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TITLE:

Production of Monoclonal Antibodies Targeting Aminopeptidase N in the Porcine Intestinal Mucosal Epithelium

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Aminopeptidase N, receptor, monoclonal antibodies, recombinant antibody protein, intestinal mucosal epithelium

Summary:

The recombinant antibody protein expressed in pIRES2-ZSGreen1-rAbs-APN-CHO cells and monoclonal antibodies produced using traditional hybridoma technology can recognize and bind to the porcine aminopeptidase N (APN) protein.

Abstract:

Porcine aminopeptidase N (APN), a membrane-bound metallopeptidase abundantly present in small intestinal mucosa, can initiate a mucosal immune response without any interference such as low protein expression, enzyme inactivity, or structural changes. This makes APN an attractive candidate in the development of vaccines that selectively target the mucosal epithelium. Previous studies have shown that APN is a receptor protein for both enterotoxigenic *Escherichia coli* (*E. coli*) F4 and transmissible gastroenteritis virus. Thus, APN shows promise in the development of antibody-drug conjugates or novel vaccines based on APN-specific antibodies. In this study, we compared production of APN-specific monoclonal antibodies (mAbs) using traditional hybridoma technology and recombinant antibody expression method. We also established a stably transfected Chinese hamster ovary (CHO) cell line using pIRES2-ZSGreen1-rAbs-APN and an *E. coli* expression BL21(DE3) strain harboring the pET28a (+)-rAbs-APN vector. The results show that antibodies expressed in

pIRES2-ZSGreen1-rAbs-APN-CHO cells and mAbs produced using hybridomas could recognize and bind to the APN protein. This provides the basis for further elucidation of the APN receptor function for the development of therapeutics targeting different APN-specific epitopes.

Introduction:

Aminopeptidase N (APN), a moonlighting enzyme that belongs to the metalloproteinase M1 family, acts as a tumor marker, receptor, and signaling molecule via enzyme-dependent and enzyme-independent pathways[1,2]. In addition to cleaving the N-terminal amino-acid residues of various bioactive peptides for the regulation of their biological activity, APN plays an important role in the pathogenesis of various inflammatory diseases. APN participates in antigen processing and presentation by trimmed peptides that bind tightly to major histocompatibility complex class II molecules[2,3]. APN also exerts anti-inflammatory effects by binding with G protein-coupled receptors participating in multiple signal transduction, modulating cytokine secretion, and contributing to Fc gamma receptor-mediated phagocytosis in the immune response[4-7].

As a widely distributed membrane-bound exopeptidase, APN is abundant in the porcine small intestinal mucosa and is closely associated with receptor-mediated endocytosis[1,5,8]. APN recognizes and binds the spike protein of the transmissible gastroenteritis virus for cell entry, and directly interacts with the FaeG subunit of enterotoxigenic *Escherichia coli* F4 fimbriae to affect bacterial adherence with host cells[9-11]. Thus, APN is a potential therapeutic target in the treatment of viral and bacterial infectious diseases.

Since the development of hybridoma technology and other strategies for monoclonal antibodies (mAbs) production in 1975, mAbs have been widely used in immunotherapy, drug delivery, and diagnosis[12-14]. Currently, mAbs are successfully used to treat diseases, such as cancer, inflammatory bowel disease, and multiple sclerosis[12,15]. Because of their strong affinity and specificity, mAbs can be ideal targets in the development of antibody-drug conjugates (ADC) or new vaccines[16,17]. The APN protein is critical for selectively delivering antigens to specific cells, and can elicit a specific and strong mucosal immune response against pathogens without any interference including low protein expression, enzyme inactivity, or structural changes[5,8,18]. Therefore, therapeutic products based on APN-specific mAbs show promise against bacterial and viral infections. In this study, we describe the production of APN-specific mAbs using hybridoma technology, and expression of anti-APN recombinant antibodies (rAbs) using prokaryotic and eukaryotic vectors. The result indicates that the APN protein was recognized by both rAbs expressed in pIRES2-ZSGreen1-rAbs-APN-CHO cells and hybridoma-derived mAbs.

Protocol:

All animal experiments in this study were approved by the Yangzhou University Institutional Animal Care and Use Committee (SYXK20200041).

1. Preparation of porcine APN protein antigen

NOTE: The pET28a (+)-APN-BL21 (DE3) strain and the APN stably expressed cells pEGFP-C1-APN-IPEC-J2 were constructed in a previous study[11].

1.1. Recover bacteria from a frozen glycerol stock and streak onto Luria–Bertani (LB) plates containing 50 μg/mL kanamycin (Km^+) for single colony isolation.

1.2. Select a single colony from the freshly streaked plate, culture in 4 mL of LB medium (10 g/L tryptone, 10 g/L sodium chloride (NaCl) and 5 g/L yeast extract, pH 7.2) supplemented with Km^+ (50 μg/mL), and leave to grow overnight (12-16 h) with agitation (178 rpm) at 37 °C.

1.3. Dilute the prepared bacteria at 1:100 in fresh Km^+ LB broth and incubate at 37 °C with shaking for 2-3 h until the OD_{600} reaches 0.4-0.6.

1.4. Add isopropyl β-d-1-thiogalactopyranoside (IPTG) to the medium to a final concentration of 0.4 mM, and incubate the cultures for an additional 10 h at 16 °C.

1.5. Consequently, centrifuge and harvest the bacteria using IPTG induction (10,000 × g, 4 °C 15 min).

1.6. Resuspend the cell pellet using 5 mL of LEW (Lysis/Equilibration/Wash) buffer (50 mM anhydrous sodium phosphate monobasic (NaH_2PO_4) and 300 mM NaCl, pH 8.0) containing 1 mg/mL lysozyme. Stir the bacterial suspension for 30 min on ice and sonicate completely (15 s pulse and 20 s off, 15 min) using an ultrasonic homogenizer.

1.7. Centrifuge the crude cell lysate at 4 °C and 10,000 × g for 30 min to remove cellular debris. Transfer supernatant into a pre-equilibrated column and incubate 1-2 min before gravity drainage. Repeat this step three times.

1.8. Wash the column using 20 mL of LEW buffer and drain using gravity. Elute the histidine-tagged APN protein using 9 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0) and collect into dialysis tubing.

1.9. Dialyze the protein solution overnight at 4 °C in sodium carbonate-sodium bicarbonate (PBS, 135 mM NaCl, 4.7 mM potassium chloride, 2 mM NaH_2PO_4, and 10 mM dodecahydrate sodium phosphate dibasic, pH 7.2) buffer

1.10. Analyze using a 12.0% SDS-PAGE gel and western blotting to assess the purity of the APN protein.

1.10.1. Load 5 μg of protein into each well of the gel and allow to run at 110 V for 1.5 h. Then, transfer protein onto a PVDF membrane for 50 min at 15 V. Determine the concentration of the purified protein using a BCA assay.

2. Animal immunization

2.1. Subcutaneous (s.c) inject female BALB/c mice, 6-8 weeks of age, with 50 μg of APN protein or PBS (negative control) mixed with adjuvants once every 2 weeks. Use complete Freund's adjuvant that contains the heat-killed Mycobacteria for initial immunization, and incomplete Freund's adjuvant for booster immunizations.

