# Purification of a polyclonal antibody against CD147 for ELISA using antigen-immunoaffinity chromatography

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Received February 23, 2016; Accepted February 20, 2017

DOI: 10.3892/mmr.2017.6523

**Abstract.** The immunoglobulin superfamily member CD147 is a widely expressed glycoprotein that occurs in both a membrane-spanning and soluble form. Sandwich ELISA is a powerful tool for analyzing soluble antigens. The aim of the present study was to obtain a highly specific polyclonal antibody against human CD147 that can be used for sandwich ELISA analysis. Expression of recombinant CD147 by a eukaryotic expression system was used to immunize rabbits to obtain antiserum. A highly specific polyclonal antibody that was able to detect soluble CD147 in sandwich ELISA was obtained by antigen-immunoaffinity chromatography purification. The purity of rabbit anti-CD147 polyclonal antibodies was ~99%, and ELISA analysis was able to determine the titer of the rabbit anti-CD147 polyclonal antibodies at 1:512,000. The lowest concentration of the standard CD147 antigen that the sandwich ELISA was able to detect was 31.25 pg/ml. The sandwich ELISA system was composed of anti-hepatoma HAb18 monoclonal antibodies and purified rabbit anti-CD147 polyclonal antibodies. The present study demonstrated that antigen-immunoaffinity chromatography may be a good technique for the purification of polyclonal antibodies, which may be used to detect antigen in sandwich ELISAs.

## Introduction

CD147, a member of the immunoglobulin (Ig) superfamily (1), is a transmembrane glycoprotein that is widely expressed in various cell types and at a high level in human tumors (2,3); its expression has been reported to be upregulated in a number of cancer types (4,5). The hepatoma-associated antigen HAb18G, which was cloned by anti-hepatoma monoclonal antibody (MAb) HAb18 screening of a human hepatocellular carcinoma cDNA library, has an identical nucleotide and amino acid sequence to CD147 (6,7). Previous reports suggested that CD147 may be shed from the cell membrane via matrix metallopeptidase-dependent cleavage, which generates a soluble form of CD147 that may contain either one N-terminal Ig-like domain or two Ig-like domains (8,9). Additional studies demonstrated that full-length CD147 may also be released via microvesicle shedding (10,11). Soluble CD147 has also been indicated as a potential marker for the detection of certain types of cancer (12,13).

ELISA is one of the basic applications of antibodies that is used to analyze soluble antigens (14); therefore, ELISA may be used to detect the concentration of soluble CD147 (15). We previously generated a murine antibody, HAb18, which targeted hepatocellular carcinoma-associated antigen HAb18G/CD147 (16). However, a successful sandwich ELISA detection system requires either MAbs that bind to independent sites on the antigen or affinity-purified polyclonal antibodies. Antibodies are widely used for the identification and localization of proteins due to their ability to bind an antigen with a high degree of affinity and specificity (17). MAbs have monospecificity, as they target a single epitope, which results in reduced cross-reaction (18). By contrast, polyclonal antibodies exhibit higher sensitivity, as a number of different epitopes are recognized (17). Owing to the various applications in which they may be used, antibodies with high specificity and sensitivity are desired. There are numerous methods used to purify antibodies, and the choice of purification procedure depends how the antibodies will be used and on the resources available (19). IgG may be purified by ammonium sulfate precipitation, ion-exchange chromatography, Protein A or Protein G affinity chromatography (20); occasionally, immunoaffinity chromatography is required to obtain more highly purified products (19). Currently, the majority of antibodies against CD147 are purified by Protein A or Protein G affinity chromatography. However, we have previously found that anti-CD147 polyclonal antibodies that are purified only by Protein A or Protein G affinity chromatography do not work well in the sandwich ELISAs to detect soluble CD147 (data not shown).

