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TITLE:

Generation of Monoclonal Antibodies Against Natural Products

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SUMMARY:

This article provides a detailed protocol for the synthesis and evaluation of hapten-carrier conjugates for use in various immunological assays. This procedure includes immunization, cell fusion, indirect competitive ELISA for positive clone screening, and monoclonal hybridoma preparation. The specifications for antibody characterization using MALDI-TOF-MS and ELISA analyses are also provided.

ABSTRACT:

The analysis of the bioactive components present in foods and natural products has become a popular area of study in many fields, including traditional Chinese medicine and food safety/toxicology. Many of the classical analysis techniques require expensive equipment and/or expertise. Notably, enzyme-linked immunosorbent assays (ELISAs) have become an emerging method for the analysis of foods and natural products. This method is based on

antibody-mediated detection of the target components. However, as many of the bioactive components in natural products are small (<1,000 Da) and do not induce an immune response, creating monoclonal antibodies (mAbs) against them is often difficult. In this protocol, we provide a detailed explanation of the steps required to generate mAbs against target molecules as well as those needed to create the associated indirect competitive (ic)ELISA for the rapid analysis of the compound in multiple samples. The procedure describes the synthesis of the artificial antigen (*i.e.,* the hapten-carrier conjugate), immunization, cell fusion, monoclonal hybridoma preparation, characterization of the mAb, and the ELISA-based application of the mAb. The hapten-carrier conjugate was synthesized by the sodium periodate method and evaluated by MALDI-TOF-MS. After immunization, splenocytes were isolated from the immunized mouse with the highest antibody titer and fused with the hypoxanthine-aminopterin-thymidine (HAT)-sensitive mouse myeloma cell line Sp2/0 -Ag14 using a polyethylene glycol (PEG)-based method. The hybridomas secreting mAbs reactive to the target antigen were screened by icELISA for specificity and cross-reactivity. Furthermore, the limiting dilution method was applied to prepare monoclonal hybridomas. The final mAbs were further characterized by icELISA and then utilized in an ELISA-based application for the rapid and convenient detection of the example hapten (naringin (NAR)) in natural products.

INTRODUCTION:

Monoclonal antibodies (mAbs), also known as mono-specific antibodies, are produced from a single B-lymphocyte clone and are composed of monovalent antibodies that all bind to the same epitope[1]. In recent years, many medicinal plant-derived natural products have been used in the treatment of various diseases[2]. Indeed, many small molecular compounds originally derived from natural products are now applied as first-line drugs, such as artemisinin for malaria and paciltaxel (taxol) for cancer[2,3]. The study of natural products has made rapid progress, largely due to the tremendous development and optimization of conventional analysis techniques, including high performance liquid chromatography (HPLC) and mass spectrometry (MS). However, there are still some limitations associated with these methods, such as their complex pretreatment protocols and associated costs with regards to time, labor/expertise, and required instruments[4].

Recently, mAb-based enzyme-linked immunosorbent assays (ELISAs) have been applied to qualitatively and quantitatively analyze food and natural products. In fact, this method has been applied for both biological samples analysis and clinical testing and has been shown to be accurate, sensitive, and highly efficient while also avoiding the tedious pretreatment steps associated with other analyses[5,6].

When using mAb-based ELISAs to study complex natural products, preparation of the monoclonal antibodies is one of the core steps. Unfortunately, the mAbs specific to the small bioactive components present in these types of substances[6-15] are often limited compared to the protein antigens. To circumvent this issue, we have developed a protocol to specifically

generate mAbs against small compounds. The protocol presented here includes artificial antigen synthesis, mouse immunization, cell fusion, indirect competitive ELISA, and monoclonal hybridoma preparation.

Notably, our research group has been studying the formation of mAbs against small bioactive compounds from traditional Chinese medicines and developing their applications for years. In our on-going studies, we have developed mAbs against baicalin[16], puerarin[17], glycyrrhizic acid[18], paeoniflorin[19], ginsenoside Re[20], ginsenoside Rh1[21], and many other small molecules. Our ELISA protocols based on these mAbs have been used in a number of studies to evaluate the pharmacokinetics of these small molecules as well as their interactions with other bioactive compounds. Moreover, using these mAbs, we have also developed immunoaffinity chromatography methods for the separation of structural analogues, including epimers. Recently, we prepared a lateral flow immunoassay using our anti-puerarin mAb that was subsequently used for rapid, on-site detection of this compound. Our results indicate that our mAb-based assays are indispensable and convenient tools for studying the biology and quality of natural-product-derived compounds, particularly those used in traditional Chinese medicines.

PROTOCOL:

All of the animal procedures performed in this study have been approved by the Ethical Review Committee at the Beijing University of Chinese Medicine (approval number 2016BZYYL00109).

NOTE: Female BALB/c mice (8 weeks old) were immunized with hapten-carrier protein conjugates. When used alone, a small molecule (<1,000 Da) cannot elicit an immune response. However, conjugating the small molecule to a carrier macromolecule results in antigen synthesis. In this context, the small molecule is labeled a hapten. Hapten conjugation is a necessary and effective strategy for mAb production. To avoid cross reactivity, two different protein carriers, such as bovine serum albumin (BSA) and ovalbumin (OVA) or keyhole limpet hemocyanin (KLH) and BSA, should be used as the immunogens (for animal immunization) and coating antigens (to coat the plate for anti-serum detection). BSA and OVA are used as an example in this protocol.

1. Preparation of the Immunogen and Coating Antigen

NOTE: For artificial antigen synthesis, use the appropriate functional group (*e.g.*, hydroxyl, sulfhydryl carboxyl acid, or amino) as the side arm for covalent binding with the carrier protein. The conjugation methods include periodate oxidation, the carbodiimide method, a mixed anhydrides reaction, a glutaraldehyde reaction, and the succinate method. This protocol uses naringin (NAR), a well-known flavanone glycoside, as an example compound. NAR is a small compound (581 Da) present in citrus fruits as well as various traditional Chinese medicines.