2.2. Detect antibody titers against APN in the sera of these mice by indirect enzyme-linked immunosorbent assay (ELISA) using a microtiter plate coated with 5 μg/mL APN protein diluted in 0.05 M PBS (pH 9.6).

3. Hybridoma technology to produce monoclonal antibodies against APN

3.1. Intraperitoneally (i.p.) inject 100 μg of APN protein into the selected mice for a final antigen boost.

3.2. Three days later, sacrifice the mice using 10% chloral hydrate and cervical dislocation.

3.3. Collect spleens, and wash with DMEM twice to remove blood and fat cells. Filter the spleen-cell suspension using a 200-mesh copper grid to remove tissue debris, and harvest spleen cells using centrifugation (1500 × g, 10 min) to remove the membrane of the spleen.

3.4. Seed mouse myeloma SP2/0 cells in a 25 cm^2 flask containing 5 mL of DMEM supplemented with 6% fetal bovine serum (FBS) and culture at 37 °C, 6% CO_2 atmosphere to maintain cell viability. After 5-6 days of culture, the cells reach 80%-90% confluence post-resuscitation and are in growth log phase. Under the microscope, the cells are round, bright, and clear.

3.5. One day before hybridization, collect macrophages from peritoneal cavities of the mice according to a previously published method[12,19].

3.6. Seed peritoneal macrophages at a density of 0.1-0.2 × 10^5/mL in 96-well plates, each well containing 100 μL of HAT medium (DMEM supplemented with 10% FBS and 1x HAT Supplement), and incubate at 37 °C, 6 % CO_2 humidified atmosphere overnight.

3.7. For hybridization, gently aspirate SP2/0 cells with a pipette from 8-10 bottles, and suspend in 10 mL of serum-free DMEM medium. Wash cells with fresh DMEM, centrifuge (1500 × g, 10 min) twice, and then re-suspend in 10 mL of DMEM.

3.8. Mix the quantified spleen cells with SP2/0 cells at a ratio of 10:1 and transfer into 50 mL tubes. Centrifuge (1500 × g, 10 min) and discard the supernatant. Collect the cell pellets at the bottom of the tubes and tap with palm to loosen the pellets prior to hybridization.

3.9. Add 1 mL of polyethylene glycol 1500 (PEG 1500), pre-warmed to 37 °C, dropwise using a dropper to the loosened cell pellet over the time period of 45 s while gently rotating the bottom of the tube.

3.10. Slowly add 1 mL of DMEM pre-warmed to 37 °C to the above mixture over the period of 90 s, followed by another 30 mL of fresh DMEM. Place the fusion tube into a 37 °C water bath for 30 min.

3.11. After incubation in the warm bath, harvest the cells and re-suspend in HAT medium. Then culture in a 96-well plate inoculated with peritoneal macrophages.

3.12. Five days later, add 100 μL of fresh HAT medium to each well, and incubate the plate for an additional 5 days, after which replace the medium with HT medium (DMEM supplemented with 10% FBS and 1x HT Supplement).

3.13. Use a microtiter plate coated with 5 μg/mL APN protein diluted in 0.05 M PBS (pH 9.6) to analyze monoclonal antibodies in the hybridoma supernatant using ELISA assay.

3.13.1. When the medium in the wells of the 96-well plate turns yellow (due to cell growth and metabolite release, pH in the medium decreases to 6.8, and phenol red turns from fuchsia to yellow) or cell clusters are observed, acquire 100 μL supernatant from the selected wells and add to the wells of the coated ELISA plate. Use a microplate reader to measure the OD450 values.

3.13.2. Use the polyclonal antibodies against APN and non-infected mouse serum as positive and negative control, respectively, and use PBS as blank control. In this study, OD450 ratio of sample to negative control (P/N) $\geq$ 2.1 was recognized as positive selection standard.

3.14. After three consecutive positive selection rounds, select the hybridoma showing increased serology response against the APN protein for a limited dilution assay.

3.14.1. Prepare peritoneal macrophages and seed in 96-well plates as described previously.

3.14.2. Suspend hybridoma cells in HT medium at an average of 0.5-2 cells per well and culture in a 37 °C, 6% CO_2 incubator. Repeat this step three or four times until the positive rate indicated by ELISA immunoassay reaches 100%.

3.15. Under the pressure of continuous freezing and thawing, select the positive hybridoma cells able to stably secrete anti-APN antibodies and proliferate normally.

3.15.1. Administer a single i.p. injection of 0.3 mL of pristane to each mouse (8-10 weeks). At 10 days after receiving pristine, inject each mouse with 2-5×10^5 hybridoma cells in 0.5 mL of PBS (pH 7.2).

3.15.2. Carefully collect peritoneal fluid from the peritoneal cavity of these mice 8 to 10 days after the injection.

3.15.3. Harvest the supernatants by centrifugation at 5,000 × g for 15 min, and purify antibodies in the supernatants using 33% saturated ammonium sulfate [$(NH_4)_2SO_4$] precipitation and protein A agarose.

4. Characterization of mAbs against APN protein

4.1. Determine the immunoglobulin subtype of the collected mAbs using an SBA Clonotyping System-HRP[20]. Use SDS-PAGE and western blotting to assess mAb purity and specificity.

4.2. Analyze mAb epitope specificity against the APN protein using ELISA[21]. Additivity value (AV) is the ratio of $OD_{mAbs\ (a+b)}$ to ($OD_{mAbs\text{-}a}$+$OD_{mAbs\text{-}b}$), which is used to evaluate whether mAbs recognize the same antigenic site; $OD_{mAbs\text{-}a}$ and $OD_{mAbs\text{-}b}$ represent the OD450 values of different monoclonal antibodies against APN alone, and $OD_{mAbs\ (a+b)}$ represent the OD450 values of a 1:1 mixture of two mAbs against APN.

4.2.1. Assess each sample at least four replicates, and repeat the whole experiment at least three times.

5. Expression of rAbs against APN

5.1. Extract total RNA from the above-mentioned hybridoma cells and spleens of APN-immunized mice (e.g., TRIzol)[22]. Synthesize complementary DNA (cDNA) using a cDNA synthesis kit per manufacturer's instructions.

5.2. Amplify variable regions of mAbs using nested PCR and determine heavy chain (VH) and light chain (VL) sequences using sequencing. Analyze the genes encoding VH and VL using the IMGT mouse genome analysis tool (http://www.imgt.org/about/immunoinformatics.php).

5.3. Combine the VH and VL genes with leader sequences and sequentially subclone them into the pET28a (+) and pIRES2-ZsGreen1 vectors, respectively, using seamless cloning technology to allow for scarless DNA fragment insertion. The specific primers are listed in **Table 1**.

5.4. Grow the pET28a (+)-rAbs-APN-BL21-transformed bacteria in the presence of 0.4 mM IPTG in orbital shakers at 37 °C for 10 h. Then induce, purify, and assess for the expression of the rAbs protein using routine protein purification.

