell lines. (D) Non-reducing SDS-PAGE analysis of purified antibodies from the ten monoclonal cell lines. M: Protein marker; Lanes 1-10: Monoclonal cell lines 1H8, 2D4, 2F9, 3C2, 3F10, 4H2, 4E10, 5G5, 5C6, and 5H11.

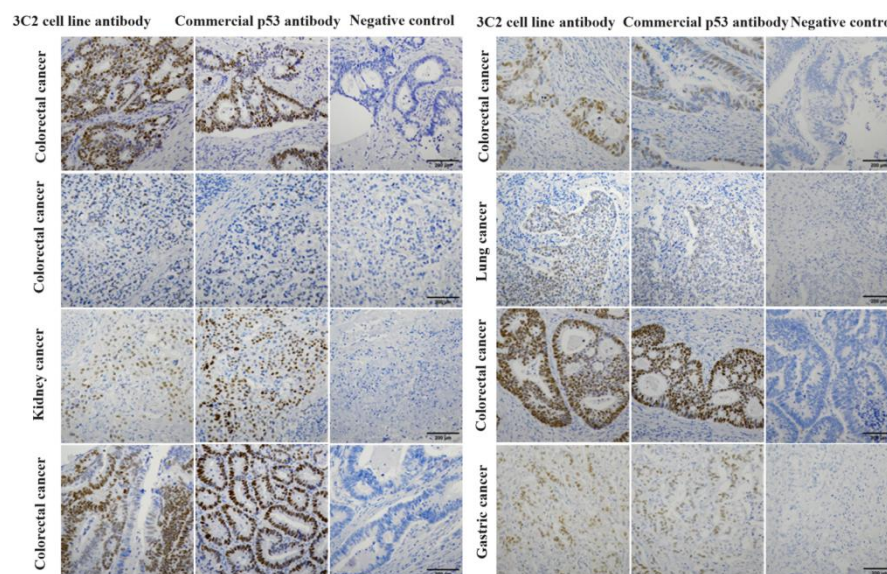


Fig 10 Multi-sample IHC detection.

The antibody expressed by the 3C2 clone was further evaluated using a broader panel of samples. A total of 65 clinical samples, including colorectal cancer, esophageal cancer, gastric cancer, renal cancer, lung cancer, and bladder cancer specimens, each from different patients, were analyzed by IHC and IF. Representative results are shown in Figure 10. The IHC and IF results were consistent. Staining intensity varied across different sample types: strong positive signals were observed in most colorectal cancer tissues, while weaker positivity was detected in gastric and lung cancer tissues.

3.6. Optimization of Antibody Expression Conditions

The high-producing 3C2 monoclonal cell line was selected for scale-up production. Because Growth A medium led to premature cell death in the bioreactor, the basal medium was switched to Media C. An orthogonal experimental design was employed to investigate the effects of three key factors on antibody expression: initial seeding density, temperature shift point, and temperature shift day. The experiments were performed in triplicate. The optimal conditions were determined to be an initial seeding density of 0.3×10^6 cells/mL, a temperature shift to 33°C, initiated on day 5 of culture. The effects of the different culture conditions on metabolic parameters (lactate, NH_4^+ , glutamine), antibody expression, cell density, and viability are shown in Figure 11. Lactate concentration decreased to its lowest level by day 7 across all conditions, before gradually increasing thereafter (Figure 11A). The NH_4^+ concentration fluctuated between 4 and 6 mmol/L throughout the cultures (Figure 11B). Glutamine accumulated continuously, with Condition 8 reaching the highest concentration of 1.50 mmol/L on day 17 (Figure 11C). The maximum cell density (10.4×10^6 cells/mL) was achieved on day 7 by Condition 1 (Figure 11E). A significant decrease in cell viability was observed between days 13 and 17; notably, viability in Condition 9 dropped to 87.2% by day 13 (Figure 11F).