1.1. Use a periodate oxidation procedure to synthesize NAR-BSA and NAR-OVA conjugates.

1.1.1. Dissolve 50 mg of NAR in water at a final concentration of 1 mg/mL[22].

1.1.2. Add 100 µL of freshly prepared sodium periodate solution (0.1 M) to 5 mL of the NAR solution. Stir the mixture at room temperature for 1 h.

1.2. Dissolve 4 mg of BSA and OVA in 2.0 mL of 50 mM carbonate buffer (pH 9.6). Add this protein solution to the NAR/sodium periodate reaction mixture.

1.3. Adjust the mixture to pH 9 with 1 M Na_2CO_3 solution and stir at room temperature for 6-8 h.

1.4. Dialyze the reaction mixture in phosphate-buffered saline (PBS) using a dialysis membrane (MWCO 10 kDa) for 3 days to exchange the CBS.

1.5. Analyze the hapten-carrier conjugate using matrix assisted laser desorption/ionization time of flight (MALDI-TOF)-MS.

1.5.1. Mix 1–10 pmol of the conjugate with a 10^3-fold molar excess of sinapinic acid in an aqueous solution containing 0.15% trifluoroacetic acid (TFA). Dry the mixture on a sample plate in air.

1.5.2. Perform the analysis using a MALDI-TOF mass spectrometer equipped with a pulsed nitrogen laser operated at 337 nm. Acquire positive ion MALDI mass spectra in the linear mode within the mass range (m/z) of 15,000–100,000 as previously described[22].

2. Immunization

NOTE: A total of 5 BALB/c female mice (8 weeks old) were used: 4 for NAR conjugate immunization and 1 for control (PBS) immunization.

2.1. For the first vaccination, mix 50 µg of conjugate (diluted in 100 µL of PBS) with an equal volume of Freund's complete adjuvant (CFA) and emulsify completely. Administer 100 µL of this mixture to each mouse *via* dorsal subcutaneous injection.

2.2. Two weeks later, deliver a booster vaccination (100 µL) *via* subcutaneous injection with the same amount of conjugate mixed with incomplete adjuvant (IFA).

2.3. Extract 200 µL of blood from the tail vein of every mouse 7 days later to obtain serum.

2.3.1. Centrifuge the blood at 3,000 x g for 10 min. Transfer the serum supernatant to a fresh tube.

2.3.2. Analyze the serum using an ELISA as previously described[21]. Choose the mouse with the highest serum titer to use for cell fusion.

2.4. In preparation for cell fusion, administer a pulsed immunization to the chosen mouse *via* intraperitoneal injection of 50 µg of the conjugate in 300 µL of PBS without adjuvant 3 days before fusion.

NOTE: This step can be omitted if the titer is high enough.

3. Preparation for Cell Fusion

3.1. Cell fusion medium preparation

3.1.1. For the hypoxanthine-aminopterin-thymidine (HAT) selective medium: Dissolve the 50x HAT media supplement in 10 mL of RPMI-1640 and add this mixture to 500 mL of RPMI-1640 containing 20% FBS.

NOTE: When 10 mL of the 50x concentrate are diluted in 500 mL of culture medium, the final concentrations of hypoxanthine, aminopterin, and thymidine are 100 µM, 0.4 µM, and 16 µM, respectively.

3.1.2. For the hypoxanthine-thymidine (HT) media: Dissolve the 50x HT media supplement in 10 mL of RPMI-1640 and add this mixture to 500 mL of RPMI-1640 containing 20% FBS. The final concentrations of hypoxanthine and thymidine are 100 µM and 16 µM, respectively.

3.2. Culture the Sp2/0-Ag14 cells.

3.2.1. At least 7 days before fusion, rapidly thaw a vial of liquid nitrogen-frozen SP2/0-Ag14 myeloma cells in warm water.

3.2.2. Transfer the cell solution into 5 mL of fresh culture medium (RPMI-1640) and centrifuge for 10 min at 1000 x g.

3.2.3. After centrifugation, remove the culture medium and resuspend the cells in fresh RPMI-1640 supplemented with 10% fetal bovine serum (FBS).

3.2.4. Transfer the cells and medium into a 25 cm^2 flask and incubate at 37 °C in an atmosphere of 5% CO_2.

3.2.5. Expand the cells to 3 or 4 75 cm^2 flasks before fusion.

3.3. Preparation of abdominal feeder layer cells

3.3.1. Sacrifice a 12-week-old albino ICR mouse by cervical dislocation 1 day prior to cell fusion and submerge it in 75% ethanol.

3.3.2. After removing the fur from the abdomen with hemostatic forceps, disinfect the area with 75% ethanol. Cut the outer skin to expose the abdominal cavity. Inject 3 mL of sterile RPMI-1640 medium into the abdomen and massage the abdomen to detach additional cells into the solution. Aspirate the feeder cell suspension into a 15 mL centrifuge tube.

3.3.3. After centrifuging the cell suspension for 10 min at 1000 x g, discard the supernatant and resuspend the feeder cells in 20 mL of HAT selective medium.

3.3.4. Transfer the cells into 96-well cell culture plates (100 µL/well). Incubate the plates overnight at 37 °C, 5% CO_2.

4. Cell Fusion

4.1. After the chosen immunized mouse has been prepared for cell fusion (see step 2.4), administer an intraperitoneal injection of 10% chloral hydrate (anesthetic) and sacrifice the immunized mouse *via* cervical dislocation.

4.2. Collect additional blood from the heart (1 mL), and prepare serum as described in step 2.3.1 to use as a positive control during hybridoma selection.

