



Production and Evaluation of Cost-Effective Polyclonal Antibodies Against Human Cytokeratin 7 and 20 for Immunohistochemical Applications

Zahra Asadi Samani¹, Hamid Bakherad², Marzieh Rezaei^{1*}

¹Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran;

²Department of Pharmaceutical Biotechnology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

ABSTRACT

Background: Cytokeratin 7 (CK7) and cytokeratin 20 (CK20) are widely used immunohistochemical (IHC) markers for determining the origin of epithelial malignancies, particularly in cases of carcinoma of unknown primary. However, access to high-quality commercial antibodies remains limited in many laboratory settings. In this study, a practical and reproducible workflow was established to generate mouse-derived polyclonal antibodies against human CK7 and CK20.

Methods: Recombinant full-length proteins and epitope-enriched Recombinant fragment fragments were expressed in *Escherichia coli*, purified, and used for immunization. Antibody responses were evaluated by ELISA, while specificity and diagnostic applicability were assessed using Western blot and IHC on formalin-fixed, paraffin-embedded tissue sections. Post-production refinement, including protein A purification and negative absorption, was applied to improve antibody performance.

Results: High-titer antibody responses were successfully generated, with epitope-based antigens eliciting stronger and more selective reactivity compared to full-length proteins. Initial antibody preparations showed broader epithelial reactivity, consistent with the conserved structure of cytokeratin proteins. However, purification and absorption steps significantly reduced non-specific staining and improved specificity. In IHC analyses, the generated antibodies demonstrated staining patterns consistent with established CK7/CK20 expression profiles and comparable interpretability to commercially available reagents.

Conclusion: This study demonstrates that diagnostically usable polyclonal antibodies against human CK7 and CK20 can be produced using a cost-effective and accessible approach. The proposed workflow provides a practical solution for laboratories with limited access to commercial antibodies and supports the continued use of IHC as a reliable tool for tumor classification.

Keywords: Cytokeratin 7, Cytokeratin 20, Polyclonal antibodies, Immunohistochemistry, Diagnostic pathology

*Corresponding author:

Marzieh Rezaei,
Department of Immunology,
School of Medicine, Isfahan
University of Medical Sciences,
Isfahan, Iran
Email: ma.rezaei@med.mui.
ac.ir

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INTRODUCTION

Cytokeratins (CKs), a family of intermediate filament proteins, constitute essential components of the cytoskeleton in epithelial cells, where they contribute to structural integrity, cellular differentiation, and intracellular signaling pathways (1, 2). Due to their tissue-specific expression patterns and altered regulation in pathological conditions, particularly malignancies, CKs have become indispensable biomarkers in diagnostic pathology. Among more than 20 identified CK isoforms, cytokeratin 7 (CK7) and cytokeratin 20 (CK20) are of particular importance because of their distinct and complementary expression profiles across a wide range of epithelial tumors (3). CK20 is characteristically expressed in gastrointestinal epithelium, particularly in the intestinal epithelium, and is also expressed in urothelial epithelium, and Merkel cells. Its immunohistochemical expression is therefore useful in the evaluation of carcinomas, particularly colorectal adenocarcinomas, urothelial carcinomas, and Merkel cell carcinomas (4). In contrast, CK7 is expressed in a broad range of epithelial tissues and is commonly detected in carcinomas of the lung, breast, ovary, and endometrium. CK7 is generally absent or expressed only focally in conventional colorectal adenocarcinomas and is typically negative in prostatic adenocarcinoma. Consequently, the combined CK7/CK20 immunophenotype can provide valuable information for distinguishing colorectal and other gastrointestinal carcinomas from tumors of several non-gastrointestinal origins (5).

The combined immunohistochemical (IHC) assessment of CK7 and CK20 expression patterns is a well-established component of the diagnostic evaluation of metastatic carcinomas and can assist in determining the likely site of tumor origin. For example, a CK7+/CK20- phenotype is commonly observed in adenocarcinomas of the lung and breast, although this

immunophenotype is not specific to these sites. In contrast, a CK7-/CK20+ phenotype is characteristic of conventional colorectal adenocarcinomas and, in the appropriate clinical and morphological context, supports a colorectal origin (3, 6).

This diagnostic approach is particularly useful in the evaluation of carcinoma of unknown primary (CUP), a heterogeneous group of metastatic malignancies in which the primary tumor site cannot be identified despite appropriate clinical radiological, and pathological evaluation (7, 8).

Accounting for approximately 1–2% of all cancer diagnoses, CUP is a clinically important cause of cancer-related mortality and has historically been reported to account for approximately 3–5% of all cancer diagnoses (8, 9). Advances in diagnostic imaging, histopathological assessment, immunohistochemistry, and molecular profiling have contributed to a decline in CUP incidence in developed nations (10–12). CUP exhibits distinctive biological and clinical characteristics, including early metastatic dissemination, heterogeneous metastatic patterns, and, in many cases, aggressive clinical course (13). A substantial proportion of patients with CUP have been reported to present with multiple metastatic sites, whereas only 15–20% exhibit favorable clinical or pathological features and may be classified into favorable subsets (14, 15). For example, pancreatic cancer presenting as CUP has been reported to show a markedly increased frequency of bone metastases compared with pancreatic cancer with an identified primary tumor (13). In a systematic review of 12 autopsy studies involving 884 patients with CUP, a primary tumor was identified post-mortem in 73% of cases; among these, the lung (27%), and pancreas (24%) were the most frequently identified primary sites, followed by the hepatobiliary tract (8%) and kidney (8%) (16).

Polyclonal antibodies comprise heterogeneous populations of immunoglobulins that recognize multiple

epitopes on a target antigen. In IHC, this multi-epitope recognition can enhance detection sensitivity and increase the likelihood of detecting antigens when some epitopes are masked or altered by fixation, tissue processing, or partial denaturation (17). Polyclonal antibodies may also be more tolerant of minor antigenic variations, such as sequence polymorphisms or conformational changes, than antibodies directed against a single epitope (18).

However, these potential advantages are accompanied by important limitations. Recognition of multiple epitopes may help preserve antibody reactivity when some epitopes are altered or masked during fixation or tissue processing; however, this potential advantage should not be interpreted as greater chemical stability under different storage conditions. Polyclonal antibody preparations may exhibit lot-to-lot variability, cross-reactivity with unrelated antigens, and increased nonspecific background staining, particularly in immunohistochemistry (IHC). Therefore, rigorous validation is essential to establish staining specificity, sensitivity, and reproducibility. Storage-related loss of antibody activity can affect both polyclonal and monoclonal antibodies and is not unique to either format. Nevertheless, locally produced and adequately validated polyclonal antibodies may offer a potentially cost-effective alternative to commercial reagents, particularly where access to validated commercial antibodies is limited.

IHC remains a cornerstone of the diagnostic evaluation of CUP, complementing histomorphological assessment by using characteristic patterns of protein expression to determine tumor lineage and narrow the differential diagnosis of the primary site. CUP encompasses a heterogeneous spectrum of histological subtypes, with adenocarcinoma being the predominant histological type, followed by poorly differentiated adenocarcinoma, (15–30%), squamous cell carcinoma, and undifferentiated neoplasms (11). In a large registry-based study, moderately

and well-differentiated adenocarcinomas accounted for 51.3% of CUP cases, followed by poorly differentiated carcinomas (23.2%) and squamous cell carcinomas (12.5%) (Ref: DOI: 10.1093/milmed/usab291). However, histomorphological classification alone is often insufficient to establish the tissue of origin particularly in poorly differentiated or undifferentiated tumors. Accordingly, IHC panels incorporating lineage-associated and organ-associated markers, including CK7 and CK20, are used to further characterize these tumors and narrow the differential diagnosis (15). Despite advances in genomic and transcriptomic profiling for predicting tumor origins, the relatively high cost and technical requirements of these approaches may limit their availability, particularly in resource-limited settings (19, 20). Consequently, IHC, integrated with morphological and clinical assessment, remains a key component of the initial and cost-effective diagnostic work-up of CUP (21).

In this context, the present study aims to develop and evaluate mouse-derived polyclonal antibodies against CK7 and CK20 using a practical and reproducible approach suitable for resource-constrained laboratory settings. By focusing on the feasibility of antibody production and its applicability to IHC, this study seeks to provide potentially accessible and cost-effective reagents for the immunohistochemical characterization of epithelial malignancies, particularly in settings where access to validated commercial antibodies is limited.

