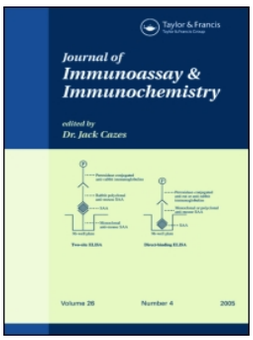

Journal of Immunoassay and Immunochemistry

ISSN: 1532-1819 (Print) 1532-4230 (Online) Journal homepage: http://www.tandfonline.com/loi/ljii20

Scale-up production and characterization of anti-human cardiac troponin I monoclonal antibody in ascitic fluid of balb/c mice

Asiabanha Rezaee Majid, Rasaee Mohammad Javad, Paknejad Malihe & Mohammadnejad Javad

To cite this article: Asiabanha Rezaee Majid, Rasaee Mohammad Javad, Paknejad Malihe & Mohammadnejad Javad (2017) Scale-up production and characterization of anti-human cardiac troponin I monoclonal antibody in ascitic fluid of balb/c mice, Journal of Immunoassay and Immunochemistry, 38:4, 389-399, DOI: 10.1080/15321819.2016.1274263

To link to this article: http://dx.doi.org/10.1080/15321819.2016.1274263

Accepted author version posted online: 27 Dec 2016.
Published online: 27 Dec 2016.

JOURNAL OF IMMUNOASSAY AND IMMUNOCHEMISTRY
2017, VOL. 38, NO. 4, 389–399
http://dx.doi.org/10.1080/15321819.2016.1274263

Taylor & Francis
Taylor & Francis Group

Scale-up production and characterization of anti-human cardiac troponin I monoclonal antibody in ascitic fluid of balb/c mice

Asiabanha Rezaee Majid[a], Rasaee Mohammad Javad[a], Paknejad Malihe[b], and Mohammadnejad Javad[c]

[a]Department of Clinical Biochemistry, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran; [b]Department of Biochemistry, Faculty of Medical Sciences, Tehran University of Medical Sciences, Tehran, Iran; [c]Department of Life Science Engineering, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran

ABSTRACT

The Human Cardiac Troponin I (hcTnI) is a 210 amino acid protein, 23 kDa in molecular weight. This biomarker is commonly used to diagnose myocardial infarction, micro injury, and acute coronary syndrome (ACS) in patients referring to emergency departments. The American Heart Association (AHA) and European Society of Cardiology (ESC) proposed troponin I as the gold biomarker for early detection of heart attack, especially in myocardial infarction (MI). Therefore, developing monoclonal antibodies against this biomarker could help in for early detection of heart attack. Hybridoma technology is a well-known technique introduced to produce monoclonal antibodies in specialized cells. The aim of this study was to produce large scale of monoclonal antibody against human cardiac troponin I using Hybridoma technology in order to design a diagnostic kit. The monoclonal antibody was produced using conventional Hybridoma technology in ascitic fluid of mouse and characterized for its ability to detect Human Cardiac Troponin I in a rapid test system. The results indicate the successful detection of Troponin I using the obtained monoclonal antibody. According to the achieved results it seems that ascites production of monoclonal antibody is very versatile, inexpensive, and economically useful for monoclonal antibody production.

KEYWORDS

human cardiac troponin I; hybridoma technology; ascitic fluid; early detection

Introduction

Hybridomas are cells that have been engineered to produce a desired monoclonal antibody in large amounts.[1] Hybridoma technology is a well-known technique introduced to produce monoclonal antibodies in specialized cells.[1b]

The Human Cardiac Troponin I (hcTnI) is a 210 amino acids protein, 23 kDa in molecular weight. As the inhibitory subunit of the troponin complex, hcTnI inhibits actomyosin ATPase activity in the absence of Ca2+.[2] This biomarker is commonly used to diagnose myocardial infarction, micro injury, and acute

CONTACT Rasaee Mohammad Javad rasaee_m@modares.ac.ir Department of Clinical Biochemistry, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/ljii.

coronary syndrome (ACS) in patients referring to emergency departments.[3] The American Heart Association (AHA) and European Society of Cardiology (ESC) proposed troponin I as the gold biomarker for early detection of heart attack, especially in myocardial infarction (MI).[4] hcTnI concentration increases drastically about 4–6 hr after chest pain occurs and remains high for 14 days.[5] The cardiac troponin I (cTnI) shows a good sensitivity as a marker of myocardial necrosis due to high specificity. Recently, due to tissue specific cTnI, cardiac troponin I has been determined as one of the good biomarkers for diagnosis of acute myocardial infarction (AMI).[6]

For mass production of the monoclonal antibody, hybridoma cells must be grown by one of the following methods: in vivo method; injection of requested clone into the abdominal cavity of a suitably prepared mouse or in vitro method; and culture of the cells in tissue culture flasks.[7]
Further processing of the mouse ascitic fluid and of the tissue culture supernatant are required to obtain mAb with the required purity and concentration. The mouse method is generally familiar, well understood, and widely available in many laboratories. The tissue-culture methods have been expensive and time consuming and often failed to produce the required amount of antibody without considerable skilled manipulations.[8]

The aim of this study was to produce large scale of monoclonal antibody against human cardiac troponin I in order to design of diagnostic kit.