The present study produced a rabbit polyclonal antibody against HAb18G/CD147, which was purified by ammonium sulfate precipitation followed by antigen-immunoaffinity

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*Key words:* soluble CD147, polyclonal antibody, purification, immunoaffinity chromatography, sandwich ELISA

chromatography. This polyclonal antibody performed well with MAb HAb18 in the sandwich ELISA, which was used to detect soluble CD147.

## Materials and methods

*Preparation of eukaryotic-expressed CD147.* Chinese hamster ovary (CHO)-derived cell line CHO-H8F8E10, that stably expresses HAb18GEP-Fc (a recombinant human protein containing the extracellular portion (EP) and the fragment crystallizable region (Fc) of HAB18G/CD147, termed hereafter CD147-Fc), preserved in our laboratory, was cultured in SFM4 medium (Hyclone; GE Healthcare Life Sciences; Logan, UT, USA) at 37˚C. The recombinant eukaryotic expression vector pcDNA5/HAb18G-Fc, which contains the extracellular Ig-like domains of HAb18G with human Fc fragment in the C-terminal domain, was produced and large-scale cell culture was accomplished. Culture suspensions were collected and separated by tangential flow microfiltration (Sartorious AG, Göttingen, Germany). CD147-Fc recombinant protein was purified by Protein A chromatography with GE HiTrap rProtein A (GE Healthcare Life Sciences), according to the manufacturer's instructions. The Fc fragment was cleaved by Human Rhinovirus (HRV) 3C Protease (Sino Biological, Inc., Beijing, China). Briefly, CD147-Fc recombinant protein in cleavage buffer (Sino Biological, Inc.) was mixed with the recombinant HRV 3C protease in cleavage buffer at a mass ratio of 100:1 and incubated at 4˚C overnight. The mixture was purified by $Ni^{2+}$ affinity chromatography with GE HisTrap (GE Healthcare Life Sciences), followed by Protein A chromatography with GE HiTrap rProtein A, according to the manufacturer's instructions, in order to remove the residual Fc fragments and HRV 3C protease which also have a polyhistidine tag. Fractions were desalted and concentrated by an ultrafiltration device (Merck KGaA Darmstadt, Germany). The concentration of purified CD147 was determined by the Bicinchoninic Acid (BCA) assay (Thermo Fisher Scientific, Inc. Waltham, MA, USA). Samples were subsequently analyzed by 12% SDS-PAGE.

*Immunization of rabbits with CD147.* New Zealand white rabbits (n=2; weight 2.4 and 2.7 kg; age, 7 months; housed at 12 h light/dark cycles at 20-26˚C and with free access to food and water) were used in the present study and handled in the Animal Center of the Fourth Military Medical University (Xi'an, China). All animal procedures were performed according to the University's Institutional Animal Care and Use Committee. The rabbits were given a hypodermic injection of recombinant purified CD147 protein (800 $\mu$g), mixed with Complete Freund's Adjuvant (Sigma-Aldrich; Merck KGaA) in a 1:1 ratio. After 3 weeks, the rabbits were boosted 2 times with CD147 (400 $\mu$g) mixed with incomplete Freund's adjuvant (1:1) at 2-week intervals. Prior to each injection, blood samples were obtained from the marginal vein of the rabbit ear, centrifuged at 2,000 x g for 10 min at 4˚C and the sera was used to determine antibody titer by ELISA.

*Antiserum titer determination by ELISA.* The titer of antiserum was determined by indirect ELISA. For each well of the 96-well plate, 1 $\mu$g of CD147 was diluted in 100 $\mu$l of 0.1 M sodium bicarbonate solution pH 9.6 and incubated overnight at 4˚C. The plates were washed 3 times with PBS + Tween-20 (PBST; 0.05% Tween-20), and then blocked with 1% bovine serum albumin (BSA; MP Biomedicals, LLC, Santa Ana, CA, USA) for 1 h at 37˚C. The plates were washed again 3 times with PBST and incubated with 100 $\mu$l rabbit antisera against CD147 at 6 different dilutions between 1:16,000 and 1:512,000 for 1 h at 37˚C. Non-immune serum was used as a negative control. Following 3 washes with PBST, plates were incubated with 100 $\mu$l diluted horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000 dilution; cat. no. 31460; Pierce; Thermo Fisher Scientific, Inc.) for 30 min at 37˚C. The reaction was developed by adding 100 $\mu$l 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich; Merck KGaA) for 12 min at room temperature. Finally, 200 M sulfuric acid was added to stop the reaction, and the absorbance was determined at 450 nm using a BioTeck Epoch microplate reader. The experiments were repeated two times.