MATERIALS AND METHODS

Antigen Design and Recombinant Constructs

The amino acid sequences of human CK7 (KRT7; UniProt accession P08729) and CK20 (KRT20; UniProt accession P35900) were retrieved from the UniProt database (<https://www.uniprot.org/>). KRT7 (UniProt P08729) comprises 469 amino acids, and its coding sequence corresponds to the canonical

transcript RefSeq NM_005556.4. KRT20 (UniProt P35900) comprises 424 amino acids, and its coding sequence corresponds to RefSeq NM_019010.3. The selected sequences represent the canonical human isoforms. Two antigen-design strategies were employed: full-length recombinant proteins and selected Protein Epitope Signature Tag (PrEST)-derived recombinant fragments. The coding sequences of full-length CK7 and CK20 were codon-optimized for expression in *Escherichia coli* and cloned into the pET28a and pET42a expression vectors, respectively. The PrEST-derived recombinant fragments were selected based on antigenic regions reported in the Human Protein Atlas (HPA) and available epitope information for commercial antibodies. For CK7, the antigenic sequence associated with HPA entry HPA007272, corresponding to the Sigma-Aldrich antibody, was selected; for CK20, the corresponding sequence associated with HPA024309 was selected. The selected amino acid sequences were reverse-translated, codon-optimized for expression in *E. coli*, and cloned into the pET42a expression vector. All recombinant constructs were commercially synthesized (Biomatik, Canada).

The selected antigenic regions were evaluated using the Immune Epitope Database (IEDB) (<https://www.iedb.org/>), ProtScale (<https://web.expasy.org/protscale>), Protein-Sol (<https://protein-sol.manchester.ac.uk>), ProtParam (<https://web.expasy.org/protparam>), BLAST (<https://blast.ncbi.nlm.nih.gov/Blast>), and AbDesigner (<https://www.abdesigner.org>) to assess predicted antigenicity, sequence accessibility, solubility, physicochemical properties, sequence uniqueness, and suitability for antibody production. Available structural information from HPA, including the AlphaFold-predicted three-dimensional structures of KRT7 and KRT20, was considered during antigen selection. The selected antigen sequences were visualized within the predicted protein structures to assess their structural context and potential accessibility for antibody

recognition.

For the CK7 PrEST-derived recombinant fragment, the HPA-derived antigenic sequence was combined with an additional 37-amino-acid sequence (AKSSRLPDIFE AQIAGLRGQLEALQVDGGRLEAELRS). This sequence was selected based on sequence-comparison analysis intended to identify a region with low similarity to other human proteins and no detectable homology to other keratins. The additional region was included in the recombinant immunogen used for mouse immunization as part of the antigen-design strategy. It was also intended to facilitate potential epitope-based affinity purification; however, affinity purification using this 37-amino-acid sequence was not performed in the present study. The additional sequence was followed by an ENLYFQS TEV protease cleavage site downstream of the PrEST-derived recombinant fragment.

The CK20 PrEST-derived recombinant fragment was based on the HPA-derived antigenic sequence associated with HPA024309. The corresponding sequence was codon-optimized for expression in *E. coli*, commercially synthesized, and cloned into the pET42a expression vector.

Preparation of Competent E. coli BL21(DE3) Cells

Competent *E. coli* BL21(DE3) cells were prepared using the MgCl₂-CaCl₂ method. Briefly, a single colony was inoculated into 5 mL of antibiotic-free Luria-Bertani (LB) medium and cultured overnight at 37°C, with shaking at 150 rpm. Subsequently, 0.5 mL of the overnight culture was transferred to 50 mL of antibiotic-free LB medium and incubated at 37°C with shaking until the culture reached an optical density at 600 nm (OD₆₀₀) of 0.4–0.6. The culture was chilled on ice, and the cells were harvested by centrifugation at 5,000 rpm for 10 minutes at 4°C.

The cell pellet was gently resuspended in 25 mL sterile 0.1 M MgCl₂ and incubated on ice, followed by centrifugation under the same conditions. The resulting pellet was

then gently resuspended in 25 mL sterile 0.1 M CaCl₂, incubated on ice for 20 minutes, and centrifuged again. Finally, the pellet was resuspended in 1.5 mL of 0.1 M CaCl₂ containing 15% glycerol. The competent cells were aliquoted into 100 µL portions, and stored at -70°C until use.

Competency was assessed by transformation with an appropriate control plasmid and subsequent selection on antibiotic-containing LB agar. Growth of transformed cells on selective medium was used to confirm successful acquisition of the plasmid. Competency was assessed by transformation with an appropriate control plasmid (pET-28a(+), which carries a kanamycin-resistance marker), followed by selection on antibiotic-containing LB agar. Growth of transformed cells on the selective medium was used to confirm the transformation competence of the prepared cells.

Bacterial Transformation and Protein Expression

Competent *E. coli* BL21(DE3) cells were transformed with the recombinant plasmids by heat shock at 42°C for 2 min. Following recovery, the transformed cells were plated on LB agar containing kanamycin and incubated at 37°C. Kanamycin-resistant colonies were selected for subsequent recombinant protein expression. Protein expression was induced by addition of IPTG to a final concentration of 0.5 mM, and induction was evaluated at 25°C and 37°C for different time periods to determine the optimal expression conditions (23). Protein expression was assessed by SDS-PAGE. Based on the SDS-PAGE results, induction at 37°C for 16 h produced the highest level of recombinant protein expression and was therefore selected for subsequent large-scale protein production.

Protein Extraction and Purification

Recombinant proteins were recovered using two complementary extraction approaches: Recombinant proteins were recovered using two complementary extraction approaches:

Denaturing extraction with urea buffer: Cell pellets were resuspended in 8 M urea buffer, and subjected to sonication for 7 cycles of 30 s each. The lysates were then incubated at room temperature for 24 h, and centrifuged at 15,000 rpm for 15 min. The resulting supernatants containing solubilized recombinant proteins were collected for subsequent purification by Ni-NTA affinity chromatography (24).

Inclusion body (IB) washing and alkaline treatment: For IB washing and removing residual host-cell proteins and other cellular contaminants cell pellets were resuspended in 50 mM Tris buffer containing 25% (w/v) sucrose (Merck, Germany) and 10 mM 2-mercaptoethanol (2-ME), followed by cell lysis in buffer containing 1% Triton X-100. Cell disruption was performed by sonication for seven cycles of 30 s each. The lysates were centrifuged at 12,000 rpm for 20 min at 4°C to recover the insoluble inclusion-body fraction. The resulting pellet was extracted with 50 mM Tris-HCl buffer adjusted to pH 12. After washing, the samples were centrifuged at 12,000 rpm for 20 min at 4°C. Under these alkaline conditions, the recombinant target protein was released from the inclusion-body fraction and recovered in the supernatant. The supernatant was collected and analyzed by SDS-PAGE, and the fraction showing the strongest band corresponding to the target recombinant protein was selected for subsequent purification. Protein-containing fractions obtained from both extraction approaches were purified by Ni-NTA affinity chromatography based on the presence of the recombinant His tag. For urea-extracted samples, urea concentration was gradually reduced during the purification process. Low concentrations of imidazole were used during the washing step to remove nonspecifically bound proteins, followed by elution of the His-tagged and recombinant proteins with increasing concentrations of imidazole (100–500 mM).

The purity of the purified proteins was assessed by SDS-PAGE, and protein concentrations were determined using the

Bradford assay.

SDS-PAGE and Western Blot Analysis

Protein expression and apparent molecular integrity were assessed by SDS-PAGE followed by Coomassie Brilliant Blue staining. For Western blot analysis, proteins were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes at 80 V for 1 h in transfer buffer. The membranes were blocked with 5% skim milk in PBST (PBS containing 0.1% Tween 20) and incubated with an HRP-conjugated anti-His-tag antibody (Sigma, Germany). Following washing with PBST, the membranes were developed using 3,3'-diaminobenzidine (DAB) substrate for 15 min. SDS-PAGE and Western blot analyses were each performed independently in triplicate.

Protein Dialysis

Purified recombinant proteins were dialyzed against PBS to remove imidazole and exchange the protein into a suitable buffer for subsequent applications. Briefly, 1 mL of sample was transferred into a dialysis membrane with a molecular-weight cutoff (MWCO) of 10 kDa. Before use, the dialysis membrane was prepared according to the manufacturer's instructions. The sample was dialyzed against 200 mL of PBS with gentle shaking at 4°C for 2 h after which the PBS was replaced twice in total, including an overnight dialysis step at 4°C. Following dialysis, glycerol was added to a final concentration of 60% (v/v), and the protein preparations were aliquoted and stored at -70°C.

Animal Immunization and Serum Collection

Twenty female BALB/c mice (6–7 weeks old) were randomly assigned to four groups (n = 5 per group). Each group was immunized intraperitoneally with one of the following recombinant antigens: full-length human CK7, CK7PrEST-derived recombinant fragment, full-length CK20, or CK20 PrEST-derived recombinant fragment. For the primary

immunization, recombinant antigens (40–50 µg/mL; 300 µL per mouse) was emulsified with complete Freund's adjuvant. Three booster immunizations were subsequently administered at 2-week intervals using the corresponding antigen (30 µg/mL, 150 µL per mouse) emulsified with incomplete Freund's adjuvant. Blood samples were collected before the initial immunization and after each booster immunization to monitor the development of antigen-specific antibodies. At the end of the immunization schedule, the mice were euthanized according to the approved animal-study protocol and blood samples were collected. Sera were separated by centrifugation and stored at -70°C until further analysis.