4.3. Remove the skin and muscle tissue using scissors to expose the spleen.

4.4. Isolate the spleen with tweezers carefully and wash the spleen with RPMI-1640. Cut the spleen into pieces and slowly pound the spleen to triturate. Prepare a spleen cell suspension by pressing the spleen tissue through an 800 mesh cell strainer into a 50 mL centrifuge tube.

4.5. Wash the spleen cell suspension with RPMI-1640 medium. Harvest the spleen cells by centrifugation (1000 x g, 10 min, and 4 °C), and then discard the supernatant. Repeat this washing step three times.

4.6. Remove the cultivated and amplified myeloma cells from the incubator and gently tap/shake the flasks to obtain a cell suspension. Wash this suspension with RPMI-1640 medium twice to remove the FBS.

NOTE: This is an essential step as FBS may influence cell fusion.

4.7. Count the cells and adjust the concentration before mixing with the spleen cells. The final ratio of spleen cells to myeloma cells should be between 1:5 and 1:10.

4.8. Mix the spleen cell suspension and myeloma cells. Centrifuge the cell mixture (1000 x g, 10 min, and 4 °C), and remove the supernatant.

4.9. Add 1 mL of 50% polyethylene glycol (PEG) solution to the cells and gently stir at 37 °C for 1 min. Let stand for 30 s.

NOTE: The PEG solution used in this step should contain 50% (w/v) polyethylene glycol 1500 and 10% (v/v) dimethyl sulfoxide (DMSO) in PBS without calcium (Ca^{2+}).

4.10. Add 2 mL of RPMI-1640 medium for 2 min to terminate the reaction. Add an additional 2 mL of RPMI-1640 medium for another 1 min, and then add an additional 10 mL of RPMI-1640 medium.

4.11. Centrifuge the cells at 800 x g for 10 min. After discarding the supernatant, add HAT solution and continue to cultivate the cells. Then add 100 µL of the cell suspension to each well of the feeder layer plates and incubate the plates for 7 days at 37 °C, 5% CO_2.

NOTE: Under these conditions, the unfused myeloma cells will die while the hybridoma cells will continue to grow in the HAT medium. After 7 days, monoclonal hybridoma will form as a single cluster of cells in the well, while wells containing polyclonal hybridoma will have multiple clusters of cells.

4.12. Aspirate the supernatant for analysis and replace the medium with HT medium.

NOTE: Be sure to substitute the HAT medium with HT medium first as switching directly to unsupplemented medium is detrimental to the hybridoma.

5. Indirect Competitive ELISA (icELISA)

5.1. Coat a 96-well plate with NAR-OVA (1 µg/mL, 100 µL/well) and incubate for 1 h at 37 °C or overnight at 4 °C.

5.2. Block the plate with 300 µL of GPBS (PBS containing 1% m/v gelatin) for 1 h at 37 °C to prevent non-specific adsorption.

5.3. Wash the plate three times with TPBS (PBS containing 0.05% v/v Tween-20).

5.4. Dilute unconjugated NAR into a series of concentrations with 10% methanol. Add 50 µL of these dilutions to separate wells. In the other wells, add 50 µL of anti-serum or hybridoma supernatant (containing mAbs). Incubate the plate for 1 h at 37 °C.

5.5. Add 100 µL of peroxidase-labelled anti-mouse IgG into each well and incubate for an additional 1 h.

5.6. After washing the plate three times with TPBS, add 100 µL of substrate solution (0.1 M citrate buffer (pH 4) containing 0.015% v/v H_2O_2 and 2 mg/mL of 3, 3', 5, 5'- tetramethyl benzidine (TMB)) to each well and incubate for 15 min.

5.7. Stop the reaction by adding 50 µL of 1 M H_2SO_4 to each well.

5.8. Measure the absorbance using a microplate reader at 450 nm. Choose the hybridomas that secreted the highest concentrations of NAR antibodies into their supernatant for further preparation.

6. Preparation of Monoclonal Hybridomas

6.1. Use the limiting dilution method to prepare the monoclonal hybridomas.

6.1.1. After removing the HT medium from the selected hybridomas (*i.e.,* those positive for NAR antibody secretion), resuspend the cells in RPMI-1640 and count them.

6.1.2. Dilute the cells to a concentration of 1 cell, 2 cells, and 4 cells per well in 100 µL of RPMI-1640 in a 96-well plate. Incubate the plate at 37 °C, 5% CO_2.

6.1.3. After 7-10 days, detect the NAR antibodies in the culture supernatant using the icELISA protocol (step 5). Transfer cells from the hybridomas that are positive for NAR antibodies to 24-well plates, 25 cm^2 flasks, and 75 cm^2 flasks for cultivation.

6.2. Cryopreserve the monoclonal hybridomas.

6.2.1. Harvest the cells and transfer them to centrifuge tubes.

6.2.2. After centrifuging the cells (800 x g, 10 min), remove the supernatant and resuspend the cells in freezing medium (RPMI-1640, 20% fetal calf serum (FCS), and 10% DMSO). Transfer the cell suspensions to cryotubes.

6.2.3. Store the cryotubes in a gradient cooling box at -80 °C for 24 h. Then transfer the cryotubes to liquid nitrogen for long-term storage.

REPRESENTATIVE RESULTS:

Generation of monoclonal hybridomas

The molecular weight of the hapten-carrier conjugate was confirmed by MALDI-TOF-MS analysis. As the molecular weight of both BSA and the NAR are known, the number of small molecules conjugated with BSA could be calculated. **Figure 1** shows representative spectral results for NAR-BSA[22], which displays a broad peak at *m/z* 77,058. As the average molecular weight of BSA is 66,430, it appears that at least 18 NAR molecules (MW 581) were conjugated with the BSA (molar coupling ratio (NAR:BSA) = 18:1).

icELISAs were performed to determine the anti-serum titers following mouse immunization[22]. It appears that the serum antibody titers of the four mice immunized with the NAR-BSA conjugate were significantly higher than that observed for the control mouse (**Figure 2**). The mouse with the highest titer (over 1:5,000) was used for cell fusion.

