

Generation of Monoclonal Antibodies for Sensitive Detection of Pro-Inflammatory Protein S100A9

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Abstract: The calcium-binding protein S100A9 regulates inflammatory processes and the immune response. It is overexpressed in a variety of inflammatory and oncologic conditions. In this study, we produced a recombinant human S100A9 (hS100A9) antigen with high yield and purity and used it to generate a hybridoma cell culture-based monoclonal anti-hS100A9 antibody. We selected five anti-hS100A9 antibodies from cell supernatants that showed high antigen binding efficiency and identified the nucleotide sequences of three antibodies: two with high effective concentration values and one with the lowest value. The antigen and antibody development procedures described herein are useful for producing large amounts of monoclonal antibodies against hS100A9 and other antigens of interest. The nucleotide sequences of the anti-hS100A9 monoclonal antibody revealed herein will be helpful in the generation of recombinant antibodies or antibody fragments against hS100A9.

Keywords: S100A9; recombinant protein; monoclonal antibody; inflammation



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1. Introduction

S100A9 is a member of the calcium-binding S100 protein family, which participates in cytoskeleton rearrangement and arachidonic acid metabolism [1]. As S100A9 is secreted at high concentrations from immigrated and activated phagocytic cells during inflammation [2], it plays a critical role in modulating the inflammatory response by stimulating leukocyte recruitment or inducing cytokine secretion [1]. S100A9 mediates the inflammatory process in myeloid cells and is involved throughout the initial development of cancer cells and metastatic disease progression [3,4]. Moreover, S100A9 is involved in the formation of amyloid aggregates, integrating the amyloid-neuroinflammatory cascade in Alzheimer's disease, and enhancing the neurodegenerative effects of Parkinson's disease [5–8]. Elevated levels of S100A9 have also been noted in chronic bronchitis, cystic fibrosis, systemic lupus erythematosus, rheumatoid arthritis, colitis ulcerosa, Crohn's disease, inflammatory bowel disease, colorectal cancer, multiple sclerosis, and human immunodeficiency virus infection [9–12]. Detection strategies are needed if S100A9 is to be considered a biomarker of these diseases.

Antibody assays are the method of choice for disease diagnosis and treatment because they strongly bind to the target antigen, even in complex blood or tissue samples. Antibodies are classified as polyclonal or monoclonal, and hybridoma technology is a traditional and robust method for generating antibodies. Antibody detection tests are cost effective and are neither labor intensive nor technically demanding compared with

the more expensive and complex phage display technology. Polyclonal antibodies are purified from the serum of an immunized animal, and monoclonal antibodies are secreted by immortalized B cells from the spleen of an immunized animal. In order to generate a unique monoclonal antibody of the desired specificity, the splenic B cells are fused with myeloma cells, resulting in immortal hybridomas [13]. Although polyclonal antibodies can be generated more rapidly, at less expense, and with less technical skill than is required to generate hybridomas and, subsequently, monoclonal antibodies, the principal advantages of monoclonal antibodies are their homogeneity and consistency, whereas polyclonal antibodies are heterogeneous. Specifically, as a monoclonal antibody recognizes a single epitope of an antigen, its affinity and selectivity to the target antigen are greater than those of a polyclonal antibody. Another advantage of monoclonal antibodies is that once the desired hybridoma has been generated, it becomes a constant and renewable resource. In contrast, polyclonal antibodies generated to the same antigen using multiple animals will differ between individuals, and their avidity may change as they are harvested over time. Therefore, the concentration and purity levels of specific antibodies are higher in monoclonal than in polyclonal antibodies. A few commercially available purified anti-S100A9 monoclonal antibodies have been developed. However, to the best of our knowledge, no study has been published on the production and sequencing of monoclonal anti-S100A9 antibodies.

We aimed to generate and sequence a monoclonal antibody against S100A9. The sequence information can be used for the development of recombinant antibodies or antibody fragments against S100A9. Additionally, the detailed methods for antigen production, antibody generation, and sequencing will support other efforts to produce monoclonal anti-S100A9 antibodies for use as a biological reagent to bind S100A9 in diagnostic and therapeutic approaches.