Antibody Titer Determination

Antibody titers against the corresponding recombinant antigens were determined by indirect enzyme-linked immunosorbent assay (ELISA). Microplates were coated with recombinant antigens at a concentration of 0.5 µg/mL in carbonate-bicarbonate coating buffer (pH: 9.5) and incubated under the specified coating conditions. Following washing, wells were blocked with 1% bovine serum albumin (BSA). Mouse sera were serially diluted (1:16,000, 1:32,000, 1:64,000, and 1:128,000) and added to the antigen-coated wells. After incubation and washing, bound mouse antibodies were detected using an HRP-conjugated anti-mouse secondary antibody (Sigma, dilution 1:3000). Following further washing TMB substrate was added, and the enzymatic reaction was measured at 450 nm.

In addition, two types of control wells were included. Antigen-coated wells containing the HRP-conjugated anti-mouse secondary antibody in the absence of mouse serum were used to assess background signal contributed by the secondary antibody and other assay components. Uncoated wells containing the corresponding mouse serum and the secondary antibody were included to assess non-specific binding to the plate or other assay

components. All samples were analyzed in technical triplicate. The ELISA cut-off was defined as the mean optical density (OD) of the preimmune sera before immunization plus two standard deviations (Mean preimmune serum + 2SD). The endpoint antibody titer was defined as the highest serum dilution yielding an OD value equal to or greater than the predefined cut-off. For each sample, the mean OD of the technical triplicates was used to determine positivity at each dilution.

Antibody Purification and Pre-Absorption

Sera were subjected to Protein A affinity chromatography to enrich the IgG fraction. Serum samples were filtered and applied to Protein A resin equilibrated with binding buffer (100 mM sodium phosphate, pH 7.5). The serum was allowed to bind to the resin for 2 h under room temperature with shaking.

The resin was subsequently washed with binding buffer, and bound IgG was eluted using 50 mM sodium citrate buffer [pH 3.0]. Eluted fractions were immediately neutralized with 1 M Tris buffer.

To reduce non-specific binding and cross-reactivity, the purified IgG fractions were subjected to sequential negative absorption. For anti-CK20 antibodies, the IgG fraction was incubated with preparations containing CK7-PrEST-derived recombinant fragment, MCF7 cell lysates, and non-transformed bacterial lysates. For anti-CK7 antibodies, the IgG fraction was similarly absorbed using CK20-PrEST-derived recombinant fragment, PrEST-derived recombinant fragment, HT29 cell lysates, and non-transformed bacterial lysates. To further reduce cross-reactivity between CK7 and CK20, anti-CK7 and anti-CK20 IgG fractions were separately absorbed against the corresponding non-target cytokeratin antigens to remove antibodies recognizing shared or homologous epitopes between the two cytokeratins. (All cell lines and bacterial strain used in this study were obtained from the Pasteur Institute of Iran. MCF7 and HT29 cell lines were selected for negative absorption based on the absence of

the target cytokeratin in these cell lines, with the aim of minimizing nonspecific antibody binding while preserving target-reactive antibodies).

Following each absorption step, the unbound IgG fractions were recovered and retained for subsequent analyses.

Immunohistochemical Validation of Antibody Performance

The performance and specificity of the generated antibodies and their quality in each preparation was evaluated by IHC using formalin-fixed, paraffin-embedded human tissue sections. Tissue sections were incubated at 45–50°C overnight, followed by deparaffinization in xylene for 30 min and rehydration through graded ethanol solutions. Sections were subsequently washed in Tris-buffered saline (TBS, pH 7.4). Heat-induced antigen retrieval was performed using Tris-EDTA buffer (pH 9.0) in a pressure cooker for 20 min. Following antigen retrieval, endogenous peroxidase activity was blocked, and the sections were incubated with 10% goat serum for 40 min to reduce non-specific antibody binding. Immunohistochemical staining was performed using the Master Polymer Plus Detection System IHC kit (Master Diagnostica, Spain) according to the manufacturer's instructions.

Anti-CK7 and anti-CK20 antibodies (Immun serum and purified and pre-absorbed IgG) were used as primary antibodies. Anti-CK7 antibodies generated against the full-length-protein and CK7 PrEST-derived recombinant fragment were evaluated at a dilution of 1:3000, whereas anti-CK20 antibodies generated against the full-length-protein and CK20 PrEST-derived recombinant fragment were evaluated at a dilution of 1:1000 in PBS. Sections were incubated with primary antibodies for 1.5 h at room temperature. After washing, a binding enhancer and HRP-conjugated secondary antibody Master Diagnostica, Spain) were applied according to the manufacturer's instructions. Immunoreactivity was visualized

using DAB chromogen for 5 min, followed by counterstaining with hematoxylin. Sections were subsequently dehydrated, cleared, and mounted for microscopic evaluation. All IHC experiments were performed in triplicate (25).

Human ovarian tissue was used as a positive control for CK7, and human colonic tissue was used as a positive control for CK20. For negative control assessment of CK7, only areas of liver tissue showing no CK7 expression were considered, while skeletal muscle and endometrial tissues were used as negative controls for CK20.

Specificity and Cross-Reactivity Assessment

Antibody specificity and potential cross-reactivity after Protein A-purification and negative absorption were evaluated by Western blot analysis using cell lysates from cell lines with characterized CK7 and CK20 expression profiles. Cell lysates from HT-29 cells, which lack CK7 expression but express CK20, were used to evaluate CK20 reactivity and the absence of non-specific reactivity toward CK7-negative samples. MCF-7 cell lysates, which lack CK20 expression, were used to assess CK7 reactivity and the absence of non-specific reactivity toward CK20-negative samples. MSC (mesenchymal stem cell) lysates, which lack both CK7 and CK20, were used as a negative control for both antibodies. In addition, SW1116 cell lysates, which lack CK7 expression, were included to further assess the specificity of the anti-CK20 antibody in a CK7-negative cellular background.

For Western blot analysis, cell lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking, the membranes were incubated with the purified anti-CK7 or anti-CK20 antibody at a dilution of 1:100, followed by incubation with an appropriate HRP-conjugated secondary antibody at a dilution of 1:3000. Finally, protein bands were visualized using a 3,3'-diaminobenzidine (DAB) detection system.

The reactivity patterns obtained by Western blot were compared with the

characterized CK7 and CK20 expression profiles of the corresponding cell lines. Detection of the expected target protein in cell lysates expressing the corresponding keratin, together with the absence of specific bands in cell lysates lacking the target keratin, was used to assess antibody specificity and potential cross-reactivity.

Statistical Analysis

ELISA data was analyzed using GraphPad Prism software (version 10.0) and are presented as mean $\pm$ standard deviation (SD). Data distribution was assessed using the Shapiro–Wilk test, which confirmed normality of the variables. Statistical significance among groups was determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant.

RESULTS

Bioinformatics Analysis of B-Cell Epitopes and Key Protein Properties

BepiPred 2.0 analysis identified two candidate linear B-cell epitope regions within the selected CK7 antigenic region, corresponding to residues 37–53 and 92–102, with prediction scores of 0.531 and 0.567, respectively. For the CK20 antigenic region, two candidate linear B-cell epitope regions were identified at residues 5–32 and 44–75, with prediction scores of 0.614 and 0.578, respectively (Table 1). These regions were considered computationally predicted candidate epitopes and were subsequently evaluated experimentally.

Protein-Sol analysis yielded predicted solubility scores of 0.851 for the CK7-derived sequence and 0.729 for the CK20-PrEST-derived recombinant fragment, both exceeding the predefined threshold of 0.45 (Table 1). These values represent computational predictions of solubility and were considered as supporting information during antigen selection rather than

Table 1. Predicted B-Cell Epitopes and Their Antigenic and Physicochemical Properties

Protein	Sequence	Bepipred Linear Epitope Prediction 2.0 (IEDB)	Amino acid number	Score threshold= 0.5	Solubility (Protein-sol) threshold ≥ 0.45	Theoretic al pI	Alpha tic index	The instability index	Grand average of hydropathicity (GRAVY)
Ck7 (HPA007272)	RQEESEQIKTLNNKFASFID KVRFLQKQNKLETKWTLL QEQKSAKSSRLPDIFEAQIA GLRGQLEALQVDGGRLEAE LRSMQDVVEDFKNKYEDEI NHRTAANEFEVVLKKDVD	TLLQEQKSAKSSRLP DI	37-53	0.531	0.851	5.01	85.65	46.7	-0.806
		KYEDEINHRTA	92-102	0.567					
Ck20 (HPA024309)	MDFSRRSFHRSLSLQAPV VSTVGMQRLGTTSPVYGG AGGRGIRISNRHTVNYGS DLTGGGDLFVGNEKMAM QNLND	RRSFHRSLSLQQA PVVSTVGMQRLG TT	5-32	0.614	0.729	10.23	65.32	48.3	-0.403
		IRISNRHTVNYGS DLTGGGDLFVGN EKMAMQ	44-75	0.578					

experimental evidence of protein solubility.