After the spleen cells from this mouse were fused to the abdominal feeder layer cells, the hybridomas were grown for 7 days in selection medium. Using icELISAs, the cell culture supernatant was tested, and the hybridomas positive for NAR mAbs were recloned and expanded. The images in **Figure 3** illustrate the conventional outcomes for stable monoclonal and polyclonal hybridoma cell lines.

Screening and application of the anti-NAR mAb

The critical point of this experiment is the screening of mAb specificity. The results in **Table 1** demonstrate that the mAb in this experiment reacted with NAR but not the blocking buffer or carrier proteins[22]. Furthermore, the specificity of the mAb was further evaluated by testing its cross-reactivity with structurally related compounds. **Table 2** shows the calculated cross-reactivity rates. As the cross-reactivity rate for the other flavonoids tested were all less than 2%, it is clear that this mAb is specific for NAR[22].

An icELISA was developed using the anti-NAR mAb and highlights the application of this methodology. Using solutions containing known concentrations of NAR, a standard curve was plotted using the absorbances of these solutions and the linear range of the S model curve was calculated (shown in the upper-right figure inset). The linear regression equation ($y=-0.176 \ln(x) + 1.1243$, $R^2 =0.9978$) calculated for this curve can be applied to quantitative analysis of unknown samples.

Production and characterization of a mAb against glycyrrhizic acid

Using this splenocyte/myeloma cell fusion protocol, a monoclonal hybridoma secreting anti-glycyrrhizic acid mAb, named DF5, was also established[18]. The subtype of this DF5 mAb was

identified as IgG1 with a kappa light chain (**Table 3**). Similar to the analysis above, the mAb was used for additional screening and applications, while the antigen-specific monoclonal hybridomas were expanded and cryopreserved in liquid nitrogen for long-term storage.

FIGURES AND TABLE LEGENDS:

Table 1. Reactivity of the anti-NAR mAb with NAR-OVA and carrier proteins.

Table 2. Cross-reactivity (%) of the anti-NAR mAb against NAR and its related compounds.

Table 3. Isotype analysis of mAb DF5.

Figure 1: MALDI-TOF-MS analysis of the NAR-BSA conjugate. $[M + H]^+$ and $[M + 2H]^{2+}$ indicate the single- and double-protonated molecules of NAR-BSA, respectively. This figure has been modified from Qu *et al.*, 2016[22]

Figure 2: Analysis of anti-serum titer by icELISA. Mice 1- 4 were immunized with the NAR-BSA conjugate, while the control was immunized with vehicle (PBS). The data represent the mean ± standard deviation (SD) (n = 3). This figure has been modified from Qu *et al.*, 2016[22].

Figure 3: Representative images of stable monoclonal (A) and polyclonal (B) hybridomas.

Figure 4: Application of the anti-NAR mAb in an icELISA. Various concentrations of NAR were incubated with mAb in wells pre-coated with NAR–OVA (1 mg/mL). A is the absorbance in the presence of NAR, while A_0 is the absorbance in the absence of NAR. The data represent the mean ± standard deviation (SD) (n = 3). This figure has been modified from Qu *et al.*, 2016[22].

DISCUSSION:

Here, we present a protocol for the successful production of mAbs against natural product-derived small molecules. The essential steps in the procedure have been outlined, and we have demonstrated the utility of this protocol using NAR as an example small molecule. The example spectra, reactivity analyses, and icELISA results all show representative experimental and control data that is obtained using this protocol. Example images of the hybridomas provide a visual representation of what the researcher should be looking for when differentiating between the monoclonal and polyclonal hybridomas. Taken together, we have demonstrated that the mAb production, characterization, and application strategy presented here results in the creation of an effective mAb against a small molecule as well as a novel ELISA based on the particular mAb that can be used to test the expression of the target molecule in other natural products.

Working with any type of antibody, the most common issue that may arise during this procedure is associated with the sensitivity of the mAb. Indeed, there is a high risk that the mAb

will not work as expected due to low sensitivity, high cross-reactivity, or other factors. As the whole procedure takes at least 4 months to perform in full, it is important to take care during the initial screening of the mAbs secreted from the hybridomas. One essential aspect to avoid creating an ineffective mAb is to screen the anti-serum by icELISA before cell fusion to confirmed reactive with the hapten but not the carrier. This is performed by using two different protein carriers (in this case BSA and OVA) as the immunogen and coating antigen carriers, respectively. During immunization with the hapten-BSA conjugate, the animals will produce antibodies against both the hapten (*e.g.*, NAR) and BSA. Thus, to avoid the false-positive detection of anti-BSA antibodies during screening, hapten-OVA should be used to detect the anti-hapten antibodies specifically. The creation of these two protein carriers as well as when to use them is explicitly highlighted in the protocol.

It is also important to note that during preparation of the monoclonal hybridomas, the limiting dilution method often needs to be repeated several times until all of the wells containing the monoclonal cells are positive. This repetition helps to confirm that every clone stably secrets the specific antibody.

The limitations of this method include the complicated process of hybridoma generation and the time needed for selection of the desired antibody-producing hybridoma. However, once the mAb is obtained and the icELISA is developed, the detection of the target compound in natural products can be performed quickly and efficiently. Notably, this protocol does avoid some of the costs associated with other analysis techniques and does not require the use of expensive instruments repeatedly for every natural product tested.