5.5. Seed 100 µL 0.5 x 10^5 CHO cells per well into a 96-well plate and incubate at 37 °C in a 6% CO_2 atmosphere for 18-24 h. When the cells reach 80-90% confluence, dilute the pIRES2-ZsGreen1-rAbs-APN plasmid with Opti-MEM to a final concentration of 0.1 µg/µL, and incubate 5 min at room temperature before using for transfection.

5.6. Gently mix 50 µL of diluted pIRES2-ZsGreen1-rAbs-APN plasmid with 1 µL of Lipofectamine 2000 and 49 µL of Opti-MEM, and incubate the mixture for an additional 20 min at room temperature. Add 100 µL of mixture to each well of a 96-well plate containing CHO cells and incubate at 37 °C in 6% CO_2 atmosphere for 4–6 h.

5.7. At 4-6 h post-transfection, replace the medium with DMEM-F12 medium supplemented with 10% FBS, and incubate the plate for another 48 h. Then, add 400 µg/mL G418 to each well to select the stably transfected cells.

5.8. After 10 days of selection using DMEM-F12 medium supplemented with 10% FBS and 400 µg/mL G418, sort the cells (3.0×10^7 cells/mL) by fluorescence-activated cell sorting. Approximately 10-15% of the cell population were positive.

5.9. Serially dilute harvested positive cells, seed at an average of 0.5-2 cells per well in a 96-well plate, and culture in a 37 °C, 6% CO_2 incubator. Maintain the stably transfected pIRES2-ZsGreen1-rAbs-APN-CHO cells using selection with G418 (200 µg/mL).

5.10. FBS concentration in the above-described cell-culture medium decreases gradually from 10% to 0% during the logarithmic growth phase over the time period of 3 weeks. Then, adapt the adherent CHO cells to suspension growth in a serum-free medium.

5.11. Culture the seeded pIRES2-ZsGreen1-rAbs-APN-CHO cells in the logarithmic growth phase in serum-free medium at a density of $0.8\text{-}1.0 \times 10^5$ cells/mL in shake flasks at 80-110 rpm shaking speed and 37°C, 6% CO_2.

5.12. Collect the cell suspension every 12 h to determine changes in cell viability and vitality using a cell counting kit (e.g., CCK-8) per manufacturer's instructions.

5.13. Antibody expression reaches peak levels when cell viability decreased to 80% and cell density reaches $1.0\text{-}2.0 \times 10^6$ cells/mL. Harvest cell supernatants using centrifugation, filter using a 0.22 µm polytetrafluoroethylene membrane filter, and purify using protein A agarose.

5.14. Confirm production of APN-specific antibodies using indirect immunofluorescence assays (IFA).

5.15. Determine antibody titers and binding affinities using ELISA assay as described previously.[23] Calculate the equilibrium dissociation constant (K_D value) of the antibodies with a four-parameter logistic equation using software.

Representative Results:

In this study, the purified soluble APN protein (2.12 mg/mL) was used for mouse immunization. Mice immunized with the APN protein four times at 14-day intervals exhibited a higher antibody titer against APN in their sera. Although 14 hybridomas were obtained using the

fusion experiments, only 9 hybridomas survived the three continuous freeze-thaw cycles, resulting in 9 stable clones that secreted antibodies against APN. All these cells are round, bright, and clear (**Figure 1**). The purified mAbs possessing heavy and light chains (50 kDa and 25 kDa, respectively) were confirmed by SDS-PAGE and found in the purified ascites (**Figure 2**). The titers of these anti-APN mAbs in culture supernatants and ascites are shown in **Table 2**.

The result of mouse mAbs isotyping revealed that mAbs derived from clones 5B31, 5B36, 3C48, 5C51, and 6C56 possessed IgG2b subclasses, while APN-2A20 was an IgG2a κappa-(κ) type antibody, and mAbs APN-3FD9, -3F10, and -10F3 belonged to IgM type and processed κ light chains (**Table 3**). As shown in **Table 4**, most of these mAbs showed AV values of over 50%, indicating that they targeted different epitopes in the APN, while the APN-5C51 antibody recognized antigenic epitopes similar to those recognized by APN-3C48, -5B31, and -6C56 mAbs.

APN-5B36 showed considerably higher antibody titer compared with those of other mAbs. Therefore, the APN-5B36 VH-VL gene was amplified and ligated into a pET28a (+) or pIRES2-ZsGreen1 vector to construct the recombinant expression plasmids pET28a (+)-rAbs-APN and pIRES2-ZsGreen1-rAbs-APN, respectively (**Figure 3**). The antibodies expressed by both pET28a (+)-rAbs-APN-BL21 (DE3) and pIRES2-ZsGreen1-rAbs-APN-CHO cells were purified and analyzed using ELISA and IFA assays. However, as shown in **Figure 4**, only the antibody expressed in the supernatant of pIRES2-ZsGreen1-rAbs-APN-CHO cells recognized the APN protein, as did hybridoma-derived mAbs. This recombinant antibody consisted of IgG2b heavy chains and lambda light chains, and showed a titer of 2.56×10^5 as determined using ELISA. The binding of APN-5B36 mAbs to APN proteins reached an equilibrium earlier than rAbs did (**Figure 5**), showing K_D value of $(4.232\pm0.475) \times 10^{-9}$ and $(2.201\pm0.367) \times 10^{-8}$ mol/L, respectively.

Table 1. The specific primers used in this study.

Table 2. The ELISA titers of APN mAbs.

Table 3. Isotypes of hybridoma-derived APN mAbs.

Table 4. Discrimination of antigen-epitope specificity of APN-specific mAbs. AV values greater than 0.5 indicate that these two mAbs recognize different antigenic sites; AV values less than 0.5 indicate that these two mAbs recognize a similar antigenic site.

Figure 1. Image of hybridomas. Under microscopic analysis, hybridomas are round, bright, and clear.

Figure 2. Recombinant antibody expression levels and ascites analyzed using SDS-PAGE. (**A**) Lane M, protein marker; lane 1, purified pET28a (+)-rAbs-APN-BL21 (DE3) lysate; lane 2, pET28a (+)-rAbs-APN-BL21 (DE3) supernatant; lane 3, ascites fluid purified by 33%

$(NH_4)_2SO_4$ precipitation. (**B**) Lane M, protein marker; lane 1, ascites fluid purified using protein A agarose. In this assay, 3-5 μg of total protein was loaded into each lane of the gel.

Figure 3. Recombinant expression plasmids pET28a (+)-rAbs-APN and pIRES2-ZsGreen1-rAbs-APN analyzed using agarose gel electrophoresis. Lane M, Trans 2K plus DNA marker; lane 1, pET28a (+) vector (5369 bp); lane 2 and 5, VH-VL gene combined with APN-5B36 leader sequence; lane 3, pET28a (+)-rAbs-APN plasmid expressed in BL21 (DE3) *E. coli*; lane 4, pIRES2-ZsGreen1 vector (5283 bp); lane 6, pIRES2-ZsGreen1-rAbs-APN plasmid expressed in DH5α *E. coli*.