*Purification of rabbit anti-CD147 IgG.* The polyclonal antibodies against CD147 were purified from the rabbit immune sera by ammonium sulfate precipitation followed by antigen-immunoaffinity chromatography. Briefly, rabbit blood was collected from the carotid artery following anesthesia with 40 mg/kg pentobarbital sodium and prior to sacrifice. Approximately 100 ml blood was collected from each rabbit and ~30 ml immunized rabbit serum was collected by centrifugation at 1,000 x g for 10 min at 4˚C. Serum proteins were then precipitated in 50% ammonium sulfate at 4˚C overnight and centrifuged at 2,000 x g for 20 min at 4˚C. The pellet was subsequently dissolved in PBS (pH 7.4). To improve specificity, the polyclonal antibodies were purified by antigen-immunoaffinity chromatography (CNBr-activated sepharose 4B; GE Healthcare Life Sciences), according to the manufacturer's protocol. The purity and reactivity of the anti-CD147 polyclonal antibodies were analyzed by 10% SDS-PAGE [as described previously (21)] and indirect ELISA, respectively. The indirect ELISA was performed similar as the antiserum titer ELISA described above, with the purified polyclonal antibodies used in 6 serial dilutions from 40 to 0.625 ng/ml.

*Preparation of recombinant CD147 protein for antibody purification.* As large amounts of coupling antigen are required for antigen-immunoaffinity chromatography, a prokaryotic expression system for CD147 was prepared. The extracellular domain of HAb18G/CD147 was amplified by polymerase chain reaction (PCR) using the expression plasmid pBluescript KS(+)/HAb18G as the template for the amplification. The primers used encompassed the entire transcript with *SphI* and *MluI* cloning sites added to the forward (CCCAAGCTT ATGGCGGCTGCGCTGTTCGTGCTG) and reverse (CGC GGATCCTCAGGAAGAGTTCCTGGCGGA) primers, respectively. PCR products were purified using the Wizard PCR preps kit (Promega Corporation, Madison, WI, USA). Following restriction endonuclease digestion (*SphI* and *MluI*; New England Biolabs, Inc., Ipswich, MA, USA), the fragment was inserted into the pGEX-6p-1 prokaryotic expression vector (GE Healthcare Life Sciences), which has a C-terminal glutathione S-transferase (GST) tag. Positive *Escherichia coli* BL21 (GE Healthcare Life Sciences) clones containing

recombinant plasmid pGEX-6P-1/HAb18GEP were selected by growth on ampicillin-containing agar plates. A single colony of transformed *E. coli* was selected and cultured overnight at 37˚C in Luria-Bertani (LB) medium (Thermo Fisher Scientific, Inc.) supplemented with 100 $\mu$g/ml ampicillin. The culture mixture was transferred to fresh LB medium (1:100 dilution) containing 100 $\mu$g/ml ampicillin and incubated at 37˚C with continuous shaking until the absorbance at 600 nm reached 0.6-0.8. Expression conditions were optimized, and expression of the CD147-GST fusion proteins was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside at 16˚C for 5 h. Following induction for 5 h, the cells were harvested by centrifugation at 4,000 x g for 20 min at 4˚C. The supernatant was discarded and the pellet was resuspended in PBS (pH 7.4), and lysed by sonication on ice with 4-sec pulses at high intensity and a 7-sec cooling period between each burst for 120 cycles. The suspension was centrifuged at 12,000 x g for 50 min at 4˚C to remove insoluble debris. The resultant supernatant was subsequently added to a GSTrap column (GE Healthcare Life Sciences) pre-equilibrated with PBS and the AKTA program was performed according to the GSTrap protocol. The flow-through was collected for SDS-PAGE analysis, as aforementioned, and the column was washed with PBS (pH 7.4). Finally, the bound protein was eluted with elution buffer (reduced glutathione; Amresco, LLC, Solon, OH, USA) and analyzed by 10% SDS-PAGE and western blot analysis. Fractions were desalted and concentrated by an ultrafiltration device with PBS (pH 7.4). The concentration of CD147-GST was determined by BCA assay.