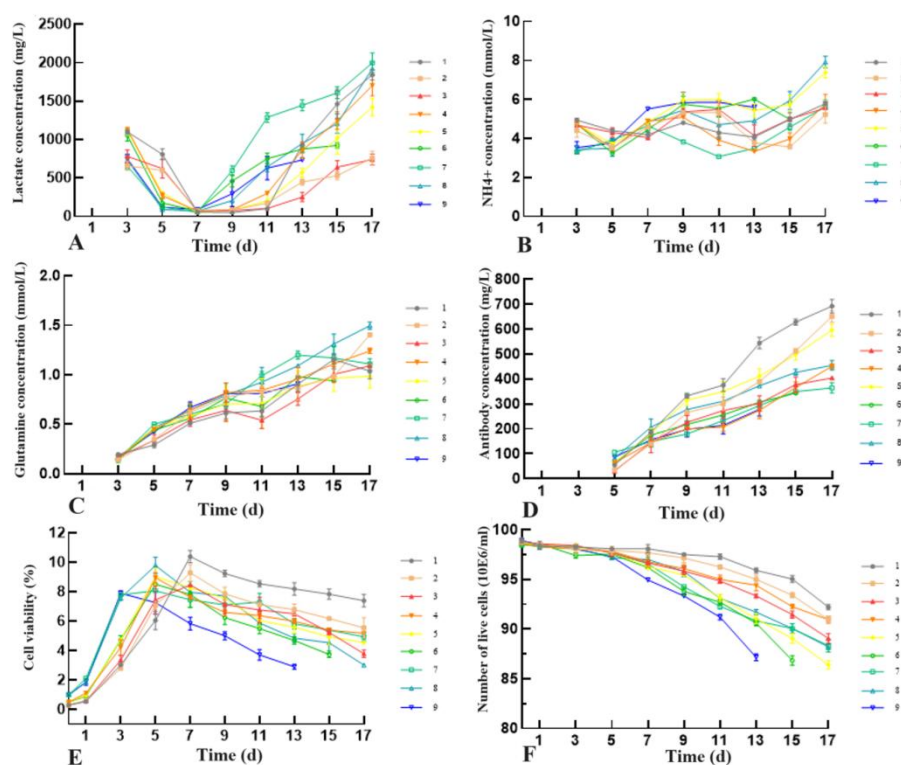


Fig 11 The impact of different culture conditions on cell metabolism and antibody production. (A) Lactate concentration; (B) NH_4^+ concentration; (C) Glutamine concentration; (D) Antibody expression level; (E) Cell density; (F) Cell viability.

3.7. Scale-up Production of the Antibody

The antibody was produced at a larger scale in a bioreactor using the optimized conditions. Key metabolic parameters and growth indicators during the culture process are shown in Figure 12. The lactate concentration exhibited a dynamic profile: it accumulated initially, was subsequently consumed, re-accumulated, and was finally consumed again at a slower rate (Figure 12A). Specifically, lactate accumulated gradually from days 1 to 5, was rapidly consumed from days 6 to 9, accumulated again from days 10 to 15, and decreased rapidly on days 16 and 17. The NH_4^+ concentration showed a continuous accumulation during the early phase (days 1-12), rising quickly to 5 mmol/L within the first 4 days, followed by a slow decline in the later phase (days 13-17; Figure 12B). The glutamine

concentration increased steadily throughout the culture, reaching a final concentration of 1.58 mmol/L on day 17 (Figure 12C). The antibody concentration also increased over time, reaching a final titer of 1006.17 mg/L on day 17 (Figure 12D). The peak cell density of 10.25×10^6 cells/mL was achieved on day 7 (Figure 12E). A noticeable decrease in cell viability occurred around day 13, and the culture was terminated on day 17 when viability dropped to 94.8% (Figure 12F).