Figure 4. Expression of recombinant antibody protein and ascites analyzed using indirect immunofluorescence. pEGFP-C1-APN-IPEC-J2 cells (green fluorescence) stably expressing APN were treated with (**A**) PBS, used as control treatment; (**B**) purified protein expressed by pET28a (+)-rAbs-APN-BL21 (DE3); (**C**) APN polyclonal antibody (1:500); (**D**) purified ascites fluid (1:500); E) purified supernatant obtained from pIRES2-ZsGreen1-rAbs-APN-CHO cells (1:500). DAPI was used as nuclear counterstain in confocal microscopy. The cells incubated with the DyLight 549-conjugated goat anti-mouse IgG secondary antibody (1:200) and treated with purified ascites and purified supernatant from pIRES2-ZsGreen1-rAbs-APN-CHO cells showed a robust red-fluorescence signal indicative as the APN polyclonal antibody did.

Figure 5. **Determination of antibody relative binding affinities using the ELISA[22].** Absorption of samples containing APN-5B36 mAbs or rAbs, in the absence and presence of APN protein, was measured at the wavelength of 450 nm. Binding curve was plotted using a four-parameter logistic curve fit; x-axis shows the logarithmic concentration of antibodies, and y-axis shows the absorbance.

Discussion:

Induction of mucosal immunity is one of the most effective approaches in counteracting pathogens and in prevention and treatment of various diseases. APN, a highly expressed membrane-bound protein in the intestinal mucosa, is involved in the induction of adaptive immune response and in receptor-mediated viral and bacterial endocytosis[1,5,8]. APN is used as antigen particulate in many formats of antigen loading and vaccine delivery. The oral administration of APN-targeted antibodies can also elicit effective immune responses[18,24,25]. However, monoclonal antibodies targeting different APN-specific epitopes require further investigation.

The methods described here were employed to produce monoclonal antibodies against APN using both traditional hybridoma and recombinant technologies. This approach can be used in the production of other mAbs. First, we followed a previously mentioned protocol to obtain nine mAbs derived from different hybridoma clones. Although the titers of these mAbs in cell supernatants and ascites were different, all the mAbs contained the 50 kDa heavy chain and 25 kDa light chain and showed specific binding with the porcine APN protein. The results of isotyping and identification of antigen epitopes showed that most of the mAbs targeted

different epitopes and belonged to different antibody types. These results indicate that traditional hybridoma technology remains an effective choice in the production of mAbs.

Approaches used for recombinant-antibody production can increase mAb production efficiency and minimize labor- and time-associated costs. Therefore, these approaches have grown in popularity, especially in the development of antibodies for diagnostic and therapeutic applications[16,17,26]. rAbs show several advantages over hybridoma-derived mAbs. First, rAbs can be produced in vitro by cloning antibody genes into expression vectors, thereby eliminating animal use in antibody production. Additionally, using eukaryotic or prokaryotic expression systems to produce rAbs results in low batch-to-batch variations and increases reliability and stability of the final product. In contrast, mAbs produced using hybridomas often do not recognize or bind to the targeted antigen epitopes, and are affected by hybridoma cell-line drift, contamination, and gene loss and mutations. Presently, hybridoma-derived mAbs are mostly used in diagnostic or therapeutic immune-reagents, highlighting the need to produce stable and reliable antibodies in a limited production period. For diagnostic and therapeutic applications, recombinant antibody technology is a better choice than traditional hybridoma-based approach. This is because recombinant antibody technology allows us to modify the sequence of rAbs to switch immunoglobulin isotypes, thereby increasing the binding specificity of the antibody[14,16,17].

In this study, we used antibody-engineering technology to obtain APN-targeted recombinant antibodies. We found that both the spleens of immunized mice and hybridoma cells were similarly effective for amplifying the mAb heavy- and light-chain sequences. The antibody expressed by pET28a (+)-rAbs-APN-BL21 (DE3) did not effectively recognize APN in either ELISA or indirect immunofluorescence assays. However, rAbs expressed by the pIRES2-ZsGreen1-rAbs-APN-CHO cell suspension and hybridoma-derived mAbs did recognize and effectively bind the APN protein. The methods described in this study can be used to develop APN antibody-based ADC and other therapeutic products targeting different APN-specific epitopes. This study will also aid in further clarifying the role of APN in the prevention and treatment of various diseases. However, strategies to improve the affinity and yield of rAbs require further investigation.

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Disclosures:
The authors declare no conflict of interest. All the authors approved and gave their explicit consent for publication of the manuscript.