Once produced and screened, the hapten mAb can be widely used in a variety of analyses. In this protocol, we focused on the use of the mAb in an ELISA-based method that was used to study the biology of the small molecule as well as its pharmacokinetic interactions[20,22]. Other mAbs created with this protocol have also been used to establish an mAb-based immunoaffinity chromatography method for the separation of structural analogues[18], including epimers[21], as well as a lateral flow immunoassay[23] for rapid and on-site detection of the target molecule. These studies highlight the broad application of mAbs produced using the protocol outlined here. Thus, this procedure, and the mAbs created, acts as a foundation for the development of various target mAb-based immunoassays that can be effectively utilized as analytical tools for the evaluation and quality control of nature products.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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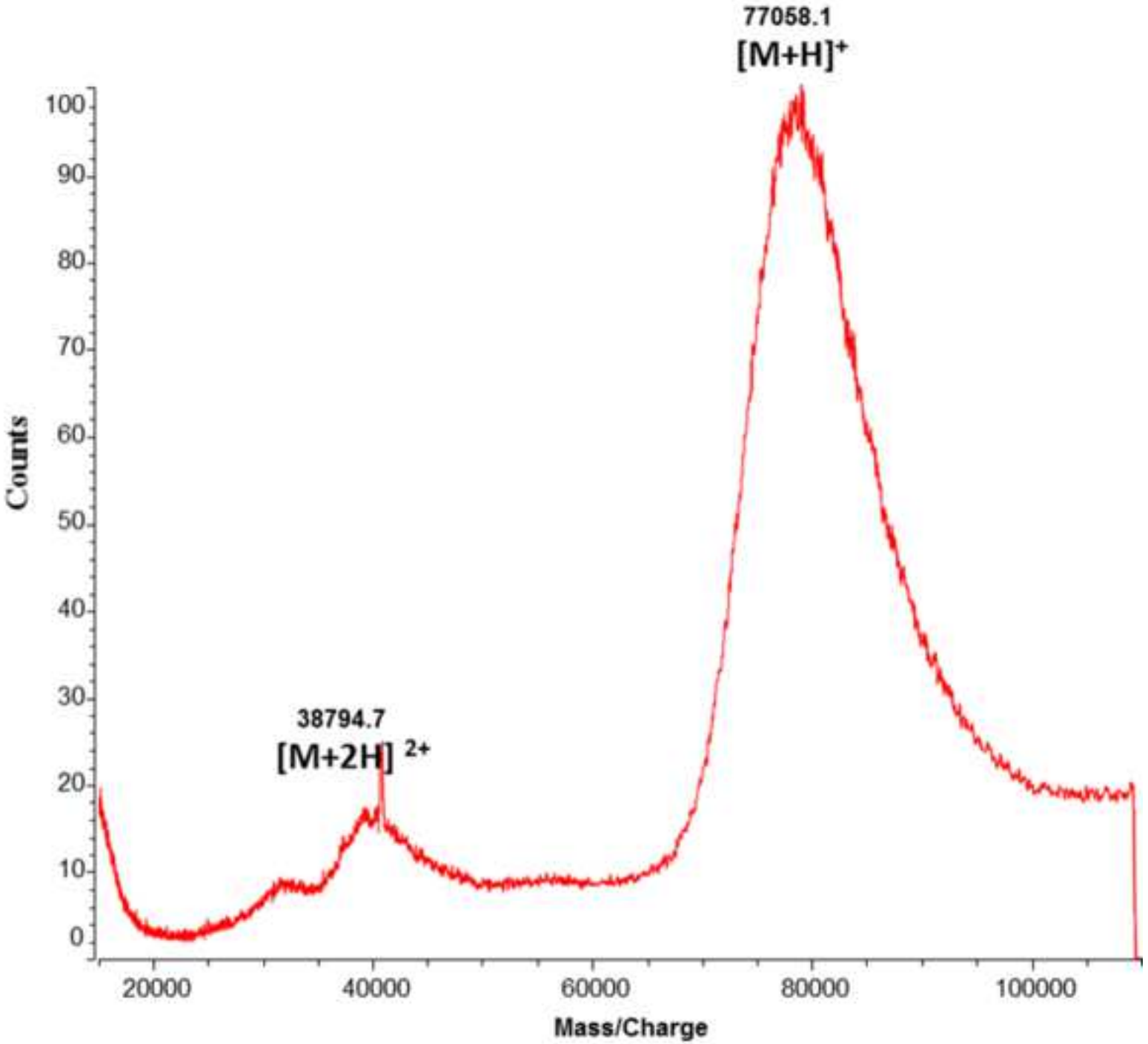