2. Materials and Methods

2.1. Construction of Human S100A9 Expressing Gene

A gene encoding S100A9 was PCR amplified using the primers 5'-atatggatccgatgacttgc aaaatgtcg-3' and 5'-aattaagcttagggggtgccctccc-3' and *Herculase II* fusion DNA polymerase to add a His-tag at the N- or C-terminus of the expressed protein. The product was digested with restriction enzymes, *BamHI* and *HindIII*, and the product was inserted into *BamHI*- and *HindIII*-digested pET-Duet1 (Addgene) using a ligation high, resulting in pET-Duet (S100A9). The obtained plasmid was prepared using the plasmid miniprep system (Genesgen, Pusan, Korea), and the entire coding-region sequence was confirmed by sequencing.

2.2. Expression and Purification of Recombinant Human S100A9

BL21 (DE3) cells were transformed with expression vector pET-Duet (S100A9) and cultured at 37 °C for 16 h in LBA medium (LB medium containing 100 µg/mL ampicillin) and 1.5% agar, and a single colony was picked and grown at 37 °C in 4 mL of LBA medium overnight, from which 1 mL was used to inoculate 50 mL of LBA medium at 37 °C to an OD₆₀₀ of 0.6. After that, 1 mM isopropylthio-β-galactopyranoside was added and incubated for an additional 20 h at 18 °C for protein induction. After centrifugation at 3500 rpm for 20 min at 4 °C, the pellet was resuspended in 5 mL of purification buffer (50 mM potassium phosphate (pH 7.4), 150 mM sodium chloride (NaCl), 10 mM imidazole, 0.5 mM PMSF), followed by sonication. After centrifugation at 3500 rpm for 20 min at 4 °C, the supernatant was purified using an Ni-NTA His-tag purification kit. The Ni-NTA-bound protein was washed with 20 mL of purification buffer and further washed with 40 mL of the same buffer containing 20 mM imidazole followed by 10 mL of the same buffer containing 50 mM imidazole. The protein was eluted with 3.2 mL of the same buffer containing 200 mM imidazole and collected using a disposable gravity column. The eluent was concentrated with an ultra-filtration device and equilibrated with PBS buffer (10 mM Na₂HPO₄, 2 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and then concentrated to

2 mg/mL. Protein expression and purification were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and protein concentration was determined using NanoDrop.

2.3. Confirmation of the Activity of Recombinant Human S100A9

Indirect ELISA for confirming the activity of purified rhS100A9 was performed as follows: several concentrations of purified rhS100A9 or commercially available purified hS100A9 protein (Sino Biological, Beijing, China, 11145-H08B) in PBS were immobilized on a 96-well plate for 16 h at 4 °C, and the wells were filled with 200 µL of PBST (PBS buffer with 0.05% Tween20) containing 2% BSA for 1 h at 37 °C and washed three times with 200 µL of TBST. Subsequently, 150 ng of anti-hS100A9 antibody (Sino Biological, 11145-MM01) in TBST was added and incubated for 1 h at 25 °C. After washing three times with 200 µL of TBST, the bound protein was probed with 5000-fold diluted HRP-conjugated goat anti-mouse antibody in TBST for 1 h at 25 °C. The wells were washed three times with 200 µL of TBST and developed with 100 µL of tetramethylbenzide (TMB) solution. After incubation for 15 min, the reaction was stopped with 25 µL of 1 N sulphuric acid, and the absorbance was read at 450 nm using a microplate reader Model 680 (Bio-Rad). As a negative control, 200 ng BSA was used instead of antigen, and the titer was used as a baseline. A well coated with 200 ng BSA only was used as a negative control to use its titer as a baseline signal.