*SDS-PAGE and western blot analysis*. Eukaryotic-expressed CD147 was separated by 12% non-reduced SDS-PAGE; prokaryotic-expressed CD147 and purified polyclonal antibodies were both separated by 10% non-reduced SDS-PAGE. All the samples were analyzed for protein concentration by BCA assay (Thermo Fisher Scientific, Inc.), and 10 $\mu$g was loaded in each lane. Coomassie brilliant blue R250 (Sigma-Aldrich; Merck KGaA) was used to stain the gels. Quantification of gel staining was performed with GeneSnap software, version 4.0 (SynGene, Frederick, MD, USA). All the purification experiments described above were analyzed by coomassie brilliant blue staining, with the exception of the purified prokaryotic-expressed CD147 that was also analyzed by western blot analysis. Briefly, samples were transferred to a polyvinylidene fluoride microporous membrane (Merck KGaA) and probed with primary antibody HAb18 (22) overnight at 4˚C and secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:3,000 dilution; cat. no. A16072; Thermo Fisher Scientific, Inc.) for 50 min at room temperature. Signals were visualized by Western Blotting Detection Reagents (cat. no. 29100; Engreen Biosystem Co., Ltd., Beijing, China), using a Kodak 4000MM Image Station (Kodak; Rochester, NY, USA) and the Carestream Molecular Imaging Software, version 5.4.2 (Caresteam, Rochaster, NY, USA).

*Establishment of sandwich ELISA*. A monoclonal antibody against the extracellular domain of HAb18G/CD147, which was previously produced in our laboratory and designated HAb18 (22), was diluted in sodium bicarbonate solution (pH 9.6) at the concentration of 10 $\mu$g/ml (100 $\mu$l/well) and used to coat 96-well plates. The plates were incubated at 4˚C overnight, washed 3 times with PBST and blocked with 1% BSA (200 $\mu$l/well) for 1 h at 37˚C. Subsequently, samples to be tested, or the highly purified CD147 protein (eukaryotic expression of the extracellular domain of HAb18G/CD147) were added to the individual wells and incubated at 37˚C for 1 h. Purified CD147 was used as a standard, serially diluted in 1% BSA/PBS (1,000, 500, 250, 125, 62.5, and 31.25 pg/ml), of which 100 $\mu$l was added to individual wells. After washing 3 times with PBST, anti-CD147 polyclonal antibodies, which were purified by different methods (including ammonium sulfate precipitation, antigen-immunoaffinity choromatography with ammonium sulfate precipitation and protein A chromatography with ammonium sulfate), or a commercial antibody against CD147 (cat. no. orb42082; Biorbyt Ltd., Cambridge, UK) were added (100 $\mu$l/well) and incubated at 37˚C for 1 h. After 3 washes with PBST, HRP-conjugated goat anti-rabbit IgG was added (100 $\mu$l/well) and the plate was incubated at 37˚C for 30 min. After washing 3 times with PBST, TMB substrate was added to the wells (100 $\mu$l/well) and was measured at 450 nm using a BioTeck Epoch microplate reader. This test was repeated 15 times.