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Figure 1

Click here to access/download;Figure;Figure 1.tif

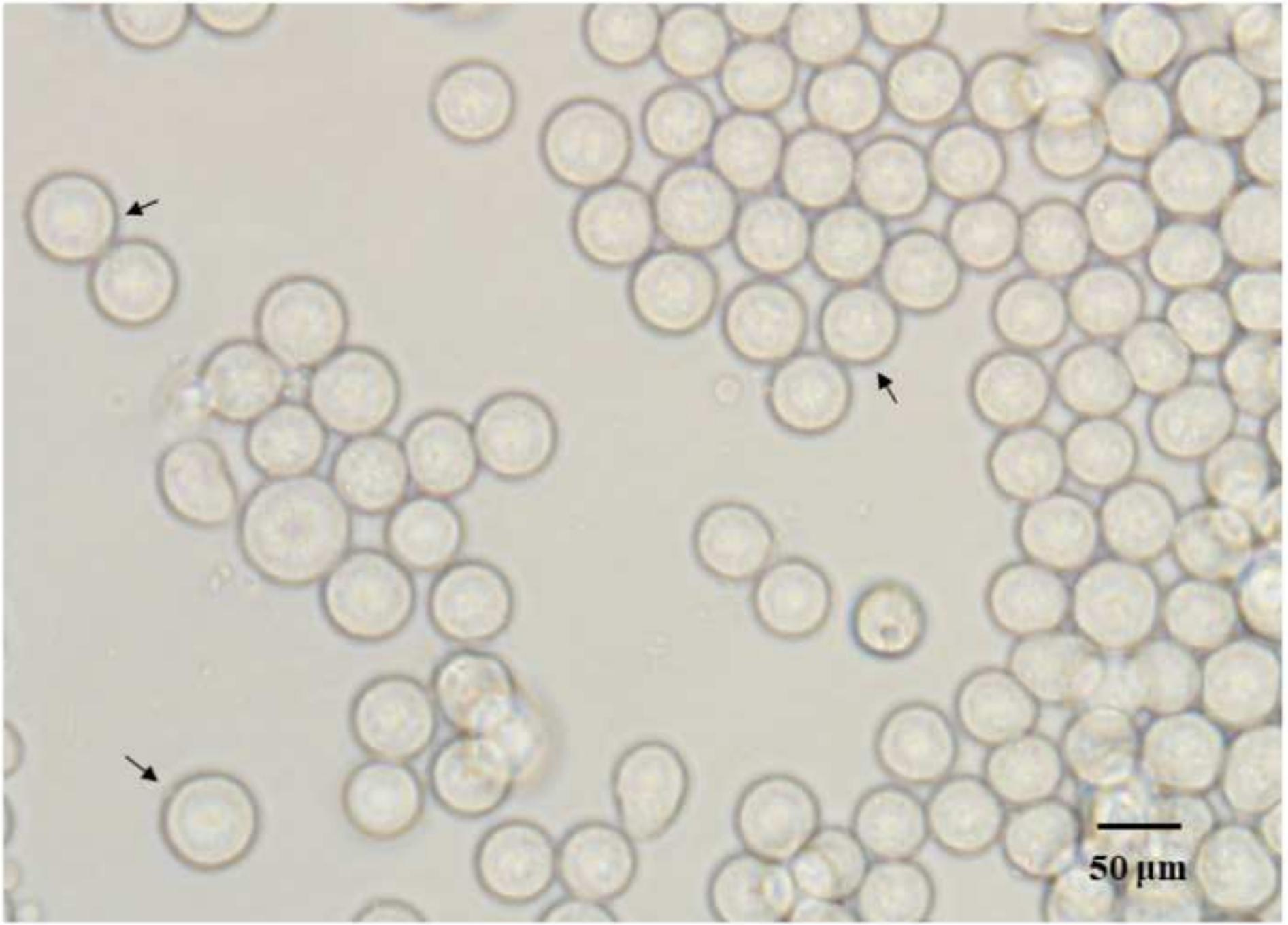

Figure 2

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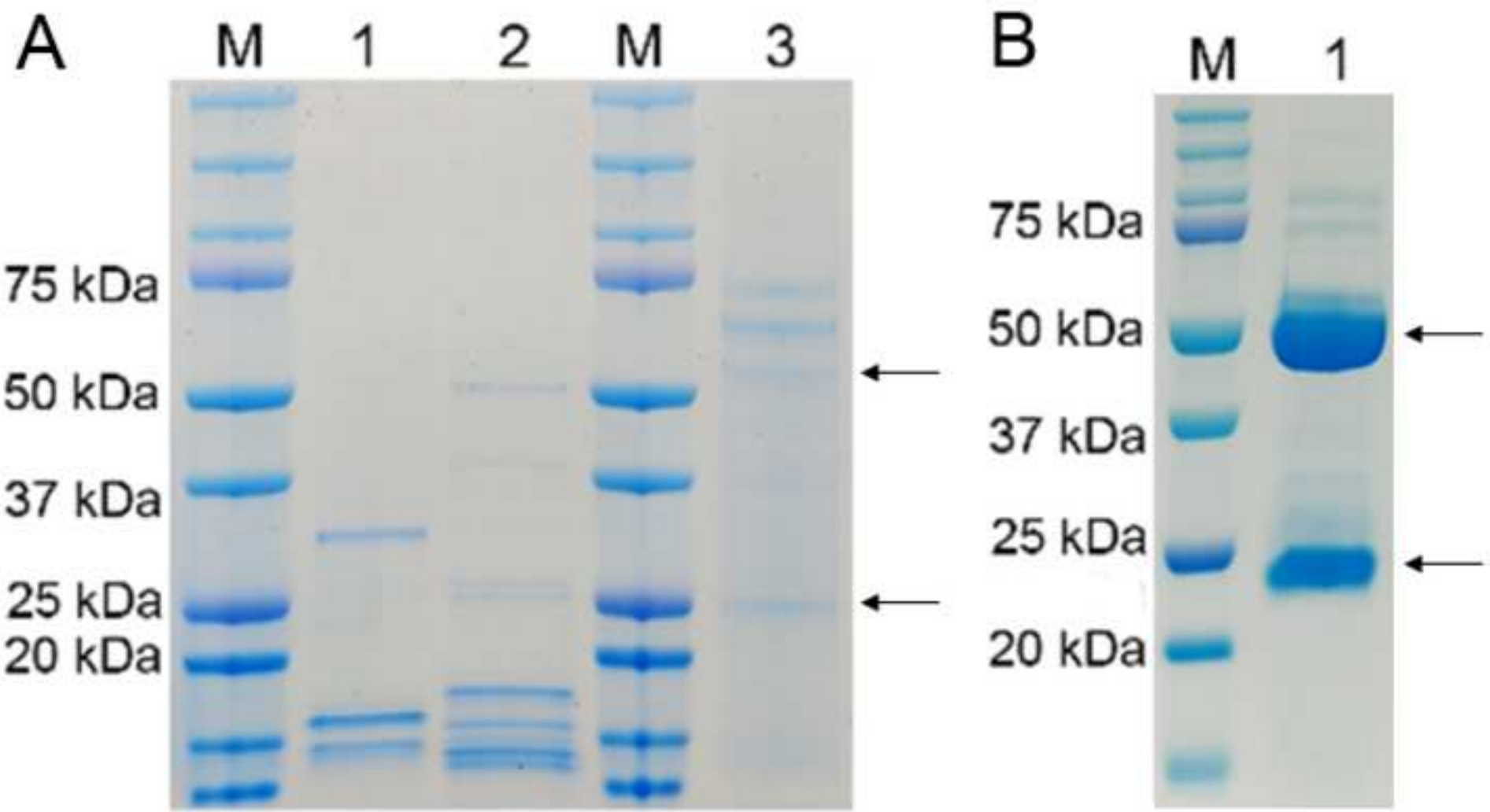