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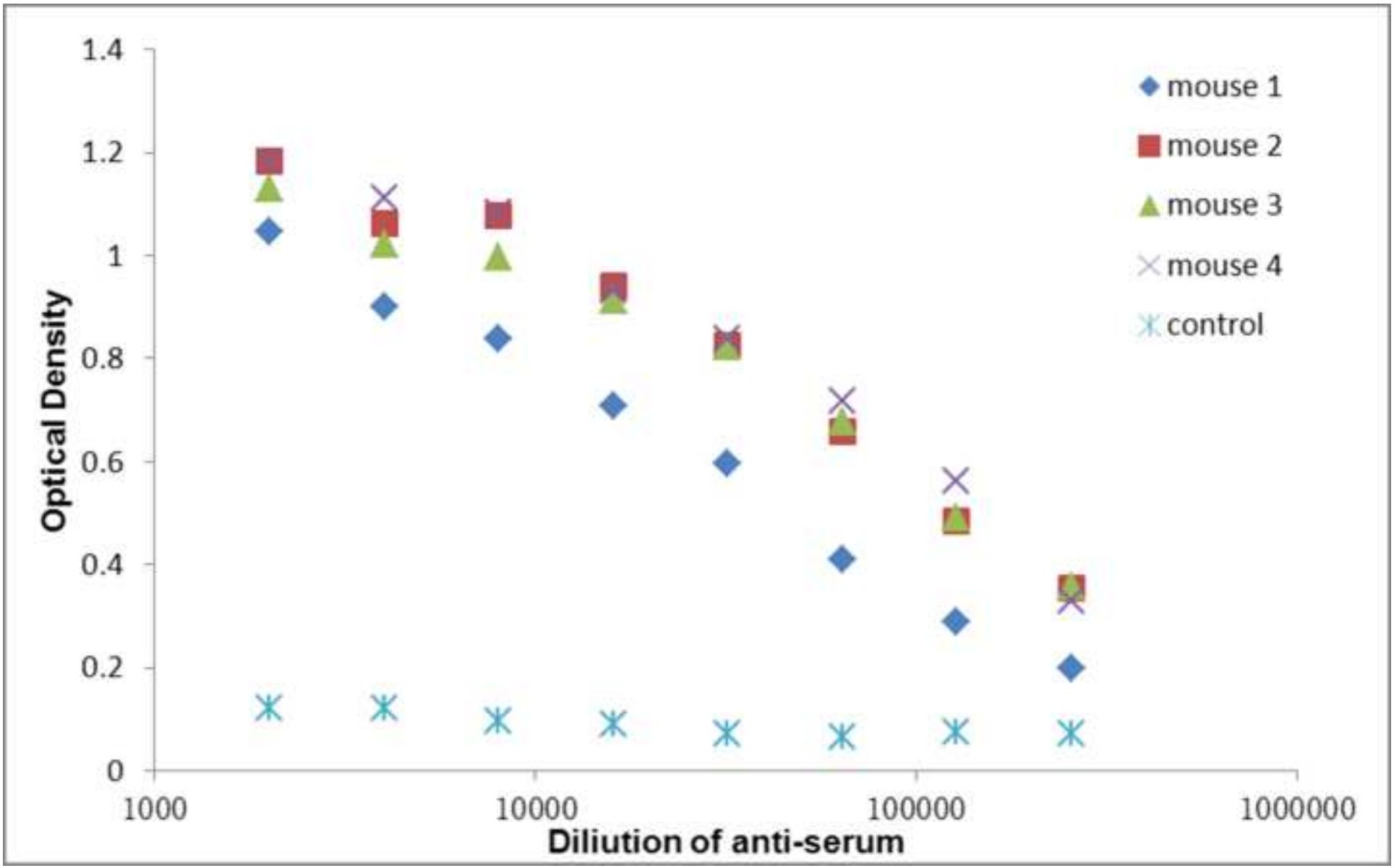

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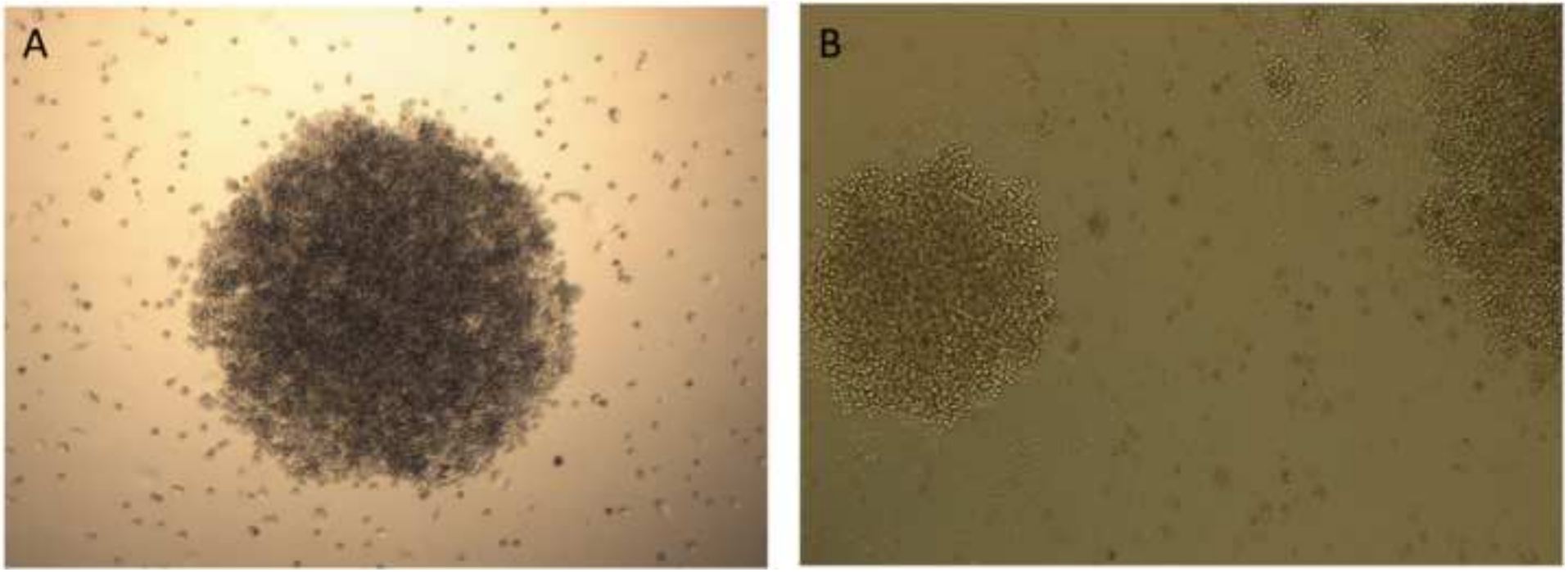