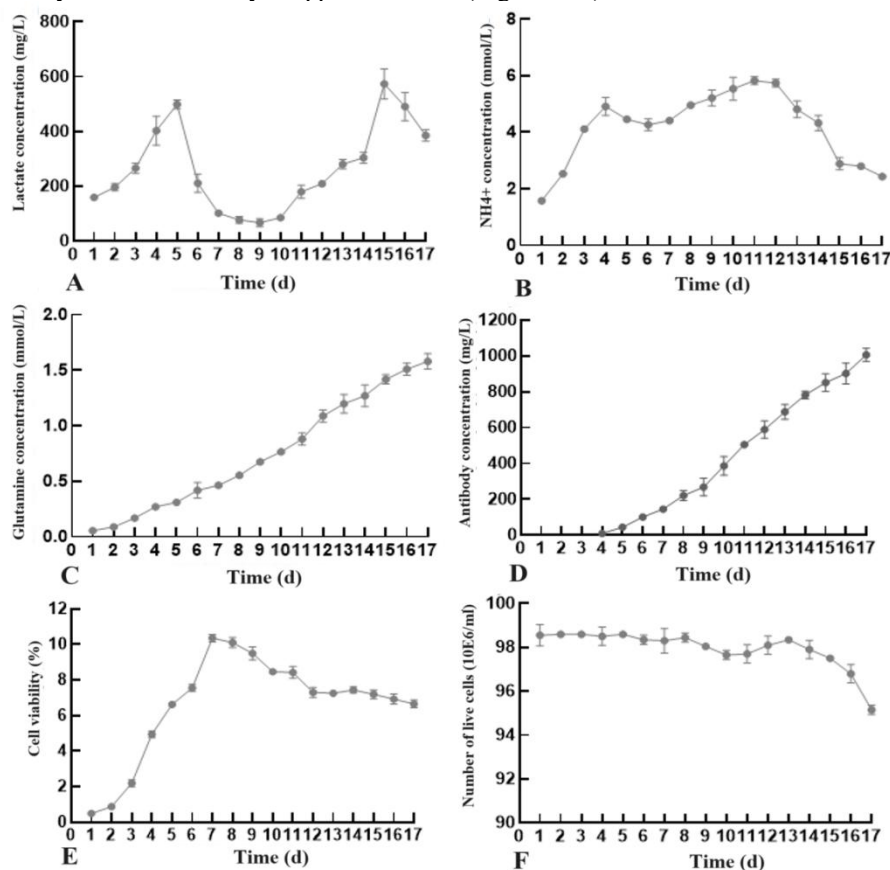


Fig 12 Metabolic profiles and growth parameters of cells in the bioreactor. (A) Lactate concentration; (B) NH_4^+ concentration; (C) Glutamine concentration; (D) Antibody titer; (E) Cell density; (F) Cell viability.

3.8. Purification and Analysis of the Antibody

Following centrifugation of the cell culture, the supernatant was collected, filtered through a 0.45 μm membrane, and purified using a Protein A gravity column. The eluted fractions were then concentrated and buffer-exchanged into PBS using a 10 kDa ultrafiltration device. The purity of the purified antibody was assessed by SDS-PAGE (Figure 13).

Figure 13A shows the analysis under non-reducing conditions, while Figure 13B shows the analysis under reducing conditions. The results indicated effective purification.

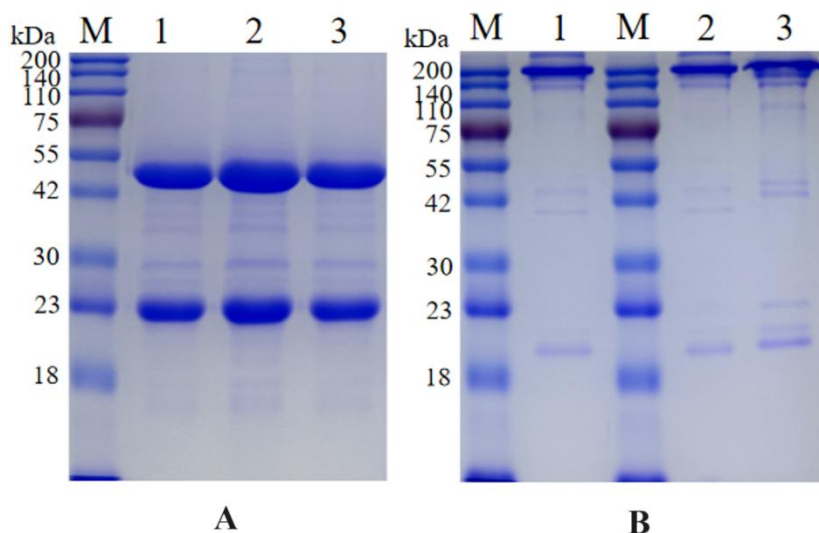


Fig 13 SDS-PAGE analysis of the purified antibody. (A) Reducing conditions; (B) Non-reducing conditions. M: Protein marker; Lanes 1-3: Three independent antibody purification samples.

Densitometric analysis using ImageJ software estimated the antibody purity to be approximately 90%. The average antibody expression titer, as measured by biochemical analysis, reached 1 g/L.

4. Discussion

Cancer remains a major threat to human health, with its incidence continuously rising. Currently, there is no complete cure for cancer, making early diagnosis critically important. For most cancer types, early detection followed by interventions such as surgical resection, chemotherapy, or radiotherapy can lead to favorable prognosis, alleviate the economic burden on patients, and prolong survival. Consequently, there is an urgent need for efficient and accurate cancer diagnostics in oncology research. Mutations in the tumor suppressor gene p53 are a significant factor in tumorigenesis and progression. The p53 gene demonstrates the highest association with human cancers among all known genes. Furthermore, as a biomarker, p53 not only facilitates exploration of disease mechanisms at the molecular level but also offers unique advantages for accurate assessment, early detection, and low invasiveness. It provides early warnings by reflecting prognostic features, recurrence risk, or disease progression, thereby offering substantial support for clinical auxiliary diagnosis.