Figure 3

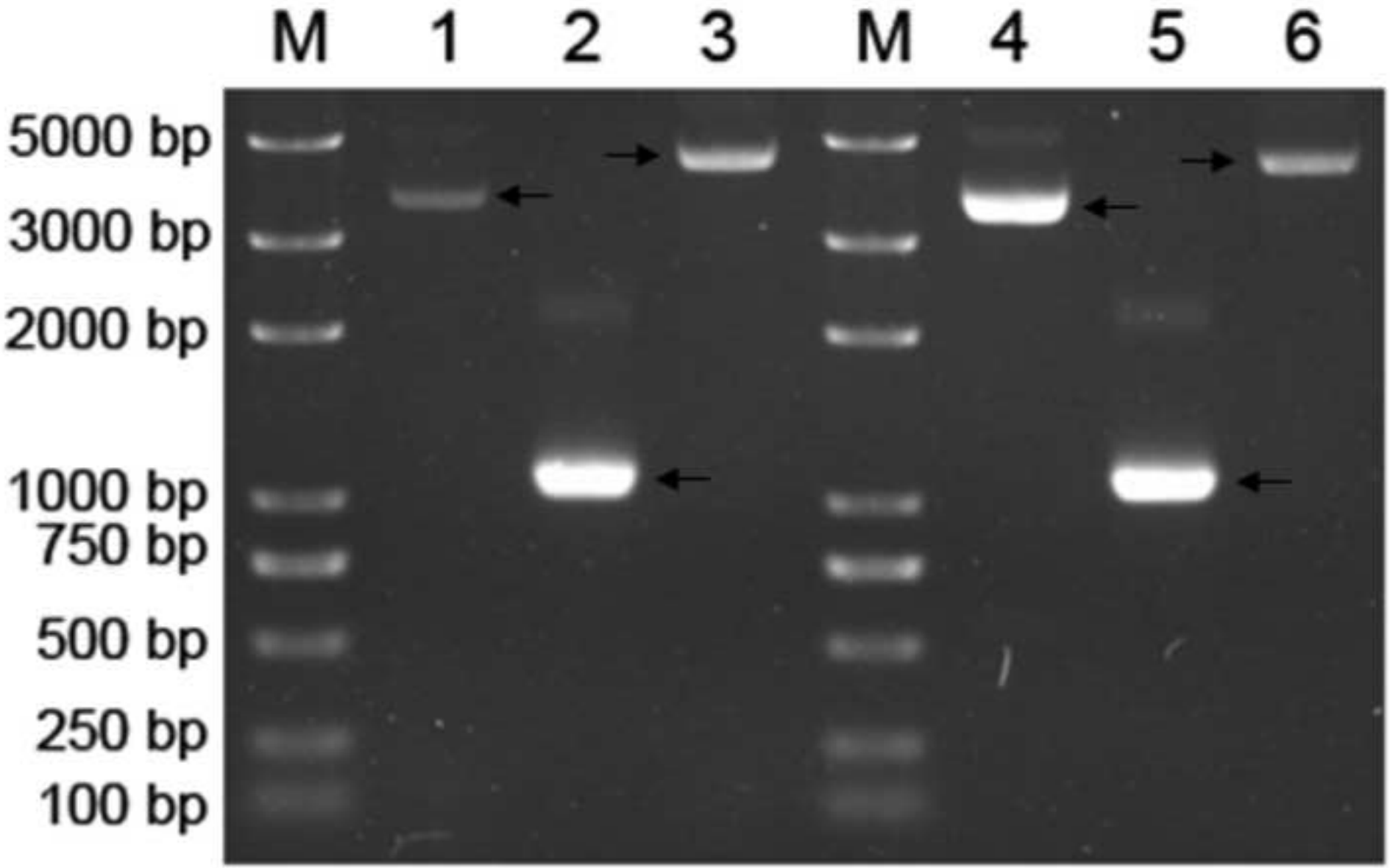

Figure 4

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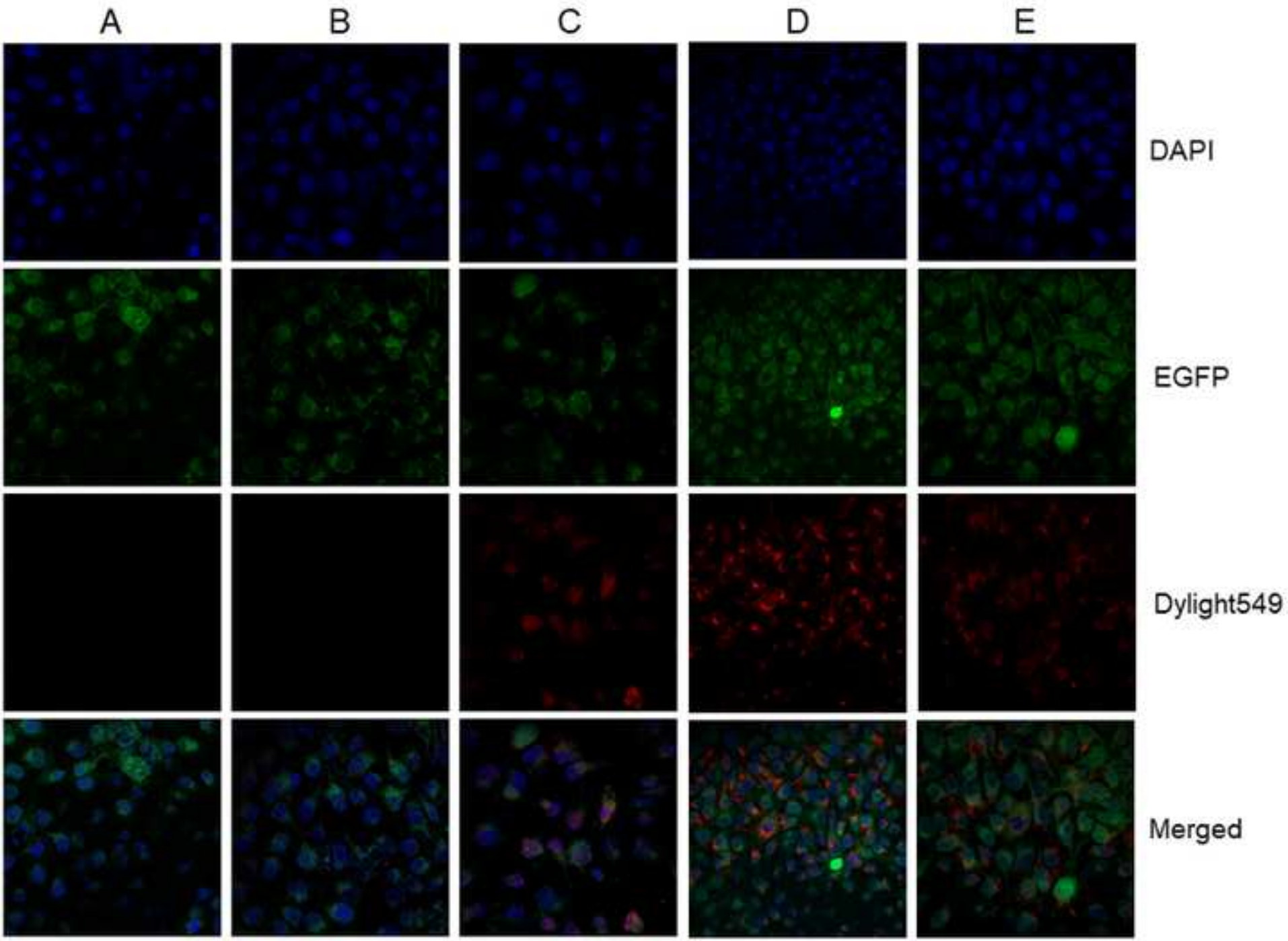