2.4. Production of Monoclonal Anti-Human S100A9 Antibodies

Monoclonal antibody was produced at A-Frontier (Seoul, Korea). Female BALB/c mice, 6 weeks old, were immunized by IP injection of 100 µg of purified rhS100A9 in PBS with complete Freund's adjuvant. After 2 weeks, the mice were tail-bled and the titers of the serially diluted antisera were measured by indirect ELISA, resulting in titers of over 1.5 at the 100- or 1000-fold diluted antisera, which indicated that no additional immunizations would be necessary prior to cell fusion. Three days after the injection, $1\text{--}1.5 \times 10^8$ spleen cells separated from the immunized mice were washed with culture medium and then fused with SP2/0 murine myeloma cells using polyethylene glycol as a fusing agent for hybridoma production. The fused cells were cultured using HAT medium and culture supernatants were screened by indirect ELISA with the use of 250 ng of rhS100A9 as a seeding antigen, TBST containing 2% skim milk as a blocking buffer, 50 µL of hybridoma supernatant, and 10,000-fold diluted HRP-conjugated mouse anti-IgG antibody as a secondary antibody. Following the above fusion ELISA test, the cells of the 24 positive wells were moved to a 24-well plate and were cultured, and the activity was confirmed by indirect ELISA as above. The cells were pipetted into a 96-well plate using an HT culture medium (HAT without aminopterin) and then cultured for 2 weeks in a CO₂ incubator at 37 °C. The single clones with the highest anti-S100A9 antibody titers using indirect ELISA were subcloned and the screening processes were repeated. The dose–response curves were fitted to a four-parameter equation, $y = a + (b - a)/(1 + (c/x)^d)$, using GraphPad Prism software. In the equation, a, b, c, and d indicate ABS in the absence of antigen, ABS in the presence of 300 ng antigen, EC50, and hill slope, respectively. The EC50 values were estimated based on the parameter c in the formula. The LOD was obtained as the estimated antigen concentration that shows the mean blank value +3 standard deviation (SD).

2.5. Purification of Monoclonal Antibodies

After the final screening, five positive subclones (5B3, 7C8, 9D9, 14E7, and 22A10) were grown in larger volumes, and the antibodies obtained from each cell supernatant were purified using Protein G agarose beads (Pierce) as follows: 10 µL of resin slurry was added to 1 mL of cell supernatant and then incubated at RT for 1 h. After washing using 500 µL of PBS three times, the antibody was eluted using 100 µL of 0.1 M glycine (pH 2.5) and the eluted sample was immediately adjusted to neutral by adding 10 µL of PBS. The antibody concentration was measured from the absorbance at 280 nm using NanoDrop.

2.6. Confirmation of the Antigen Binding Efficiency of Purified Monoclonal Antibodies

The antigen-binding activities of the five purified monoclonal antibodies were tested using indirect ELISA as follows: 100 μ L of 250 ng of commercial hS100A9 (Sino Biological) in PBS was immobilized on a 96-well plate for 37 h at 2 $^{\circ}$ C. After that, the wells were filled with 200 μ L of TBST containing 2% BSA for 1 h at 37 $^{\circ}$ C and then washed three times with TBST. Subsequently, 150 ng of purified monoclonal antibody in 100 μ L of PBS with 0.1% BSA was added and incubated for 1 h at 25 $^{\circ}$ C. After washing three times with 200 μ L of TBST, bound protein was probed with 5000-fold diluted HRP-conjugated goat anti-mouse IgG antibody in PBS with 0.5% BSA for 1 h at 25 $^{\circ}$ C. The wells were washed three times with 200 μ L of TBST and developed with 100 μ L of TMB solution. After incubation for 10 min, the reaction was stopped with 50 μ L of 1 N sulphuric acid, and the absorbance was read at 450 nm using a microplate reader.

Afterwards, an isotyping test was performed using a Rapid ELISA mouse mAb isotyping kit (Pierce), and the cells were frozen in liquid nitrogen in FBS containing 10% of DMSO. 5B3, 7C8, 9D9: H chain-IgG2b, L chain-kappa; 14C4, 22A10: H chain-IgG1, L chain-kappa.