Figure

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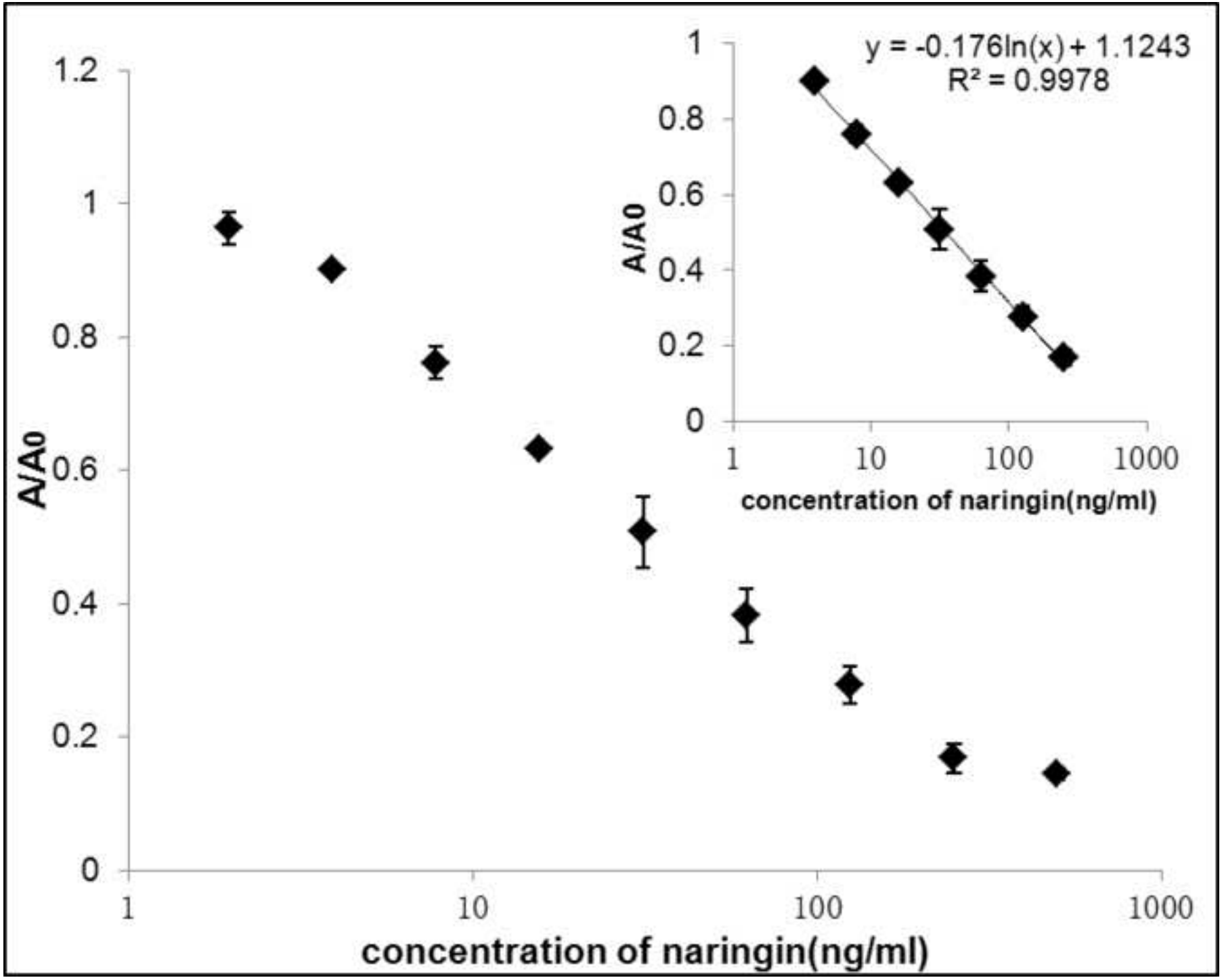

Coating substance	A_{450} value	Cross-reactivity (%)
Nar-OVA	0.97	100
OVA	0.056	<0.01
BSA	0.068	<0.01
Gelatin	0.053	<0.01
Skim milk	0.05	<0.01

Classification	Compound	Cross-reactivity (%)
Flavonoids	Naringin	100%
	Puerarin	1.26%
	Neohesperidin	18.80%
	Rutin	1.95%
	Baicalein	<0.09%
	Hyperoside	<0.09%
Terpenes	Ginsenoside Rg2	<0.09%
	Ginsenoside Rb1	<0.09%
	Ginsenoside Re	<0.09%
	Notoginsenoside	<0.09%
	Glycyrrhizic acid	<0.09%
	Glycyrrhetic acid	<0.09%
	Saikosaponin	<0.09%
	Paeoniflorin	<0.09%
	Gentiopicrin	<0.09%
Sterides	Cholic acid	<0.09%
	Deoxycholic acid	<0.09%
Anthraquinones	Rheumemodin	<0.09%
	Rheinic acid	<0.09%
Other	Salvianolic acid	<0.09%
	Curcumin	<0.09%
	Gastrodin	<0.09%
	Amygdalin	<0.09%

Table

	isotype of heavy chain					
	IgG1	IgG2a	IgG2b	IgG3	IgA	IgM
DF5	●	○	○	○	○	○

isotype of light chain	
kappa	lambda
●	○

Name of MaterA1:D28ial/ Equipment	Company	Catalog Number	Comments/Description
800 mesh (40 µm nylon) filter	FALCON	352340	
24 well culture plate	NUNC	119567	
25 cm2 Flask	Labserv	310109016	
3,3’,5,5’-Tetramethylbenzidine(TMB)	Sigma Aldrich	860336 1G	
75 cm2 Flask	Corning	430720	
96 well culture plate	NUNC	117246	
bovine serum albumin	AMRESCO	332	
cell strainer	FALCON	352340	
centrifuge tube 15 mL	Corning	430645	
centrifuge tube 50 mL	Corning	430828	
cryotubes, 1 mL	Sigma Aldrich	V7384-1CS	
cultivator	DRP-9082	Samsung	
dialysis membrane (10kDa)	Heng Hui	45-10000D	
dimethylsulfoxide	Sinopharm Chemical	DH105-10	
electronic balance	BS124-S	Sartorius	
ELISA plates, 96 well	NUNC	655101	
ethanol, 96%	Sinopharm Chemical		
Fetal bovine serum	Gibco	16000-044	
fetal calf serum	Invitrogen	10270106	
Freund´s adjuvant, complete	Sigma Aldrich	SLBM2183V	
Freund´s adjuvant, incomplete	Sigma Aldrich	SLBL0210V	
Gelatin	AMRESCO	9764-500g	
Gradient cooler container	Nalgene	5100-0001	
HAT media supplement	Sigma Aldrich	H0262-10VL	
HRP-conjugated goat-anti-mouse IgG antibody	applygen	C1308	
HT media supplement	Sigma Aldrich	H0137-10VL	
Inverted Microscope	IX73	Olympus	
keyhole limpet hemocyanin	Sigma Aldrich	H8283	