## Results

*Characterization of the CD147 antigen for immunization*. As the first step in the production of the polyclonal antibodies against CD147, a CD147 protein was prepared. To obtain antibodies that resemble those produced in the human body, the eukaryotic-expressed CD147 was chosen as the immunogen. The recombinant expression vector pCDNA5/HAb18G-Fc contains extracellular domains of HAb18G and its C-terminal domain has an Fc fragment. The Fc fragment was cleaved by HRV 3C protease to avoid the production of antibodies against the Fc fragment. The Fc fragment and HRV 3C protease have polyhistidine tags, therefore, the mixture was purified by $Ni^{2+}$ affinity chromatography followed by Protein A chromatography to obtain high-purity CD147 protein. The purified, eukaryotic-expressed CD147 was analyzed by 12% SDS-PAGE under reducing conditions (Fig. 1). The recombinant human CD147-Fc protein consists of 422 amino acids and has a calculated molecular mass of 46.8 kDa; however, as a result of glycosylation, the recombinant protein migrates at ~58-65 kDa (Fig. 1, lane 1). The recombinant protein CD147-Fc has been degraded to some degree and exhibits dimer formation. The molecular mass of the cleaved extracellular domains of CD147 is ~30-40 kDa and the molecular mass of the HRV 3C protease is ~21 kDa (Fig. 1, lane 2). Following purification by $Ni^{2+}$ affinity chromatography, a certain amount of recombinant CD147-Fc protein and HRV 3C protease remained (Fig. 1, lane 3). However, following Protein A chromatography, CD147 was of high purity and there were no residual Fc fragments or HRV 3C protease.

*Antiserum titer analysis by ELISA*. Rabbits were immunized with the purified eukaryotic CD147 immunogen, and the titer of the antiserum was detected by indirect ELISA. A total of 6 dilutions, between 1:16,000 and 1:512,000, of the antiserum were reacted with an equal amount of the

recombinant CD147 protein. The antibody titer is defined as the highest dilution of serum at which the optical density 450 (OD450) ratio (OD450 of post-immunization serum/OD450 of pre-immunization serum) is >2:1. The antibody titer was demonstrated to be ~1:512,000 (Fig. 2). Generating a specific antibody preparation from low titer antiserum is difficult, and the production of a high-titer antiserum is a basic requirement for high-quality antibodies. The CD147 antiserum titer produced in the present study was high, which indicated that a strong response has been generated, and thus, the antibodies could be purified.

*Characterization of the prokaryotic recombinant CD147 protein for antigen-immunoaffinity chromatography.* Antigen-immunoaffinity chromatography requires large amounts of CD147 protein to purify the CD147 antibodies. Therefore, a prokaryotic expression system was used to make CD147 protein instead of the eukaryotic vector, as CD147 proteins may be expressed in large amounts in a short time and has increased stability compared with eukaryotic-expressed CD147. The extracellular domain sequence of the CD147 gene was cloned in an expression plasmid with a C-terminal GST tag, and the construct was transformed into *E. coli* BL21 competent cells. The GST tag was used to purify the recombinant CD147 with a GSTrap affinity column. The purified CD147-GST fusion protein, whose expected size is ~44 kDa, was analyzed by 10% SDS-PAGE and confirmed by western blot analysis with anti-CD147 MAb HAb18 (Fig. 3). SDS-PAGE and western blot analysis demonstrated that purification of CD147 was of high purity and confirmed the presence of a corresponding band for expressed CD147-GST protein. This high-quality purified CD147 protein is required to produce high-quality purified antibodies.

*Assessment of the purity and reactivity of the anti-CD147 polyclonal antibody by SDS-PAGE and indirect ELISA.* A non-reducing SDS-PAGE was used to determine the purity of rabbit anti-CD147 IgG (120-150 kDa), which was purified by ammonium sulfate precipitation followed by antigen-immunoaffinity chromatography (Fig. 4A, lane 1). Compared with the antiserum (Fig. 4A, lane 5) and the antibodies purified only by ammonium sulfate precipitation (Fig. 4A, lane 4), the affinity-purified antibodies had a higher purity; the purity of the rabbit anti-CD147 IgG was ~99%.