Sequence-comparison analysis indicated partial similarity between the selected CK7-PrEST-derived recombinant fragment (HPA007272) and other type II cytokeratins. An additional 37-amino-acid sequence with low sequence similarity to other human proteins was incorporated into the CK7-PrEST-derived recombinant fragment as part of the antigen-selection strategy. The resulting candidate sequences were subsequently used for recombinant antigen production and experimental evaluation of antibody development, specificity, and performance. Conclusions regarding antibody specificity and performance were based on the subsequent experimental analyses rather than on the computational predictions alone.

Recombinant Expression of CK7 and CK20 Antigens

Following the transformation of the recombinant pET42a and pET28a constructs into competent *E. coli* BL21(DE3) cells, protein expression was induced with IPTG under the optimized conditions described above. Recombinant constructs encoding full-length and CK7 and CK20 PrEST-derived recombinant fragment were successfully expressed in *E. coli* BL21(DE3) cells.

Among the evaluated constructs, the pET28a-based CK7 and the pET42a-based CK20 full-length constructs showed relatively

higher expression levels and were selected for subsequent production.

Recombinant protein expression was assessed by SDS-PAGE. Distinct protein bands were observed at apparent molecular masses of approximately 53 kDa for the CK7-pET28a fusion protein and approximately 83 kDa for the CK20-pET42a fusion protein, together with the corresponding PrEST-derived fragments (Figure 1A). These molecular masses represent the apparent molecular masses of the complete recombinant fusion constructs, including vector-derived sequences and tags, and should not be interpreted as the molecular masses of the native human CK7 and CK20 proteins.

Western blot analysis using an HRP-conjugated anti-His-tag antibody detected the corresponding His-tagged recombinant products at their respective apparent molecular masses (Figure 1B), supporting the presence of the His-tagged recombinant proteins.

Protein Extraction and Purification

Recombinant CK7 and CK20 proteins were recovered using both denaturing urea-based extraction and the IB-washing/solubilization procedure described above. SDS-PAGE analysis showed a prominent band corresponding to the target recombinant proteins in the early Tris extraction fraction after alkaline solubilization (T1). Based on the

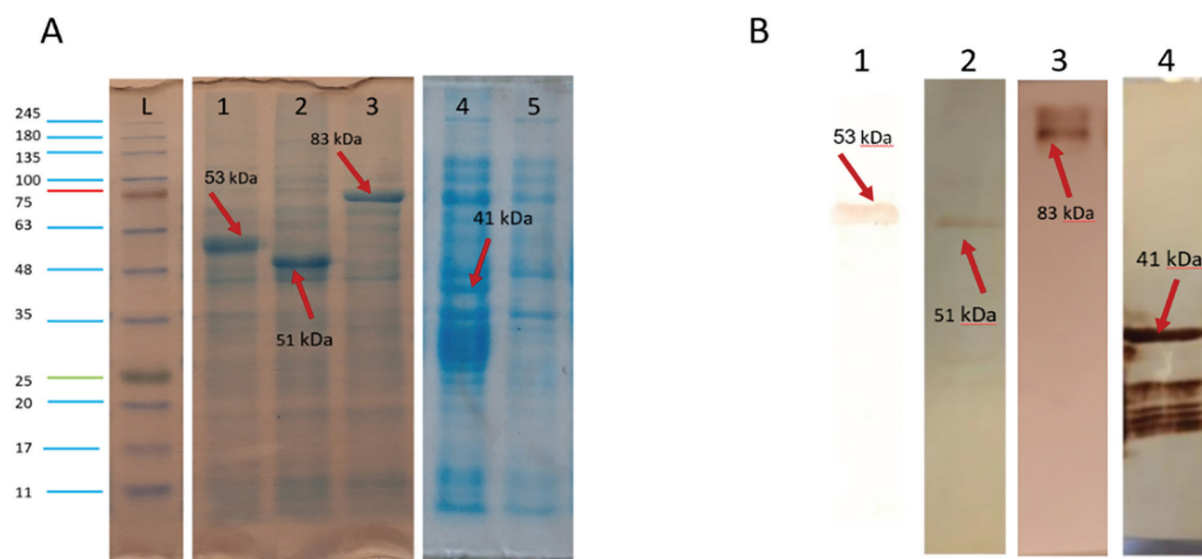


Figure 1. Expression and verification of recombinant CK7 and CK20 antigens. A) SDS-PAGE analysis of recombinant proteins stained with Coomassie Brilliant Blue. Lane L: Protein molecular weight marker (kDa), Lanes 1: Bacterial lysate containing CK7 full-length protein (~ 53 kDa); Lanes 2: Bacterial lysate containing CK7 PrEST recombinant fragment (~ 51 kDa), Lanes 3: Bacterial lysate containing CK20 full-length protein (~ 83 kDa), and Lane 4: Bacterial lysate containing CK20 PrEST recombinant fragment (~ 41 kDa), Lane 5: Uninduced bacterial lysate from *E. coli* BL21(DE3) cells carrying the CK20 PrEST recombinant construct, collected before IPTG induction, was used as the uninduced control. (B) Western blot analysis using an HRP-conjugated anti-His antibody confirming expression of His-tagged recombinant proteins. Lane. 1: CK7 full-length protein, Lane 2: CK7 PrEST recombinant fragment, Lane 3: CK20 full-length protein, and Lane 4: CK20 PrEST recombinant fragment.

observed SDS-PAGE profile, the T1 fraction was selected for subsequent purification (Figure 2A).

Ni-NTA affinity chromatography resulted in enrichment of the His-tagged recombinant proteins, as demonstrated by the prominent target-protein bands in the elution fractions (E1) (Figure 2B). Recombinant proteins obtained using both extraction approaches were recovered in sufficient quantities for subsequent applications. However, the CK20 PrEST-derived recombinant fragment obtained using the IB-washing procedure yielded a relatively low protein concentration. Therefore, the urea-extracted and Ni-NTA-purified CK20 PrEST-derived recombinant fragment preparation was selected for subsequent immunization.

Protein concentrations were determined using the Bradford assay with bovine serum albumin (BSA) as the standard. The standard curve showed good linearity over the concentration range used ($R^2 = 0.992$), and the resulting concentrations were used to

determine the amount of recombinant antigen administered for immunization.

Antibody Production and Titer Evaluation

Polyclonal antibody responses were detected in all immunized groups ($n = 5$ mice per group). Indirect ELISA demonstrated increasing reactivity to the corresponding recombinant antigens following successive booster immunizations, with detectable reactivity at serum dilutions up to 1:128,000 for most groups (Figure 3 A-D). Serial serum dilutions were examined to characterize the dilution-dependent reactivity of antibodies against the corresponding recombinant antigens. The progressive reduction in ELISA signal with increasing serum dilution supported a concentration-dependent binding pattern. Detectable reactivity was observed at dilutions up to 1:128,000 in most immunized groups. This dilution series was used to characterize the antibody reactivity profile, whereas endpoint titers were determined separately, where applicable, using the predefined positivity cut-off.