Figure 5

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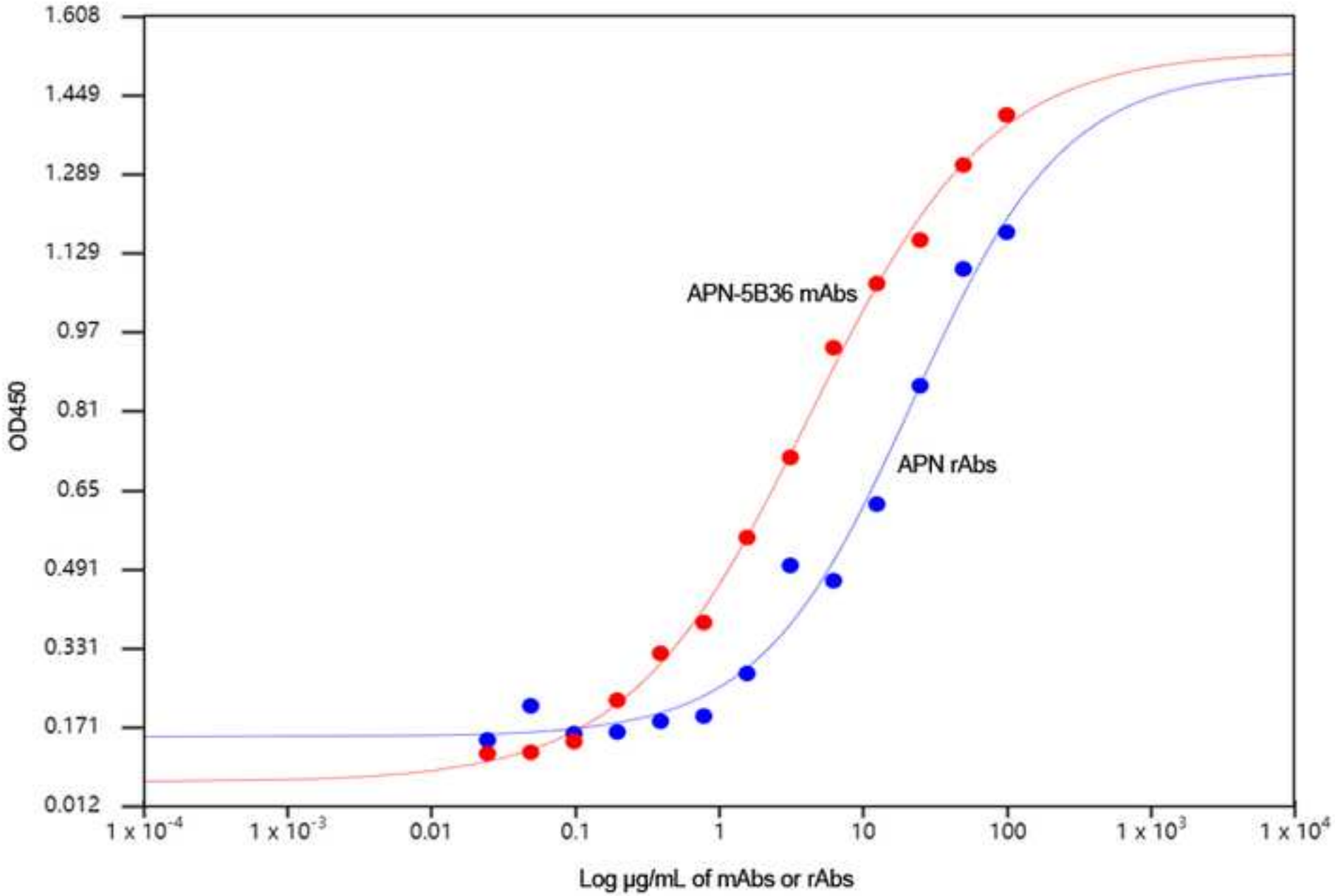

Table 1

Primer	Sequence (5'-3')
VH-VL-F	CCGGGTGGGCCGGATAGACMGATGGGGCTG
VH-VL-R	CCGGCCACATAGGCCCCACTTGACATTGATGT
pET28a (+)-F	TCCACCAGTCATGCTAGCCATAACAACGGTCGTGATTCGA
pET28a (+)-R	CTGGTGCCGCGCGGCAGCCAGTGGGATACCCGTATTACCC
pIRES2-ZsGreen1-F	CGACGGTACCGCGGGCCCGGTAACAACGGTCGTGATTCGA
pIRES2-ZsGreen1-R	GGGGGGGAGGGAGAGGGGCGGTGGGATACCCGTATTACCC

Table 2

Cells	Titers of supernatants （U/mL）	Titers of ascites （U/mL）
2A20	0.64×10^{4}	3.20×10^{5}
5B31	1.28×10^{4}	1.60×10^{5}
5B36	0.64×10^{4}	1.28×10^{6}
3C48	0.16×10^{4}	0.80×10^{5}
5C51	0.16×10^{4}	0.80×10^{4}
6C56	0.80×10^{3}	0.80×10^{4}
3FD9	0.80×10^{3}	0.80×10^{4}
3F10	0.16×10^{4}	0.16×10^{5}
10F3	0.80×10^{3}	0.32×10^{5}

Table 3

	Ig	IgA	IgM	IgG1	IgG2a	IgG2b	IgG3	Kappa
2A20	1.735	0.023	0.011	0.006	0.903	0.044	0.015	0.137
5B31	1.199	0.006	0.003	0.005	0.005	1.731	0.004	0.004
5B36	1.652	0.012	0.013	0.01	0.008	2.41	0.002	0.003
3C48	0.951	0.063	0.068	0.104	0.062	1.785	0.059	0.065
5C51	1.064	0.008	0.007	0.008	0.008	1.87	0.004	0.004
6C56	0.78	0.062	0.06	0.063	0.063	1.516	0.062	0.061
3FD9	1.474	0.007	1.678	0.003	0.016	0.081	0.002	0.519
3F10	1.21	0.002	1.454	0.009	0.008	0.054	0.003	0.414
10F3	1.179	0.058	1.562	0.152	0.131	0.179	0.044	0.359

Lambda	Summary
0.073	IgG2a, Kappa
0.413	IgG2b, Lambda
0.707	IgG2b, Lambda
0.51	IgG2b, Lambda
0.415	IgG2b, Lambda
0.387	IgG2b, Lambda
0.059	IgM, Kappa
0.096	IgM, Kappa
0.049	IgM, Kappa

Table 4

mAbs	AV (100 %)							
	2A20	5B31	5B36	3C48	5C51	6C56	3FD9	3F10
2A20	-	0.601	0.905	0.889	0.804	0.884	1.009	1.047
5B31	0.601	-	0.871	0.754	0.464	0.694	0.613	0.88
5B36	0.905	0.871	-	0.794	0.684	0.934	0.91	1.07
3C48	0.889	0.754	0.794	-	0.461	0.709	0.428	1
5C51	0.804	0.464	0.684	0.461	-	0.301	0.601	0.594
6C56	0.884	0.694	0.934	0.709	0.301	-	1.216	0.583
3FD9	1.009	0.613	0.91	0.428	0.601	1.216	-	1.737
3F10	1.047	0.88	1.07	1	0.594	0.583	1.737	-
10F3	0.914	0.989	0.959	0.787	0.852	0.389	0.744	0.682

10F3
0.914
0.989
0.959
0.787
0.852
0.389
0.744
0.682
-

Name of Material/Equipment	Company	Catalog Number
Complete Freund's adjuvant	Sigma-Aldrich	F5881
DAPI	Beyotime Biotechnology	C1002
DMEM	Gibco	11965092
DMEM-F12	Gibco	12634010
Dylight 549-conjugated goat anti-mouse IgG secondary antibody	Abbkine	A23310
Enhanced Cell Counting Kit-8	Beyotime Biotechnology	C0042
Fetal bovine serum	Gibco	10091
Geneticin™ Selective Antibiotic	Gibco	11811098
HAT Supplement (50X)	Gibco	21060017
HT Supplement (100X)	Gibco	11067030
Incomplete Freund's adjuvant	Sigma-Aldrich	F5506
isopropyl β-d-1-thiogalactopyranoside	Sigma-Aldrich	I5502
kanamycin	Beyotime Biotechnology	ST102
Leica TCS SP8 STED confocal microscope	Leica Microsystems	SP8 STED
Lipofectamine® 2000 Reagent	Thermofisher	11668019
LSRFortessa™ fluorescence-activated cell sorting	BD	FACS LSRFortessa
Microplate reader	BioTek	BOX 998
Micro spectrophotometer	Thermo Fisher	Nano Drop one
NaCl	Sinopharm Chemical Reagent	10019308
$(NH_4)_2SO_4$	Sinopharm Chemical Reagent	10002917
Opti-MEM	Gibco	31985088
Polyethylene glycol 1500	Roche Diagnostics	10783641001
PrimeScript™ 1st strand cDNA Synthesis Kit	Takara Bio	RR047
protein A agarose	Beyotime Biotechnology	P2006
Protino® Ni+-TED 2000 Packed Columns	MACHEREY-NAGEL	745120.5
SBA Clonotyping System-HRP	Southern Biotech	May-00
Seamless Cloning Kit	Beyotime Biotechnology	D7010S
Shake flasks	Beyotime Biotechnology	E3285
Sodium carbonate-sodium bicarbonate buffer	Beyotime Biotechnology	C0221A
Trans-Blot SD Semi-Dry Transfer Cell	Bio-rad	170-3940
Tryptone	Oxoid	LP0042