2.7. Identification of VH and VL Sequences

Total cellular RNA from 5B3, 9D9, and 14C4 hybridoma cells was extracted using an RNeasy kit according to the manufacturer's protocol. After that, 0.5 μ g of RNA was reverse-transcribed by 5 U of Moloney murine leukemia virus (M-MLV) RT using 50 pmol of 5'-phosphorylation-modified primer, 5'-tgcaaggcttacaaccacaatcc-3' for VH or 5'-gatatctccacttgacattgatg-3' for VL, in a total volume of 12.5 μ L at 42 $^{\circ}$ C in the presence of 10 U RNasin RNase inhibitor. Reactions were terminated after 30 min by heating to 95 $^{\circ}$ C for 15 min and subsequently adding 3 volumes of TE buffer supplemented with 4 μ g/mL RNase A. The resulting cDNA was circularized by 20 U of T4 RNL1 in the presence of 15% PEG 8000 in a total volume of 50 μ L at 37 $^{\circ}$ C for 1 h. Unreacted residual first-strand primers and cDNA were removed by the addition of 1.5 U of T4 DNA polymerase for 30 min at 37 $^{\circ}$ C. Next, 2 μ L of these intramolecularly joined 5' cDNAs was amplified by 35 cycles of PCR using inverted primer pairs as follows: IgG-inF, 5'-ccagcgagaccgtcacctg-3'; IgG-inR, 5'-agcccttgaccaggcatcc-3'; Kappa-inF, 5'-gcttctgaacaacttctacc-3'; and Kappa-inR, 5'-cagttggtgcagcatcagc-3'. Finally, an amplicon of the expected size was observed by TAE agarose gel electrophoresis, purified, and cloned into the T vector. Plasmid DNA minipreps of clones were subjected to Sanger sequencing (Bionics, Seoul, Korea). CDRs of VH and VL were determined via the IgBlast tool.

3. Results

3.1. Expression of Soluble rhS100A9 with High Yield and Purity

We produced a recombinant human S100A9 (rhS100A9) protein in *Escherichia coli* (*E. coli*) to produce a sufficient amount of highly pure antigens that can be used to boost the generation of antigen-specific antibodies in mice. We cloned the N-terminally His-tagged rhS100A9-coding gene (NM_002965.3) into the pET-Duet protein expression vector and transformed the clones into *E. coli*. The proteins were bacterially expressed and then purified by nickel affinity purification, yielding 16 mg of soluble S100A9 protein per 100 mL culture, with over 99% purity (Figure 1a). We performed an indirect ELISA using a commercial mouse anti-hS100A9 antibody and HRP-conjugated goat anti-mouse IgG antibody to confirm the antibody-binding activity of the purified rhS100A9 antigen (Figure 1b). The resulting titer revealed higher dose-dependent binding activity between the recombinant antigen and antibody than that of the commercial hS100A9 protein (Figure 1c).

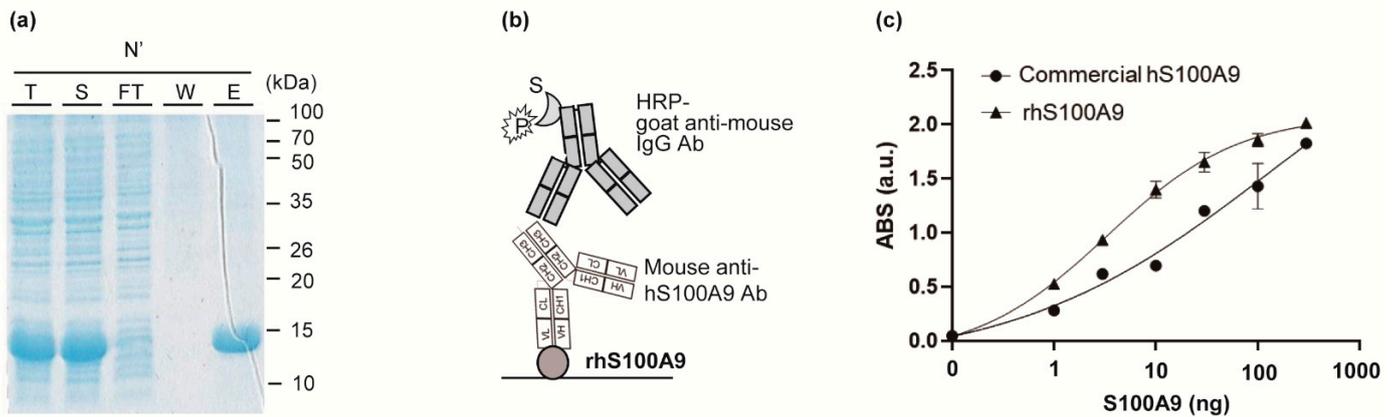


Figure 1. (a) SDS-PAGE analysis of Ni-NTA purified rhS100A9. T, S, FT, W, and E indicate total protein, soluble protein, flow through fraction, wash fraction, and elution fraction, respectively. (b) Schematic representation of the indirect ELISA for confirming the antibody-binding activity of purified rhS100A9. (c) ELISA titer with different concentrations of rhS100A9 or commercial hS100A9.