To determine the reactivity of the purified antibodies, an indirect ELISA was performed. Antibodies were purified by ammonium sulfate precipitation followed by antigen-immunoaffinity chromatography, and different dilutions (0.625-40 ng/ml) were reacted with an equal amount of CD147 protein. The result demonstrated in Fig. 4B indicated that the purified antibody had a good affinity for CD147, which indicated that the purified antibody has a high reactivity.

*Establishment of sandwich ELISA.* A sandwich ELISA was constructed with the following components: i) The HAb18 anti-CD147 MAb, previously produced in our laboratory (22), was used as the capture antibody; ii) the highly purified eukaryotic CD147 was used as a standard protein, which was serially diluted to 1000, 500, 250, 125, 62.5, 31.25 pg/ml and iii) the anti-CD147 polyclonal antibodies were used as the detecting antibody. Detection of the standard proteins was performed under optimized conditions. The present study demonstrated that the sandwich ELISA system detected the concentration of CD147 as low as 31.25 pg/ml (Table I). By contrast, the present study established other sandwich ELISA systems using various rabbit anti-CD147 polyclonal antibodies that were purified by methods other than antigen-immunoaffinity chromatography. The results demonstrated that only the system comprised of antibodies purified by antigen-immunoaffinity chromatography was able to detect CD147 protein at concentrations as low as 31.25 pg/ml (Table II).

Table I. Sensitivity test results of human CD147 sandwich ELISA.

| CD147 standard concentration (pg/ml) | Average OD450[a] ± SD |
|---|---|
| 1,000 | 1.765±0.021 |
| 500 | 0.884±0.005 |
| 250 | 0.587±0.029 |
| 125 | 0.321±0.020 |
| 62.5 | 0.193±0.005 |
| 31.25 | 0.164±0.009 |
| Blank | 0.069±0.007 |

[a]Average OD450 is based on 15 replicates. OD, optical density; SD, standard deviation.

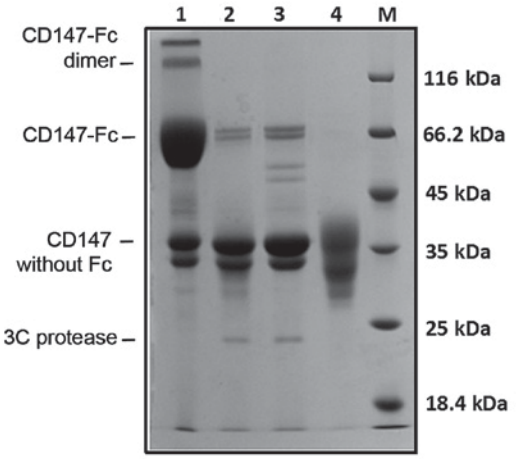


Figure 1. SDS-PAGE analysis of the CD147 antigen prior to rabbit immunization. Eukaryotic-expressed recombinant protein CD147-Fc was treated with HRV 3C protease for 16 h, and the mixture was purified by $Ni^{2+}$ affinity chromatography followed by Protein A chromatography. SDS-PAGE analysis demonstrated that CD147 was of high purity and indicated that there was no residual Fc fragment or 3C protease. Lane 1, recombinant eukaryotic-expressed CD147-Fc, which has a dimer formation and has been partially degraded; Lane 2, the recombinant CD147-Fc protein was treated with HRV 3C protease for 16 h, resulting in a mixture of CD147-Fc, CD147, Fc fragments and HRV 3C protease; Lane 3, mixture purified by $Ni^{2+}$ affinity chromatography, which is not very powerful, and a certain amount of recombinant protein and HRV 3C protease remain; Lane 4, CD147 immunogen purified by Protein A chromatography, no recombinant protein or HRV 3C protease was detected; Lane M, protein molecular weight marker. HRV, human rhinovirus.