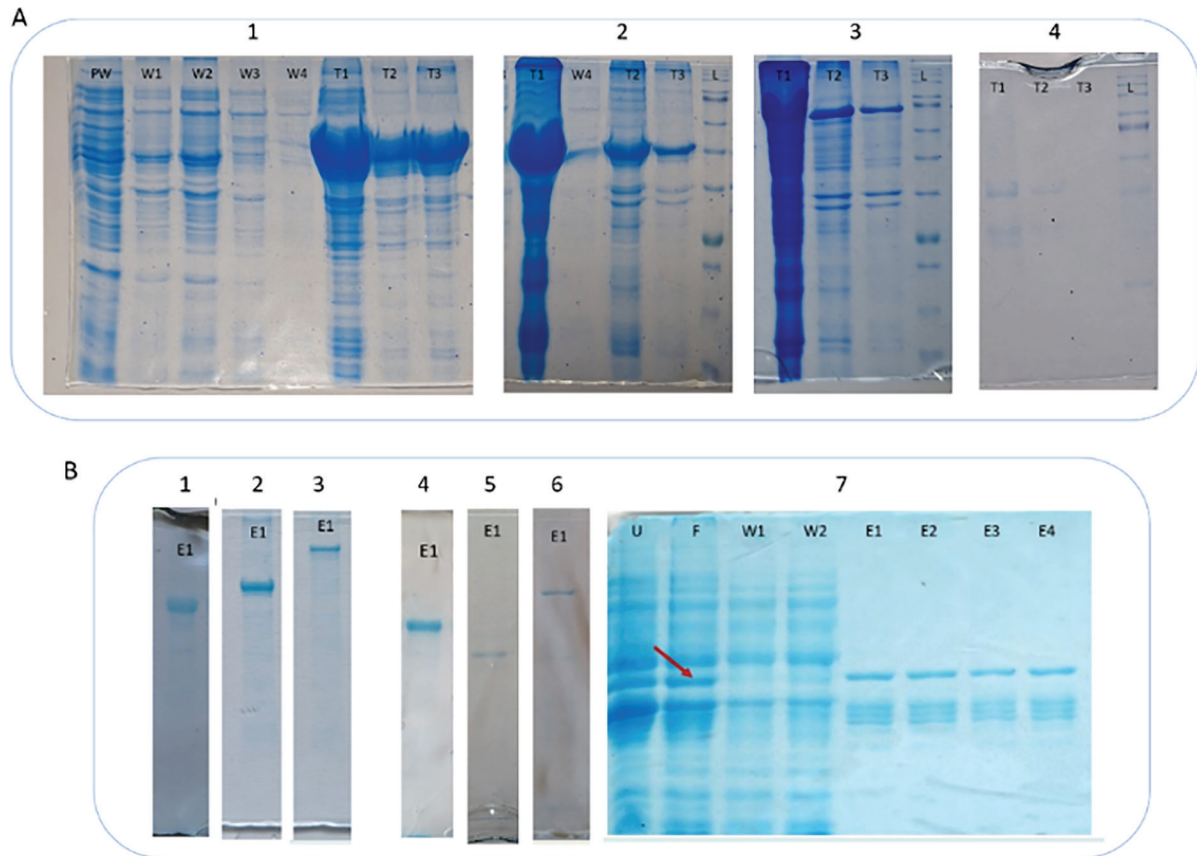


Figure 2. Extraction and purification of recombinant CK7 and CK20 antigens. (A) SDS-PAGE analysis of recombinant proteins recovered using the inclusion body (IB) washing method. Representative Tris extraction fractions (T1–T3) are shown; the strongest target protein bands were observed in the initial Tris extraction fraction (T1), which was selected for subsequent purification. 1: Extraction of CK7 full-length protein; 2: Extraction of CK7 PrEST-derived recombinant fragment; 3: Extraction of CK20 full-length protein; 4: Extraction of CK20 PrEST-derived recombinant fragment. PW: Supernatant sample before washing, W1– W4: samples from washing steps; T1–T3: samples from Tris addition steps. L: Protein molecular weight marker. (B) SDS-PAGE analysis of Ni-NTA affinity chromatography fractions. Recombinant proteins were enriched in the first elution fraction (E1), which was collected, dialyzed, and used for immunization studies. 1: Purified CK7 full-length protein; 2: purified CK7 PrEST-derived recombinant fragment; 3: purified CK20 full-length protein. Lanes 4–7 show proteins extracted by urea method, 4: purified CK7 full-length protein; 5: purified CK7 PrEST-derived recombinant fragment; 6: purified CK20 full-length protein; 7: CK20 PrEST recombinant fragment purification series. U: urea-extracted protein sample; F: column flow-through after loading the extracted protein sample; W1–W4: column wash fractions; E1–E4: elution fractions containing purified protein during imidazole addition steps. The arrow indicates the band corresponding to the CK20 PrEST-derived recombinant fragment.

At a serum dilution of 1:32,000, antibody reactivity was significantly higher in immunized mice than in their corresponding pre-immune sera (Figure 3E, F; $p < 0.05$), confirming indicating successful induction of antibodies reactive with the recombinant immunizing antigens. Among the immunized groups, mice receiving the full-length CK20 antigen showed comparatively lower ELISA reactivity than the other immunization groups, suggesting

differences in the antibody response elicited by the recombinant antigens.

Antibody Purification and Negative Absorption

Immunoglobulin fractions were enriched from immune sera by protein A affinity chromatography. SDS-PAGE analysis demonstrated enrichment of the immunoglobulin fraction following purification (Figure 4).

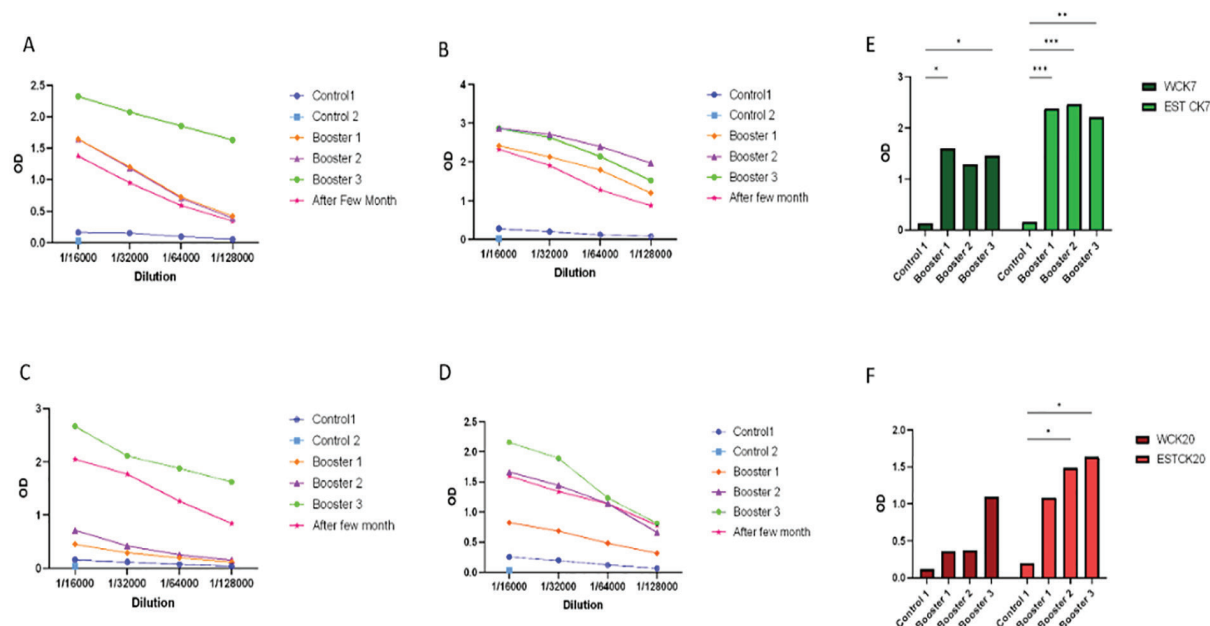


Figure 3. Immune serum titers and ELISA-based evaluation of polyclonal antibody responses against CK7 and CK20 antigens. Control 1: Pre-immunized mouse serum, Control 2: wells without primary antibody. (A) Antibody titration curve of mice immunized with CK7 full-length protein. (B) Antibody titration curve of mice immunized with the CK7 PrEST-derived recombinant fragment. (C) Antibody titration curve of mice immunized with CK20 full-length protein. (D) Antibody titration curve of mice immunized with the CK20 PrEST-derived recombinant fragment. (E) Comparison of mean ELISA absorbance values at a serum dilution of 1:32,000 for mice immunized with CK7 full-length protein (WCK7) and CK7 PrEST-derived recombinant fragment (ESTCK7). (F) Comparison of mean ELISA absorbance values at a serum dilution of 1:32,000 for mice immunized with CK20 full-length protein (WCK20) and CK20 PrEST-derived recombinant fragment (ESTCK20). The 1:32,000 dilution was selected for statistical comparison as a representative dilution within the measurable range of the antibody titration curves. ELISA measurements were performed in technical triplicates for each mouse, and the triplicate measurements were averaged before statistical analysis; Statistical significance was defined as $p < 0.05$.

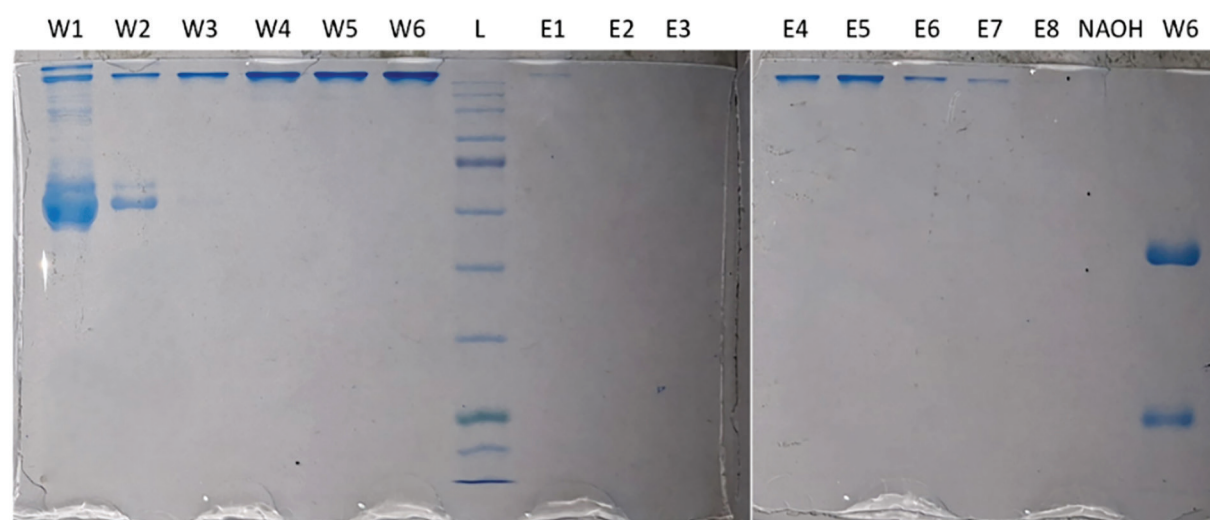


Figure 4. Purification anti-CK20 PrEST-derived recombinant fragment antibody from mouse serum via protein A affinity chromatography and confirmation by SDS-PAGE. Serum from a mouse immunized with the CK20 PrEST-derived recombinant fragment was purified over protein A affinity chromatography. W1-W6: Column wash fractions (W6 appears twice reflecting non-regenerated and regenerated forms of the column). L: Protein molecular weight marker. E1-E8: Purified serum fractions from elution steps. NaOH: Final column wash with NaOH.