This study involved the design of a p53 antigen. The p53 antigen gene was cloned into a pET32a-p53 vector and expressed in *E. coli*. A p53 monoclonal antibody was generated using hybridoma technology. Characterization showed that the p53 monoclonal antibody exhibited high specificity and titer, performing comparably to a commercial p53 murine monoclonal antibody. However, production via mouse ascites presents limitations in yield, animal ethics concerns, and risks of antibody gene loss. Therefore, this study focused on developing a recombinant antibody based on the hybridoma cells. First, antibody variable region genes were amplified from the hybridoma cells using primers designed from literature and cloned to construct the pEE12.4-p53-GS eukaryotic expression vector. After initial characterization by transient transfection, stable CHO cell lines were generated using the glutamine synthetase (GS) system. A high-producing monoclonal cell line (3C2) was selected following screening in shake flasks. The antibody from the 3C2 clone underwent extensive functional validation, including Western blot, IHC, IF, ICC, and titer determination. The results confirmed that the recombinant antibody from the 3C2 clone performed equivalently to the monoclonal antibody, with a titer of 1:2,048,000 and an expression level of 512.40 mg/L, indicating successful cloning of a high-affinity, specific antibody variable region sequence. To optimize expression conditions for the 3C2 cell line, an orthogonal experimental design was employed,

identifying the optimal conditions as an initial seeding density of 0.3×10^6 cells/mL and a temperature shift to 33°C on day 5. Using these parameters in a 3 L bioreactor, the final antibody titer reached 1 g/L.

In summary, this study successfully generated a p53 monoclonal antibody via hybridoma technology. After cloning the antibody variable region genes and constructing a eukaryotic expression vector, a high-producing monoclonal cell line was screened in CHO-GS KO cells. Process optimization increased antibody expression yield approximately 9-fold. The recombinant antibody demonstrated equivalent performance to the monoclonal antibody in applications. Large-scale, high-yield production was achieved using a bioreactor. The high-affinity antibody clone obtained in this study could potentially help reduce the market price of p53 antibodies, contribute to pathological diagnostics and in-depth p53 protein research, and provide a foundation for developing humanized p53 antibodies.

Data Availability Statement

All data generated or analyzed during this study are included in this published article and its supplementary information files. No nucleotide sequencing data were generated in this study.

Ethics Approval: All animal experiments described in this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Hubei Academy of Agricultural Sciences (approval number HBAAS-IACUC-2023-018). All experimental procedures were strictly conducted in accordance with the Guide for the Care and Use of Laboratory Animals and relevant international guidelines. The research involving human tissue samples was approved by the Medical Ethics Committee of the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (approval number UH-TJMC-2023-056). The use of all human tissue samples complied with the principles of the Declaration of Helsinki and the Measures for the Ethical Review of Biomedical Research Involving Humans (China).

Informed Consent: The human cancer tissue samples used in this study—including colorectal cancer, esophageal cancer, gastric cancer, renal cancer, lung cancer, and bladder cancer specimens—were obtained from archived surgical resection specimens. All patients or their legal guardians provided written informed consent prior to surgery, agreeing to the use of their tissue samples for subsequent medical research purposes. This study did not involve the disclosure of personally identifiable information; all samples were coded to ensure full protection of patient privacy.

Animal Welfare Statement: This study strictly adhered to the 3R principles (Replacement, Reduction, and Refinement) in the conduct of animal experiments. Female BALB/c mice (6–8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China; license no. SCXK (Jing) 2021-0006) and housed in the specific-pathogen-free (SPF) animal facility of the Experimental Animal Center, Hubei Academy of Agricultural Sciences. Housing conditions complied with national standards (temperature 20–26°C, humidity 40–70%, 12-hour light/dark cycle). For ascites collection, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The depth of anesthesia was assessed by the absence of the pedal withdrawal reflex. The mouse immunization protocol, hybridoma preparation, and ascites collection procedures were all reviewed by the ethics committee to ensure minimization of animal suffering throughout the experiments. Upon completion of the experiments, all animals were humanely euthanized by intraperitoneal overdose of sodium pentobarbital (150 mg/kg body weight), followed by cervical

dislocation, in accordance with the AVMA Guidelines for the Euthanasia of Animals. Death was confirmed by the absence of pupillary light reflex and cessation of heartbeat.

Cell Line Use Statement: The cell lines used in this study and their sources are as follows: A549 (ATCC® CCL-185™), HEK 293T (ATCC® CRL-3216™), HEK 293FT (Thermo Fisher Scientific, Cat. No. R70007), HT-29 (ATCC® HTB-38™), SW480 (ATCC® CCL-228™), U251 (ATCC® HTB-17™), T-47D (ATCC® HTB-133™), and CHO-GS KO (Horizon Discovery Ltd., Cat. No. GSKO). All cell lines were preserved in our laboratory, and their identities have been authenticated by short tandem repeat (STR) profiling.

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Conflicts of Interest: The authors declare no conflict of interest.

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