Ultrasonic Homogenizer	Ningbo Xinzhi Biotechnology	JY92-IIN
Yeast extract	Oxoid	LP0021
96-well microplate	Corning	3599

Comments/Description
Animal immunization
Nuclear counterstain
Cell culture
Cell culture
Indirect immunofluorescence analysis
Measurement of cell viability and vitality
Cell culture
Selective antibiotic
Cell selection
Cell selection
Animal immunization
Protein expression
Bactericidal antibiotic
Fluorescence imaging
Transfection
Flow cytometry
ELISA analysis
Nucleic acid concentration detection
Culture broth
Culture broth
Cell culture
Cell fusion
qPCR
Antibody protein purification
Protein purification
Isotyping of mouse monoclonal antibodies
Construction of plasmids
Cell culture
Cell culture
Western blot
Culture broth

Sample homogenization
Culture broth
Cell culture

April 30, 2021

Editor(s), *JoVE*

Dear Respected Editor (s),

Thank you very much for providing the nice comments concerning our manuscript entitled "The production of monoclonal antibodies targeting Aminopeptidase N of the porcine intestinal mucosal epithelium" (JoVE62437R1). We have revised the manuscript and have made all of the changes suggested by you and the reviewer (The changes are red-labelled in the manuscript). In advance of submission, we use LetPub language services to edit our manuscript. All authors have reviewed the final version of the manuscript and approved it for publication. These improvements to the manuscript are listed below:

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

-- Fixed as requested. We have carefully revised our paper accordingly, and the detailed change was red-labelled in the manuscript. In advance of submission, we use LetPub language services to edit our manuscript.

2. Please add additional details to the protocol.

Please provide the compositions of all buffers and solutions used.

-Fix as requested.

1.3: Harvest the bacteria how?

-The bacteria were harvested by centrifugation. The sentence changed as "Centrifuge and harvest the bacteria with IPTG induction (10,000 × g, 4 ℃ 15 min).".

1.7: Please provide PAGE parameters and Western blot parameters.

-Fix as requested. The sentence changed as "For this, load 5 μg of protein into each well of the gel and allow to run at 110 V for 1.5 h. Then, transfer protein onto a PVDF membrane for 50 min at 15 V".

2.1: How is the protein given to the animals? IP injection as in step 3.1?

- Fix as requested. The sentence changed as "subcutaneous (s.c) inject female BALB/c mice, …".

2.2: Please provide more details here.

-Fix as requested. We added the sentence "Detect antibody titers against APN in the sera of these mice by indirect enzyme-linked immunosorbent assay (ELISA) using a microtiter plate coated with 5 μg/mL APN protein diluted in 0.05 mol/L PBS (pH 9.6)" in the paper.

3.2: How are the cells harvested?

-The sentence changed as "Filter the spleen-cell suspension using a 200-mesh copper grid to remove tissue debris, and harvest spleen cells using centrifugation (1500 × g, 10 min) to remove the membrane of the spleen".

3.5: What volume is used to suspend the cells? Please specify all volumes used throughout.

-Fix as requested.

4.1: Please provide a reference here.

-Fix as requested.

5.2-5.3: How is this done?

-For 5.2, PCR is a normal protocol, and the IMGT mouse genome analysis tool is provided online. For 5.3, it is also a specific PCR reaction to allow scarless insertion of one or more fragments of

DNA into a plasmid vector. So, we provided the primers in Table 1.

5.4: As before in what step?

-The sentence changed as “Grow the pET28a (+)-rAbs-APN-BL21-transformed bacteria in the presence of 0.4 mM IPTG in orbital shakers at 37°C for 10 h, and were then induce, purify, and assess for the expression of the rAbs protein using routine protein purification”.

5.6: What 5 min incubation? Please specify all incubations conditions throughout.

Often, you specify what to do after some incubation or selection. However, please add the details on how to do those incubations and selections.

-Fix as requested.

5.9: .5-2 cells?

-The sentence changed as “The serially dilute harvested positive cells, …”.

5.10: Adapt the cells how?

-The sentence changed as “FBS concentration in the above-described cell-culture medium decreases gradually from 10% to 0% during the logarithmic growth phase over the time period of 3 weeks. Then, adapt the adherent CHO cells to suspension growth in a serum-free medium”. It is the normal protocol to convert adherent cells to suspension cells (serum dilution).

5.13: How are these actions done?

-Both protein A agarose and the CCK8 kit are purchase from company. So, we changed the sentence as “Collect the cell suspension every 12 h to determine changes in cell viability and vitality using a Cell Counting Kit-8 (CCK-8) per manufacturer's instructions”. The detail information about these two kits is provided in Table of materials.

3. Please highlight up to 3 pages of the protocol for inclusion in the protocol section of the video. This is a hard production limit to ensure that videography can occur in a single day.

-Fix as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors describe two techniques to produce monoclonals against APN namely the classical in vivo immunisation of mice and a recombinant technique in which the heavy and light genes of the antibodies are cloned into pET28a (+) and pIRES2-ZsGreen1 vectors. CHO become transfected. So although the authors mention that the advantage of the rAbs is that no animals are needed.In this setting animals are still needed.

The manuscript improved but more improvements are needed.

Major Concerns:

Still the text needs revision. The English needs improvement. I have made several corrections in the manuscript but can not submit it.

-- Fixed as requested. The detailed change was red-labelled in the manuscript. In advance of submission, we use LetPub language services to edit our manuscript.

Reviewer #2:

Manuscript Summary:

This manuscript describes production of mAbs against porcine APN using traditional hybridoma technology and recombinant antibody technology.

Major Concerns:
No major concerns.
Minor Concerns:
Significant improvement in the language and clarification of the individual method steps, there are still some typos in need of correction and sentence adjustments needed. Some of the method steps may still require more clarification to make them completely transparent to a reader trying to replicate this protocol.
-- Fixed as requested. We have carefully revised our paper accordingly, and the detailed change was red-labelled in the manuscript. In advance of submission, we use LetPub language services to edit our manuscript.

Thank you very much for taking time and considering our manuscript for publication, and any comments and suggestions will be highly appreciated.
Again, thank you for your kindly support and help!
Sincerely yours,
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