The purified antibody preparations were subsequently subjected to negative absorption against selected non-target recombinant proteins and cell/tissue lysates to reduce nonspecific and cross-reactive antibody binding.

Immunohistochemical Performance of Generated Antibodies

The immunohistochemical performance of the generated polyclonal antibodies was evaluated using a panel of formalin-fixed, paraffin-embedded tissue sections tissue sections with known CK7 or CK20 expression patterns. The generated antibodies demonstrated positive staining in CK7- and CK20-expressing epithelial tissues, whereas little or no staining was observed in the designated negative control tissues (Figures 5

and 6). Antibodies generated against PrEST-derived fragments exhibited more restricted staining patterns and lower background reactivity than antibodies raised against the corresponding full-length proteins.

Following Purification and negative absorption, nonspecific staining was further reduced. In particular, anti-CK7 antibody preparations showed reduced reactivity in colorectal and liver parenchymal cells in liver tissues, whereas anti-CK20 preparations showed reduced or absent staining in ovarian and endometrial tissues after the absorption procedure (Figure 7).

The staining patterns obtained with the generated antibodies were compared with those obtained using commercial anti-CK7 and anti-CK20 antibodies as reference antibodies.

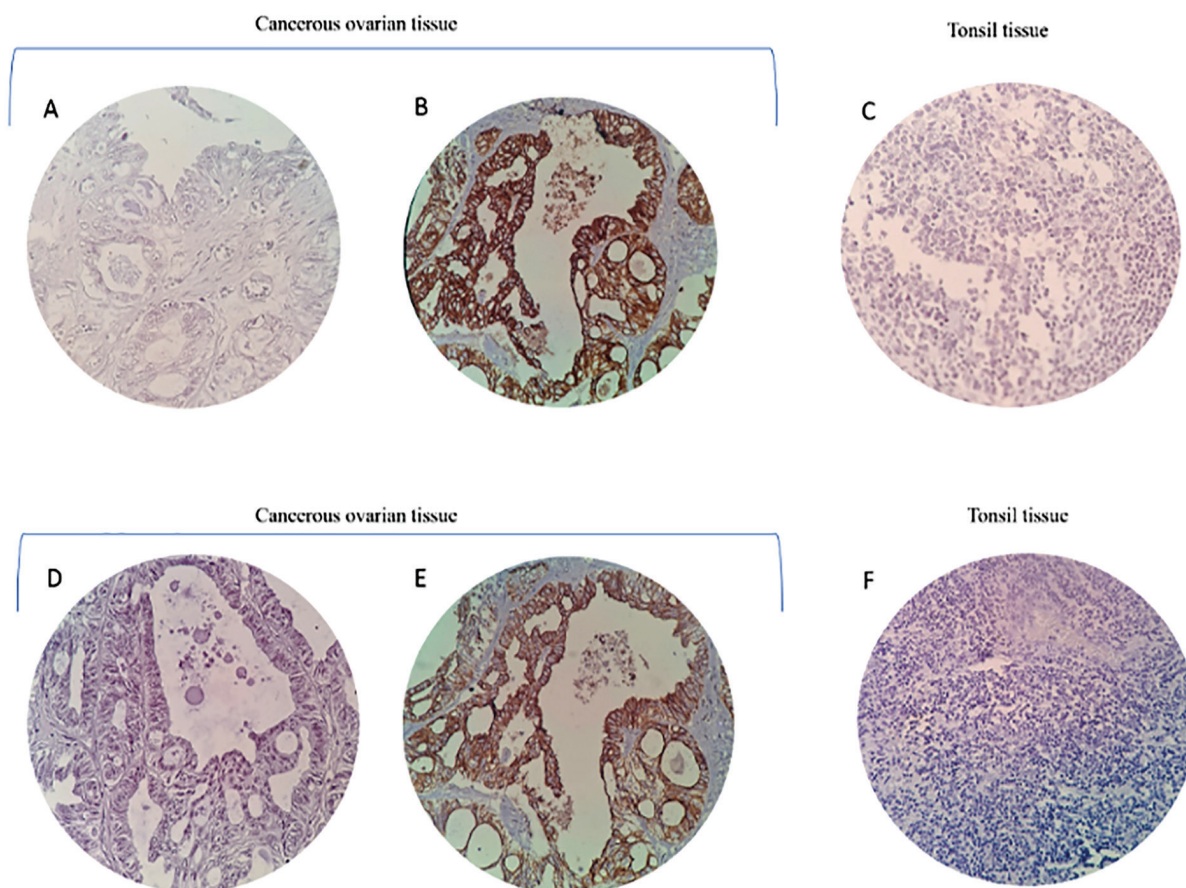


Figure 5. IHC evaluation of mouse-derived antibodies against the CK7 full-length protein and CK7 PrEST-derived recombinant fragment. FFPE tissue sections were stained using diluted mouse immune serum (1:3,000). (A and D) Ovarian carcinoma tissue stained with pre-immune mouse serum (negative control). (B and E) Positive cytoplasmic immunoreactivity in ovarian epithelial carcinoma tissue stained with serum from mice immunized with CK7 full-length and PrEST-derived recombinant fragment respectively. (C and F) Tonsil tissue stained with serum from mice immunized with the CK7 full-length and CK7 PrEST-derived recombinant fragment, respectively (negative tissue control).