Table II. Comparison of polyclonal antibodies against CD147 purified by antigen-immunoaffinity chromatography and other methods of purification.

| Source | Method of purification | Background of sandwich ELISA (OD450) | Lowest concentration of CD147 detected (pg/ml) |
|---|---|---|---|
| Present study | Antigen-immunoaffinity chromatography | 0.069 | 31.25 |
| Present study | Ammonium sulfate precipitation alone | 0.17 | 250 |
| Present study | Protein A affinity chromatography | 0.13 | 250 |
| Purchased from Biorbyt Ltd., Cambridge, UK | Protein A affinity chromatography | 0.08 | 125 |

All methods of purification included a first step of ammonium sulfate precipitation, prior to chromatography. OD, optical density.

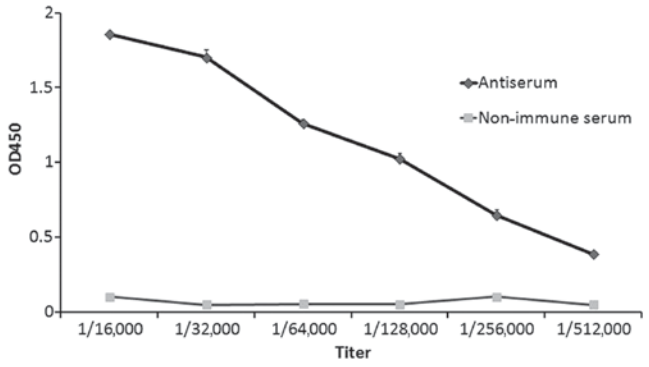


Figure 2. Analysis of antiserum titer by ELISA. The antibody titer is defined as the highest dilution of serum at which the OD450 ratio (OD450 of post-immunization serum/OD450 of pre-immunization serum) is >2:1. For non-immune serum at 1:512,000, the OD450 is 0.049, and for antiserum at 1:512,000, the OD450 is 0.387; therefore, the ratio is >2:1, and the titer of this antiserum is ~1:512,000. Data are presented as the mean + standard deviation. OD, optical density.

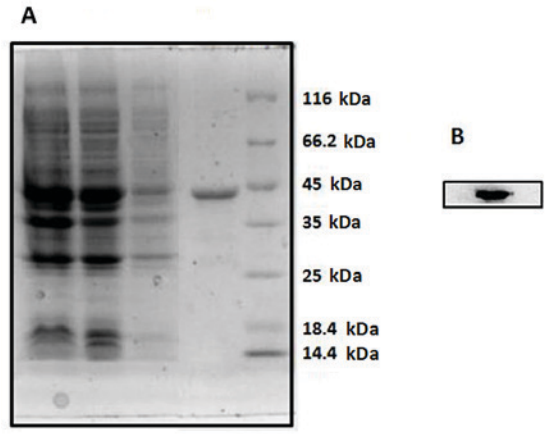


Figure 3. SDS-PAGE and western blot analysis of CD147-GST fusion protein purification and identification. The prokaryotic-expressed CD147-GST fusion protein was purified by a GSTrap column. The purified protein had a high purity. (A) SDS-PAGE analysis of purified CD147-GST. Lane 1, supernatant of the lysate of CD147-GST; Lane 2, flow through liquid of the supernatant; Lane 3, flow through liquid of the washing buffer; Lane 4, protein eluted from the column; Lane M, protein molecular weight marker. (B) Western blot analysis of purified CD147-GST. The primary antibody was HAb18, which has high specificity and affinity for CD147. The secondary antibody was goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase. Western blotting confirmed that the observed band corresponded to CD147-GST protein. GST, glutathione S-transferase.