3.2. Generating a Highly Sensitive Anti-hS100A9 Monoclonal Antibody

We used hybridoma technology to generate a monoclonal antibody by fusing an antibody-producing B cell from immunized mice with an immortal myeloma cell. First, we produced antigen-specific B cells by immunizing BALB/c mice. The mice were injected with rhS100A9 at 14-day intervals. The isolated B cells were fused with the myeloma cells to generate hybridoma cells capable of producing virtually unlimited quantities of monoclonal antibodies. The hybridoma supernatants were collected, and the anti-hS100A9 antibodies in the supernatants were screened to confirm their capacity to bind hS100A9. We selected 24 supernatants that showed positive signals with a high ELISA titer, then selected five colonies that showed a high titer (colony numbers 5, 7, 9, 14, and 22), and cultured each of them for a second screening. From the cultured cells, 96 colonies were picked and transferred to 96-well plates. After ELISA, we selected one clone from each plate that showed a high titer relative to the colony's size; that is, having a high possibility of being a single clone. We spread the cultured cells from each selected parent clone, separated each colony, and selected the best one from each plate through indirect ELISA (5B3, 7C8, 9D9, 14C4, and 22A10). After a third screening, we extracted antibodies from the five selected hybridoma cell supernatants and purified them using Protein G agarose resin. Next, we checked the activity of the antibodies through an indirect ELISA using various concentrations of commercial hS100A9 as an antigen to confirm that all of the antibodies had hS100A9 binding affinity (Figure 2 and Table 1).

Table 1. Absorbance values of monoclonal antibodies with 300 ng of rhS100A9, EC_{50} of the antibodies, and limit of detection (LOD) values of the assay.

	5B3	7C8	9D9	14C4	22A10
Absorbance (a.u.)	2.9 ± 0.1	2.9 ± 0.5	3.1 ± 0.2	3.0 ± 0.1	3.2 ± 0.5
EC_{50} (ng)	28 ± 1.4	60 ± 15	30 ± 2.3	75 ± 1.5	61 ± 12
LOD (ng)	5.6	17	4.7	20	1.5

3.3. Identification of Variable Domain Sequences

We cultured the five hybridoma clones and identified the subtypes of the IgG heavy (H) and light (L) chains by isotyping. We confirmed that the H chains of 5B3, 7C8, and 9D9 were IgG2b, and the L chains were kappa. Meanwhile, the H chains of 14C4 and 22A10 were IgG1, and the L chains were kappa. Next, we cultured three hybridoma clones: 5B3 and 9D9, which had high effective concentration (EC_{50}) and low limit of detection (LOD) values, and 14C4, which had a low EC_{50} and high LOD. The cDNA encoding the variable domains

of the heavy chain (VH) and light chain (VL) was isolated and sequenced using three-step 5'-RACE technology [14]. We prepared the mRNA of each hybridoma sample using RT-PCR with 5' phosphorylation gene-specific primers and performed cDNA circularization using T4 RNA ligase 1. Inverse PCR amplification was performed using inverted primers against a known constant region sequence. Finally, we cloned the PCR product into the T vector and obtained the VH and VL sequences (Figure 3 and Supplementary Table S1).