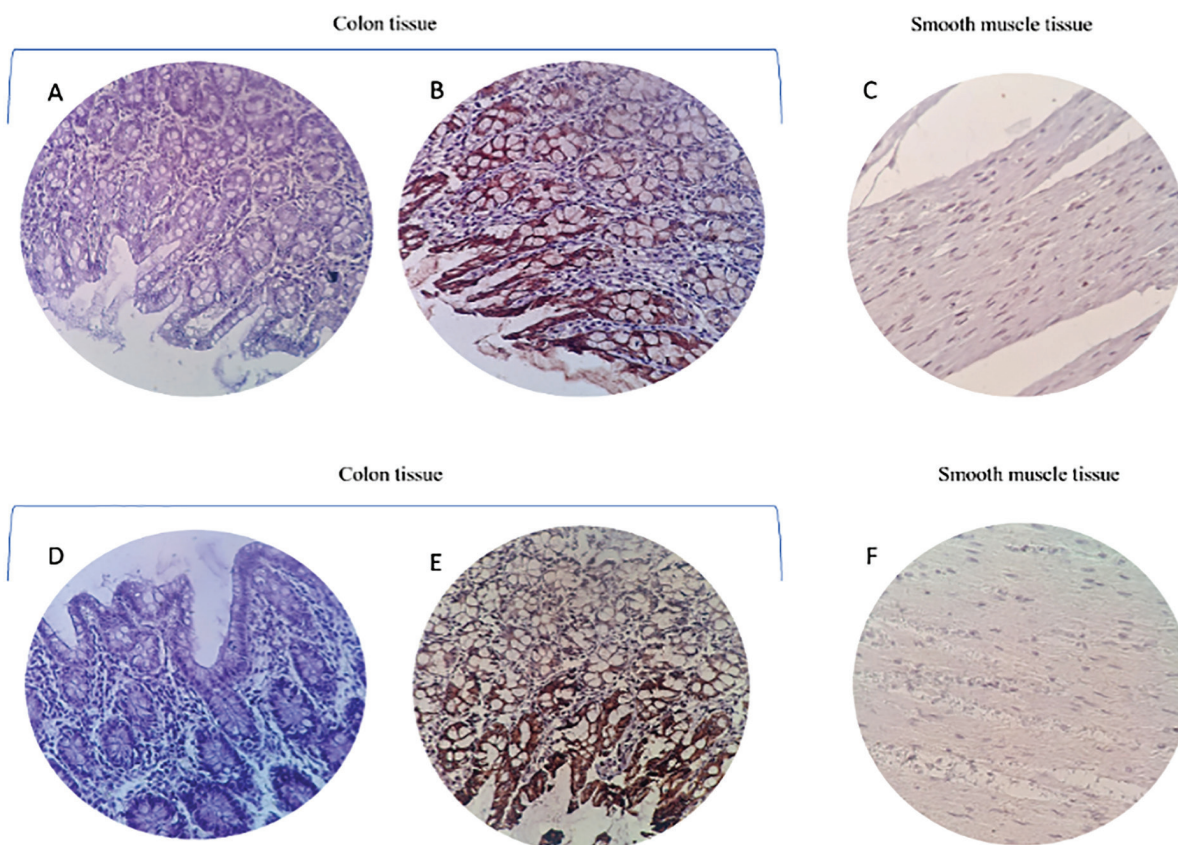


Figure 6. Immunohistochemical evaluation of antibodies generated against CK20 full-length protein and CK20 PrEST-derived recombinant fragment. FFPE tissue sections were stained using diluted mouse antiserum (1:1,000). (A and D) Normal colon tissue stained with pre-immune mouse serum (negative control). (B and E) Positive cytoplasmic staining of normal colonic epithelial cells using antiserum raised against the CK20 full-length and PrEST-derived recombinant fragment, respectively. (C and F) Smooth muscle tissue showing no immunoreactivity with antiserum raised against the CK20 full-length and PrEST-derived recombinant fragment, respectively

The generated antibodies showed broadly comparable staining patterns in the corresponding positive-control tissues, supporting their ability to detect CK7- and CK20-expressing cells by IHC. These findings indicate that purification and negative absorption improved the selectivity of the generated antibody preparations for the tested tissue panel.

Validation by Dot Blot and Western Blot

Dot blot analysis demonstrated specific binding of the purified and negatively absorbed antibody preparations to the corresponding recombinant target antigens, whereas little or no reactivity was observed with the designated negative-control antigens (Figure 8A–N). Western blot analysis further demonstrated selective recognition of the

corresponding recombinant CK7 and CK20 proteins by the purified and negatively absorbed antibody preparations. The same antibody preparations also detected the expected CK7- or CK20-reactive bands in cell line lysates, with minimal or no detectable reactivity in non-target samples (Figure 8O). Collectively, these findings support the target-specific and differential binding properties of the purified and negatively absorbed antibody preparations used for both IHC and Western blot analysis.

DISCUSSION

Cytokeratins are widely used immunohistochemical markers in surgical pathology and are important components

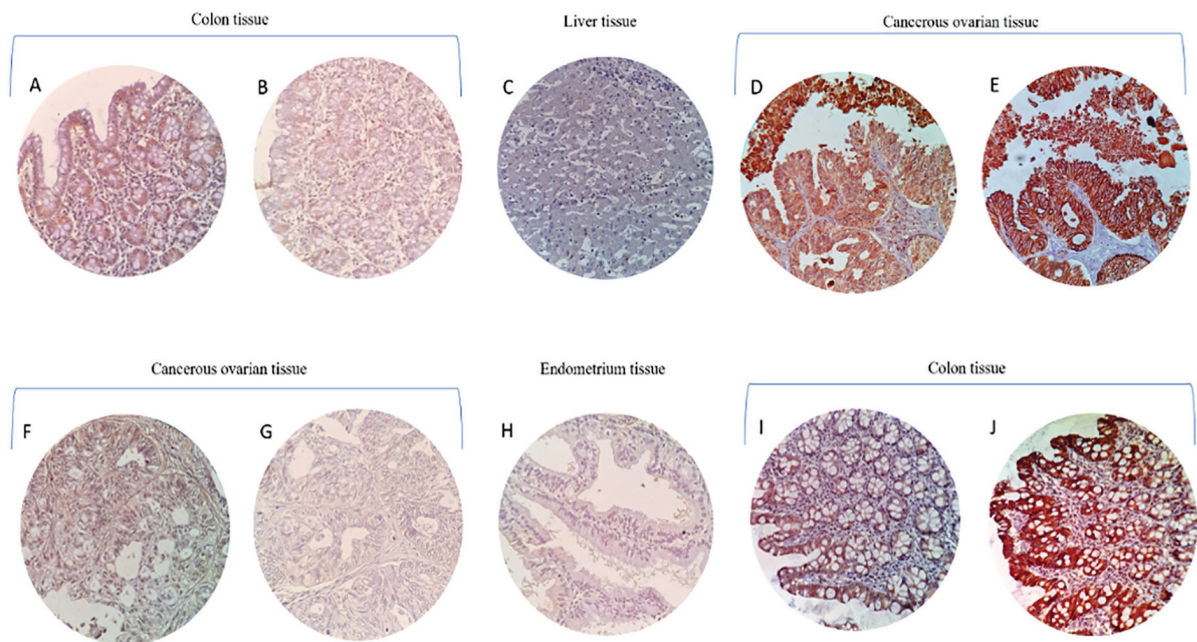


Figure 7. Effect of purification on the specificity of anti-CK7 PrEST and anti-CK20 PrEST polyclonal antibodies in IHC. FFPE tissue sections were stained with purified antibodies (3 µg/mL). (A–E) Anti-CK7 PrEST antibody before and after purification, (F–J) Staining with the anti-CK20 PrEST antibody before and after purification, (A) Unpurified anti-CK7 PrEST-derived recombinant fragment antibody showing false-positive staining. (B–D) purified and pre-absorbed IgG anti-CK7 PrEST antibody. (E) Commercial anti-CK7 antibody as a reference. (F) Unpurified anti-CK20 PrEST-derived recombinant fragment antibody showing false-positive staining. (G–I) purified and pre-absorbed IgG anti-CK20 PrEST-derived recombinant fragment antibody. (J) Commercial anti-CK20 antibody as a reference. Purification reduced non-specific staining while preserving target-specific immunoreactivity.

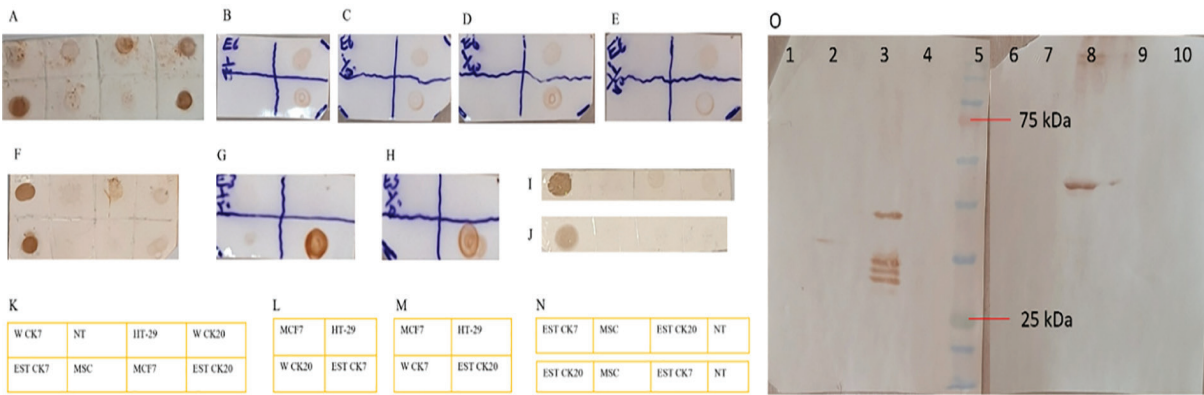


Figure 8. Evaluation of purified and pre-absorbed IgG anti-CK7 PrEST-derived recombinant fragment and anti-CK20 PrEST-derived recombinant fragment polyclonal antibody performance by dot blot and Western blot analysis. Control samples included CK7- or CK20-positive cell lines, negative cell controls, and non-transformed bacterial lysates. (A–J) Dot blot analysis before and after purification using different antibody concentrations. Purified antibodies showed strong reactivity with target antigens and minimal reactivity with negative controls. (K–N) Reference maps of sample loading positions for panels A–J. (A) Unpurified anti-CK20; (B–E, J) purified and pre-absorbed IgG anti-CK20 at different concentrations; (F) unpurified anti-CK7; (G–I) purified and pre-absorbed IgG anti-CK7 at different concentrations. (K) Reference map for A and F; (L) for G and H; (M) for B–E; and (N) for I and J. (O) Western blot validation demonstrating specific recognition of CK7 and CK20 antigens by the purified antibodies. Lanes 1–5 were analyzed with purified and pre-absorbed IgG anti-CK20 and lanes 6–10 with purified and pre-absorbed IgG anti-CK7: 1, MCF7 lysate; 2, HT-29 lysate; 3, CK20 PrEST recombinant fragment; 4, CK7 whole protein; 5, protein ladder; 6, SW1116 lysate; 7, CK20 whole protein; 8, CK7 PrEST recombinant fragment; 9, mesenchymal cell lysate; 10, HT-29 lysate.

of antibody panels for tumor classification and assessment of the likely tissue of origin, including in carcinomas of unknown primary (CUP). CK7 and CK20 exhibit distinct, although not entirely exclusive, expression patterns across epithelial malignancies and are therefore frequently used for diagnostic purposes (26-30). Despite the widespread availability of commercial antibodies, the development of locally producible antibody reagents may be valuable for research and diagnostic laboratories antibody reagents may be valuable access to commercial products is limited or associated with substantial cost. In the present study, we developed a workflow for generating polyclonal antibodies against CK7 and CK20 using both full-length recombinant proteins and selected PrEST-derived recombinant fragments, followed by evaluation of their antigen reactivity, selectivity, and immunohistochemical performance.