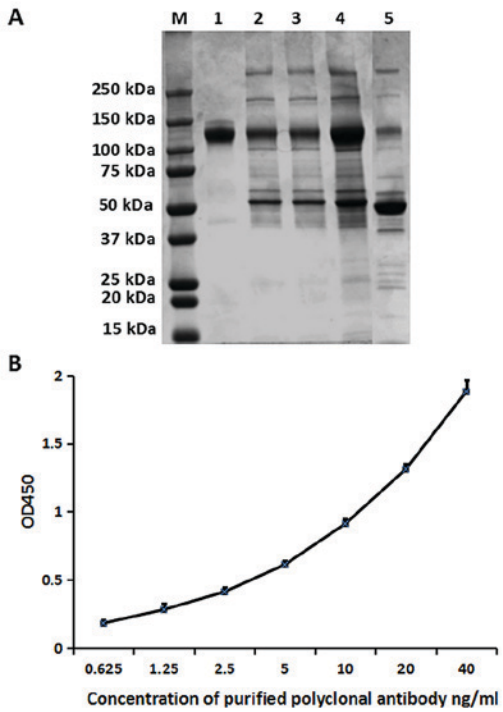


Figure 4. Characterization of the purified anti-CD147 polyclonal antibody. (A) SDS-PAGE analysis of purified polyclonal antibody. The rabbit antiserum against CD147 was purified by ammonium sulfate precipitation followed by antigen-immunoaffinity chromatography. Lane M, protein molecular weight marker; Lane 1, polyclonal antibody purified by antigen-immunoaffinity chromatography; Lane 2, washing liquid of antigen-immunoaffinity chromatography; Lane 3, flow liquid of antigen-immunoaffinity chromatography; Lane 4, polyclonal antibody purified by ammonium sulfate precipitation only; Lane 5, antiserum against CD147. (B) The reactivity of the antibodies purified by antigen-immunoaffinity chromatography was determined by indirect ELISA. The results demonstrated that the purified antibody had a high reactivity.

## Discussion

The present study obtained high-titer antiserum against human CD147 and subsequently purified the antiserum by ammonium sulfate precipitation followed by antigen-immunoaffinity chromatography. The polyclonal antibodies generated by this strategy were evaluated by indirect ELISA and proved to be useful (23). As a high titer of antiserum is the basis for high-quality antibodies (3), a titer of 1:512,000 determined in the present study

was high enough to produce high quality antibodies. The purification of immunoglobulins presents certain practical complications, particularly for polyclonal antibodies (24). There are various types of methods for the purification of antibodies (4); however, the choice of the purification method depends on the application of the antibodies (3). In the present study, polyclonal antibodies against CD147 were produced for sandwich ELISA to detect soluble CD147 antigen in serum; therefore, antigen-immunoaffinity chromatography was used to purify polyclonal antibodies to obtain highly specificity anti-CD147 antibodies. Immunoaffinity chromatography uses biologically associated binding agents and is used to selectively purify or analyze a target compound (25). In the current study, polyclonal antibodies against CD147 were purified by antigen-immunoaffinity chromatography following ammonium sulfate precipitation. The conditions of the antigen-immunoaffinity chromatography were optimized, including the buffer type and pH, and particularly the concentration of the reactors and reaction time (25,26). Following purification, antibodies with a purity of ~99% were obtained. In an indirect ELISA against CD147, the reactivity of the polyclonal antibodies purified by antigen-immunoaffinity chromatography was demonstrated to be high. The polyclonal antibody against CD147 and the MAb HAb18 made up the basic system of the sandwich ELISA for detecting soluble CD147. The lower limit of the ELISA method used for detecting the CD147 standard was 31.25 pg/ml. By contrast, with antibodies purified by Protein A or ammonium sulfate precipitation, the sensitivity of the ELISA using antibodies purified by antigen-immunoaffinity chromatography was the highest.

The polyclonal antibody purified by antigen-immunoaffinity chromatography may be a novel tool for further investigation of soluble CD147 in human serum. The sandwich ELISA kit described in the present study, which includes the antigen-immunoaffinity chromatography purified polyclonal antibody, may be used to detect the presence of CD147 in several types of cancers to investigate whether soluble CD147 is a biomarker in certain cancers. Meanwhile, the purification method discussed in the present study may be applied to the purification of various other antibodies.

## Acknowledgements

The present study was supported by grants from the National Science and Technology Major Project (grant no. 2012AA02A301).

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