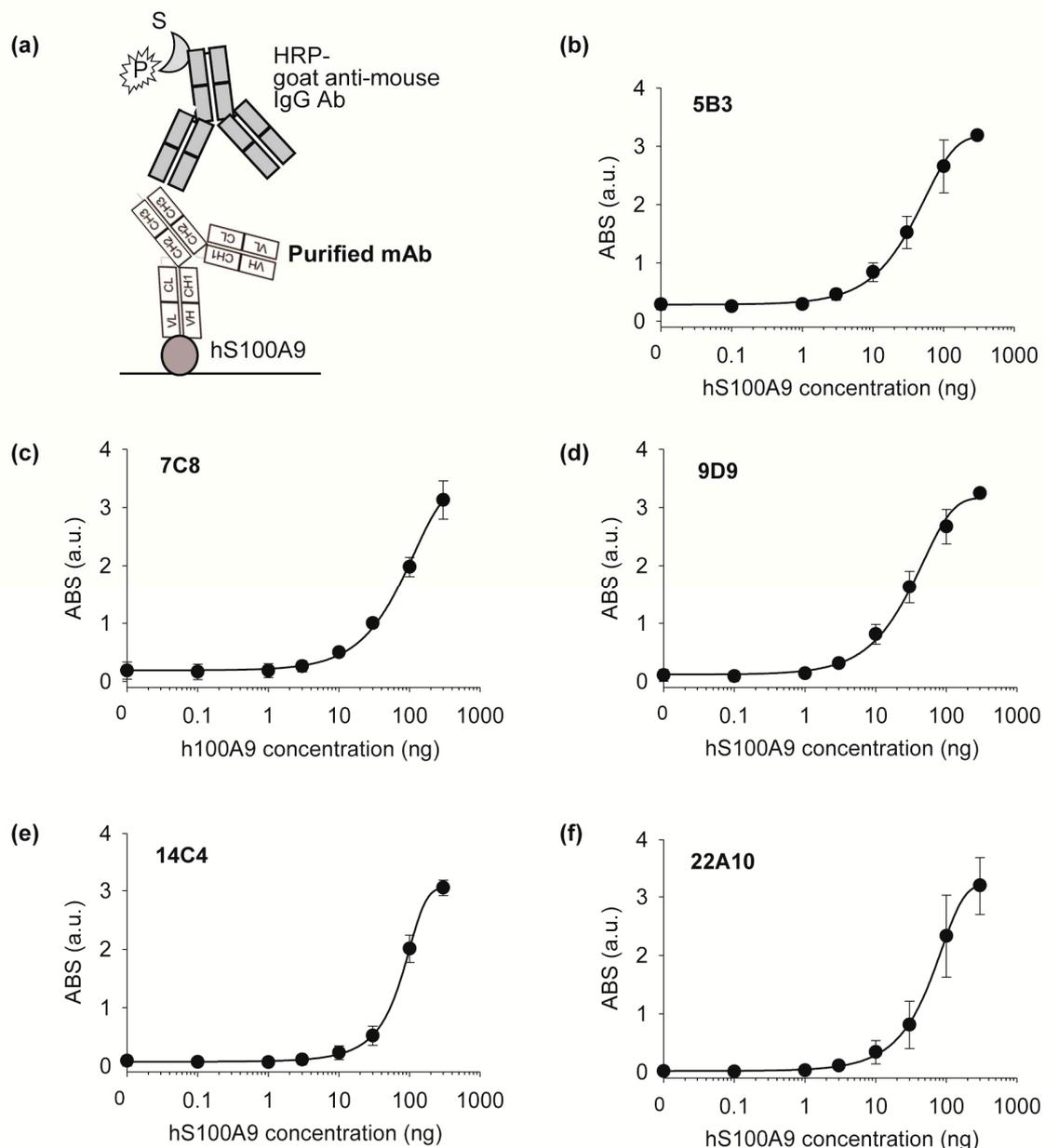


Figure 2. (a) Schematic representation of the indirect ELISA for confirming the dose-dependent antigen binding efficiency of selected five monoclonal antibodies. (b–f) ELISA signals of monoclonal antibodies with various concentrations of hS100A9. The points represent absorbances at the indicated antigen concentrations. The lines indicate dose–response curves, which were fitted to a four-parameter equation, $y = a + (b - a)/(1 + (c/x)^d)$, using GraphPad Prism software, where a, b, c, and d indicate ABS in the absence of antigen, ABS in the presence of 300 ng antigen, EC50, and hill slope, respectively. The EC50 values were estimated based on the parameter c in the formula. The LOD was obtained as the estimated antigen concentration that shows the mean blank value +3 SD. SD = standard deviation.