The production of polyclonal antibodies involved immunization of mice with full-length recombinant CK7 and CK20 proteins or selected PrEST-derived recombinant fragments, followed by Protein A affinity purification to enrich the IgG fraction from immune sera. The purified IgG preparations were subsequently subjected to negative absorption to reduce nonspecific and potentially cross-reactive antibody reactivity. This strategy is consistent with established approaches for generating polyclonal antibodies (31-33). The immunization approach was also based on previously described protocols for eliciting antibody responses in mice (34).

The rationale for employing PrEST-derived fragments as immunogens was to enrich the antibody response toward predicted regions of CK7 and CK20 relatively low sequence similarity to other cytokeratin family members. Previous studies have suggested that antibodies generated against carefully selected peptide or recombinant fragment antigens can exhibit improved target selectivity compared with antibodies raised

against full-length proteins, particularly for members of highly homologous protein families (35-37). In the present study, the recombinant fragments were designed to encompass selected regions of CK7 and CK20 identified through bioinformatic analyses of predicted B-cell antigenicity and sequence uniqueness. Compared with full-length proteins, the use of selected fragments was intended to restrict the repertoire of available epitopes and reduce the likelihood of generating antibodies against conserved regions that could contribute to cross-reactivity. The selected fragments also showed physicochemical characteristics considered favorable for recombinant antigen production.

For CK7, an additional sequence selected for its relatively low similarity to other type II cytokeratins was incorporated into the recombinant construct with the aim of further reducing potential cross-reactivity. Consistent with this design rationale, the antibodies generated against the PrEST-derived fragments showed more restricted staining patterns and lower background reactivity in the IHC assays.

The target reactivity and selectivity of the generated antibodies were evaluated using multiple complementary assays, including ELISA, IHC, and Western blotting. The generated antibodies demonstrated strong reactivity toward their corresponding recombinant antigens by ELISA, confirming successful induction of antigen-reactive humoral responses in the immunized mice. Antibodies generated against the PrEST-derived antigens generally exhibited higher ELISA reactivity than those generated against the corresponding full-length proteins.

IHC analysis further demonstrated that the generated antibodies could recognize CK7 and CK20 in formalin-fixed, paraffin-embedded tissue sections. IHC analysis further demonstrated that the generated antibodies could recognize CK7 and CK20 in formalin-fixed, paraffin-embedded tissue sections. Mouse-derived anti-CK7 antibodies

produced staining in CK7-expressing ovarian carcinoma sections, whereas anti-CK20 antibodies showed the expected staining of CK20-expressing epithelial cells in colorectal tissue.

Specificity was the primary objective of this study. IHC analysis demonstrated selective staining of CK7 and CK20-expressing tissue sections by the generated mouse-derived antibodies. Notably, antibodies generated against the CK7 and CK20 PrEST-derived recombinant fragments exhibited more restricted staining patterns and lower cross-reactivity with non-target tissues than antibodies generated against the corresponding full-length CK7 and CK20 proteins. This observation is consistent with the rationale for fragment-based antigen selection, in which relatively unique and antigenic regions are selected to reduce the likelihood of generating antibodies against conserved regions shared among related proteins. The reduced cross-reactivity observed in recombinant fragment-derived antibodies suggests that these antibodies target epitopes unique to CK7 and CK20, thereby improving their diagnostic utility, particularly in IHC, where accurate discrimination among closely related cytokeratins is essential. In contrast, immunization with Full-length CK7 and CK20 proteins is likely to elicit a broader immune response, directed against multiple epitopes, including those that are less specific or shared with other proteins. This broader reactivity increases the risk of cross-reactivity, as antibodies may recognize conserved domains present in other cytokeratins or structurally similar proteins, potentially leading to false-positive results in diagnostic applications. These observations are consistent with earlier studies that underscore the advantages of recombinant fragment-based immunization for generating highly specific antibodies, especially for proteins with high sequence homology, such as cytokeratins (35, 38).

Additional improvement in antibody selectivity was achieved through sequential

negative absorption. Following Protein A-based IgG purification, the antibody preparations were subjected to absorption with selected non-target cellular and bacterial lysates and recombinant cytokeratin antigens to reduce unwanted and potentially cross-reactive antibody reactivities. In particular, the anti-CK7 preparation was absorbed against CK20-derived antigens, whereas the anti-CK20 preparation was absorbed against CK7-derived antigens, with the aim of reducing antibody populations recognizing shared or homologous cytokeratin epitopes. Absorption with bacterial lysates was additionally used to reduce reactivity toward host-cell or bacterial components associated with recombinant antigen production.

This approach complements Protein A purification, which enriches the IgG fraction but does not selectively distinguish target-specific from nonspecific antibodies. In contrast, negative absorption selectively depletes antibody populations that recognize predefined unwanted antigens. Similar absorption-based strategies have been used to reduce cross-reactivity and improve the selectivity of polyclonal antisera (39). In the present study, the absorption procedure was associated with reduced background staining in IHC and decreased nonspecific reactivity in Western blotting while preserving detectable recognition of their intended the intended CK7 or CK20 target.

Although the generated antibodies demonstrated favorable performance in the assays examined, several limitations should be acknowledged. As with all polyclonal antibody preparations, biological variability among immunized animals and the heterogeneous composition of polyclonal antibody populations may contribute to variation in antibody performance and potential lot-to-lot variability (43). In addition, the relatively small serum volumes obtainable from individual mice may limit antibody yield and necessitate the use of multiple animals when larger quantities are required. In addition, the present study primarily provided

preliminary analytical characterization using ELISA, IHC, dot blot, and Western blot assays. The relatively limited tissue panel and absence of a large, clinically characterized cohort preclude definitive assessment of diagnostic sensitivity and specificity. Further studies using a broader panel of normal and neoplastic tissues, together with direct comparisons with well-validated commercial antibodies, are required to determine the diagnostic performance of these reagents. Finally, long-term stability and potential changes in antibody activity during storage were not evaluated and should be investigated in future studies.

Despite these limitations, the present findings demonstrate that PrEST-guided antigen selection combined with Protein A-based IgG purification and negative absorption can generate polyclonal antibodies with improved target selectivity in the experimental assays evaluated. Rather than establishing clinical diagnostic utility, these findings provide a basis for further development and validation of the generated CK7- and CK20-reactive antibodies as locally producible research reagents and potential candidates for subsequent diagnostic evaluation.

Evaluation in additional immunodetection platforms, including immunofluorescence and multiplex immunohistochemistry, may also determine whether the generated antibodies have broader applications beyond conventional chromogenic IHC.

CONCLUSION

The present study demonstrates the successful generation of polyclonal antibodies against human CK7 and CK20 using both full-length recombinant proteins and selected PrEST-derived recombinant fragments. Antibodies generated against the PrEST-derived fragments exhibited more restricted reactivity and lower non-specific background than those generated against the corresponding full-

length proteins.

Following Protein A-based IgG purification and sequential negative absorption, the antibody preparations demonstrated target-reactive binding in ELISA, Western blot, dot blot assays and showed appropriate staining of CK7- and CK20-expressing tissues by IHC. Collectively, these findings support the feasibility of combining rational antigen-region selection with negative absorption to improve the selectivity of polyclonal antibodies directed against closely related cytokeratin family members and may offer a practical platform for the development of both research and diagnostic reagents.

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ETHICAL APPROVAL

All animal experiments were conducted in accordance with applicable institutional and ethical guidelines for the care and use of laboratory animals. The study was approved by the Ethics Committee for Biomedical Research involving Laboratory Animals of Isfahan University of Medical Sciences (approval No. IR.MUI.AEC.1402.104).

GENERATIVE AI STATEMENT

The authors acknowledge the use of AI-assisted tools for language editing and refinement of the manuscript. All scientific content, data analysis and interpretation, and conclusions were independently developed, reviewed, and verified by the authors.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AVAILABILITY OF DATA AND MATERIALS

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

AUTHORS' CONTRIBUTIONS

ZA, HB and MR contributed to the conceptualization and design of the study. ZA performed the experiments, analyzed the data, and conducted the statistical analysis. ZA drafted the manuscript and prepared the figures. MR conceived the original research idea, supervised the study, and critically revised the manuscript. All authors reviewed and approved the final version of the manuscript.

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