MALDI-TOF-MS	Axima-CFR plus	Axima
Microplate Reader	BioTex	ELX-800
mouse	Vital River	BALB/c
ovalbumin	Beijing BIODEE	5008-25g
PEG	Sigma Aldrich	RNBC6325
Penicillin&Streptomycin solution	Hyclone	SV30010
Pipette 10 mL	COSTAR	4488
Pipette 25 mL	FALCON	357525
RPMI 1640	Corning	10-040-CVR
skim milk	applygen	P1622
sodium periodate	Sinopharm Chemical	BW-G0008
Sulfo-GMBS	Perbio Science Germany	22324
TipOne Tips 1,000 µL	Starlab	S1111-2021

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Author(s): Yue Zhang, Peng Cao, Xin Yan, Binggian Jiang, Jinjun Cheng, Huihua Liu

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Institution: Beijing University of Chinese medicine

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Dear editor

Thank you very much for your reviewing and replying to our manuscript (ID: JOVE57116R4). We have revised the manuscript and video accordingly. We also responded point by point to the comments and modified the video as the editor's comments. We sincerely hope this revised manuscript is more acceptable for publication.

Thank you again and all the reviewers for the kind advice and look forward to hearing from you soon.

Yours sincerely,

Huihua Qu

Beijing University of Chinese Medicine

E-mail: quhuihuadr@163.com.

Point by point response to the comments

1. How does Figure 3B show polyclonal hybridoma?

Response: we have modified Fig.3 as follows:

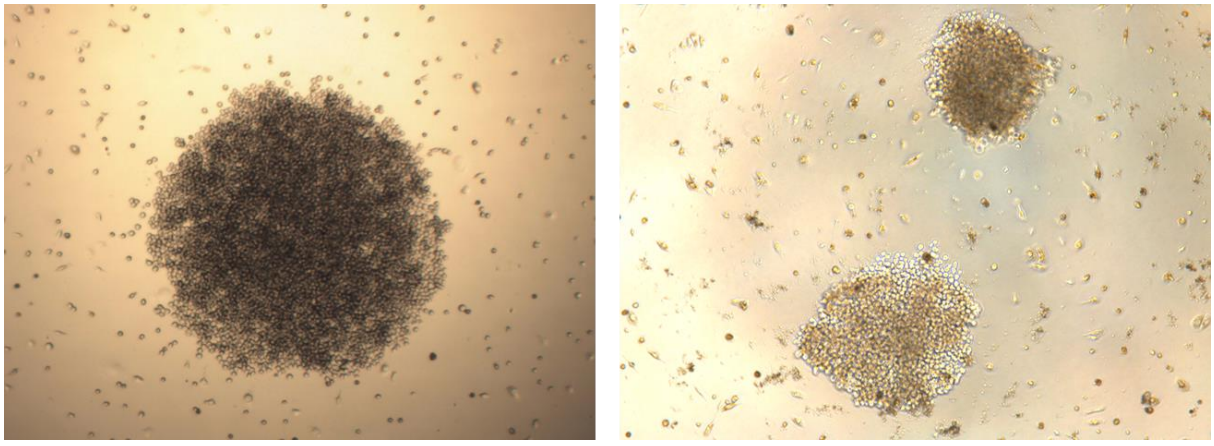

Figure 3: Representative images of stable monoclonal (A) and polyclonal (B) hybridomas.

2. 2:00 onwards - The narration of the video in the protocol does not match the written protocol in the manuscript. Please re-record the narration in the

video by reading word for word the written protocol text. This will meet our video publication standard for grammar and clarity. The written protocol text is currently acceptable, but the video narration is not. However, please exclude the sacrificing of the mouse from the video and the video narration.
Response: We have re-recorded the entire protocol narration. Please check.

3. 2:00 onwards - Please ensure that the video is matched to the narration as well. If you are talking about adding 100 uL of sodium periodate, the video should show this and the narration should state this. There are many instances where the video does not agree with the narration.
Response: We have re-recorded the entire protocol narration. Please check.
4. Another example is that it is very hard to follow the Immunization step of the protocol. The narration skips around from step 2.1 to 2.2 and then back to step 2.1.
Response: We have re-recorded the entire protocol narration. Please check.
5. step 3.2.1 says that the 50x HAT media was dissolved in 10 mL of RPMI-1640 but the video narration says that it was only 5 mL. Which is it?
Response:10 mL should be right. We have modified the 3.2.1.
6. 4:57: What is meant by percussion the cells? This is not an English sentence. This is repeated many more times. Please revise and include this in the written manuscript as well.
Response: We have modified the words and re-recorded the entire protocol narration.
7. Please do not use the words "sucked up". Use aspirate instead.
Response: We have modified the words in video.
8. Preparation is misspelled incorrectly in the protocol section headers: 3:35: Preparation of cell fusion; 9:08 - Preparation of monoclonal hybridomas,
Response: Sorry for the fault. We have modified the words in video.
9. Please re-record the entire protocol narration by reading the written text of the written manuscript word for word. However, please exclude the sacrificing of the mouse from the video and the video narration.
Response: We have modified the video, please check.
10. While showing the representative results in the results section of the video, please use screen-capture to show the results instead of filming the computer screen and pointing to it with a finger.
Response: We have modified the video, please check.

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