		<u>CDR1</u>	
5B3-VH	MRWSWIFLFLLSITAGVHCQVQLQQSGPELVKPGASVKMSCKASGYTFTSYIISWVKQRP		60
9D9-VH	MRWSWIFLFLLSITAGVHCQVQLQQSGPELVKPGASVKMSCKASGYTFTSYIICWVKQRP		60
14C4-VH	MRWSWIFLFLLSITAGVHCQVQLQQSGPELVKPGASVKMSCKASGYTFTSYIHHWVKQRP		60

	<u>CDR2</u>	<u>CDR3</u>	
5B3-VH	GQGLEWIGWIYPGDGITYKNEKFKGKTTLTADKSSSTAYMLLSSLTSEDSAIVYCARSR		120
9D9-VH	GQGLEWIGWIYPGDGITYKNEKFKGRTTLTADKSSSTAYMLLSSLTSEDSAIFYCARSR		120
14C4-VH	GQGLEWIGWIYPGDGFITYKNEKFKGKTTLTADKSSSTAYMLLSSLTSEDSAIFYCARSGK		120
	*****:*****:*****.****.		
5B3-VH	<u>YY</u> AMDYWGQGTSVTVSSAKTT		141
9D9-VH	<u>YY</u> AMDYWGQGTSVTVSSAKTT		141
14C4-VH	<u>WY</u> AMDYWGQGTSVTVSSAKTT		141
	:*****		
		<u>CDR1</u>	
5B3-VL	MKLPVRLLVLMFWIPASSSDVVMQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWY		60
9D9-VL	MKLPVRLLVLMFWIPASSSDVVMQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWY		60
14C4-VL	MKLPVRLLVLMFWIPASSSDVVMQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWY		60

	<u>CDR2</u>	<u>CDR3</u>	
5B3-VL	LQKPGQSPKLLIYKVSNRFSGVPRDFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHPY		120
9D9-VL	LQKPGQSPKLLIYKVSNRFSGVPRDFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHPY		120
14C4-VL	LQKPGQSPKLLIYKVSNRFSGVPRDFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHPY		120

5B3-VL	<u>TF</u> GGGTKLEIK		131
9D9-VL	<u>TF</u> GGGTKLEIK		131
14C4-VL	<u>TF</u> GGGTKLEIK		131

Figure 3. Amino acid sequences of VH and VL of anti-S100A9 monoclonal antibodies. An asterisk indicates positions which have a single, fully conserved residue.

The 5B3, 9D9, and 14C4 clones differed at the seventh amino acid of the complementarity-determining region (CDR)2 and the fourth to the sixth amino acids of CDR3, thus suggesting that these residues might be the source of the difference in antigen binding efficiency.

4. Discussion

S100A9 overexpression is elevated in several inflammatory and oncologic conditions, including ulcerative colitis and colorectal cancer. We generated and sequenced a novel anti-hS100A9 monoclonal antibody with high antigen-binding activity. We produced a large amount of highly pure, soluble rhS100A9 using a standard *E. coli* expression system and showed its high activity, suggesting that the recombinant antigen maintained its structural integrity. *E. coli* expressions are known for their simplicity and are highly cost effective for antigen production.

We performed ELISA using a commercial anti-S100A9 antibody and determined its EC₅₀ (135.4 ng) and LOD (0.11 ng). Although the LOD values were lower than those of a commercial antibody, the DNA sequence of the commercial antibody has not been published. We identified the variable domain sequences and the antibody subtypes. The sequence information will be broadly useful for the development of recombinant antibodies or antibody fragments targeting S100A9. The detailed methods for producing antigens, generating antibodies, and identifying the antibody sequence can be used to produce monoclonal antibodies against other antigens of interest and anti-S100A9 monoclonal antibodies.

The high target specificity and selectivity of monoclonal antibodies make them a promising diagnostic option. The anti-hS100A9 antibody produced in this study can be used to specifically detect hS100A9 by conjugating a fluorescent dye to the antibody. The

sequence information is a good example of applied research and will be useful for the development of recombinant antibodies or antibody fragments.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11104659/s1>, Table S1: Sequences of VH and VL of anti-S100A9 monoclonal antibodies.

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