Materials and methods

Production of monoclonal antibody in ascitic fluid of mouse

Balb/c female mice (4–6 weeks old) were provided from Pasteur institute of Iran. 0.5 mL Pristane (2, 6, 10, 14 tetra methyl pentadecane, Sigma) was injected intraperitoneally into each mouse. Ten days after priming with Pristane, the cells of a suitable mono clone in density of 1–2 × 10^6 cells/0.5 mL PBS was injected intraperitoneally into each mouse. Having injected the hybridoma cell, the mice were surveyed daily for production of ascitic fluid. About ten days after the injection of cells, abdomens of the mice were completely enlarged and their skins were extended. Using 19 gage needles, their ascitic fluids were harvested. After 4 days, the harvesting procedure was repeated centrifuged and the related supernatants were collected for characterization.[9]

Titration of antibody

The titer of monoclonal antibody was assessed using ELISA method. Wells of ELISA plate (Nunc, Germany) were coated with 100 µL of human cardiac troponin I recombinant (8 µg/mL in PBS) overnight at 4°C. The next day the plate was washed 3 times with PBS-T containing 0.05% Tween 20 for 5 min.

Non-specific sites of the plate were blocked with 5% skim milk and incubated at 37°C for 90 min. The wells were then washed 3 times as above and ascitic fluid was added to the wells in two-fold serial dilutions starting from 1:500. The plate was incubated at 37°C for 1.5 hr and washed again with PBS-T. At the next step, 100 µL of 1:1000 dilution of HRP-conjugated rabbit anti-mouse Ig (Razi institute, Iran) was added to the wells and incubation was continued for 1.5 hr at 37°C. After washing, 100 uL of Tetramethylbenzidine (TMB) substrate was added to each well and the plate was incubated at room temperature in a dark place. After 20 min, the reaction was stopped by adding 100 µL of stopping solution (0.16 M H2SO4) to each well. The Optical Density (OD) of the reactions was measured at 450 nm by an ELISA reader (STAT FAX 2100).[10]

Determination of mAb isotype

IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche, Germany) was used for determination the class and subclass of the mAbs.

First, an antibody-containing sample is pipetted into a development tube, then the tubes were agitated so that the colored latex beads are completely re-suspended. After that, an isotyping strip is placed in the tube. Within 1–5 min, a blue band appears in either the kappa or lambda section of the strip, as well as in one of the class or subclass sections, indicating the class or subclass and light-chain composition of the monoclonal antibody. These blue bands will intensify as the sample moves up the strip. The positive control bands on each side of the isotyping strip should also appear, indicating the antibody-coated latex beads are functional and have traveled up the strip. After 5 min, the results can be interpreted.[11]

Antibody purification

First the ascitic fluids were diluted with PBS two times with 40% saturated ammonium sulfate. After several times of washing with 40% ammonium sulfate, the fraction was centrifuged for 15 min in 5000 g. The precipitated fraction was dialyzed against 10 mM PBS, pH 7.4 and purified using Sepharose beads conjugated with Protein G column affinity chromatography equilibrated with 5–10 column volumes with the same buffer.

Mouse IgG2 was eluted with 0.1 M sodium phosphate buffer in pH 6.0. Confirmation of the purified fractions was monitored by SDS polyacrylamide gel electrophoresis and western blotting. Finally, the purified fractions were kept for conjugation with gold nanoparticles.[12]

Confirmation of the mAb purity by SDS-PAGE

Purity of the monoclonal antibody was checked by SDS polyacrylamide gel electrophoresis in non-reducing condition and reducing form. 10 µg of purified mAb was mixed with 10 µL of sample buffer. The samples were boiled for 10 min at 100°C. Electrophoresis was done in a 12.5% SDS-PAGE gel with a mini-PROTEAN electrophoresis instrument (Bio-Rad Laboratories, Hercules, CA, USA) 100 V for 1 hr. The gel was stained with Coomassie Brilliant Blue R-250 (Sigma).[13]

Western blotting

Western blotting was used to confirm the result of SDS-PAGE and to determine the pattern of specificity and cross-reactivity of anti-troponin I monoclonal antibody (with tissue homogenate of rat cardiac, liver, muscle, and human cardiac) was subjected to SDS-PAGE and then immune-blotting assay. Briefly, the nitrocellulose membrane and several thicknesses of whatman chromatography papers were soaked in the transfer buffer (25 mMTris, 192 mM glycine, 20% V/V methanol, pH 8.3). The wet nitrocellulose membrane was over laid on the wet Whatman sheets by taking precaution to avoid bubbles. Then, the SDS-PAGE gel was placed on the wet nitrocellulose membrane, where several wet Whatman papers were placed on. Transfer of the proteins from the gel to the nitrocellulose membrane was done in 100V for 3 hr. Then, non-specific sites were blocked with a 2% BSA solution. After three rounds of washing, the membrane was cut into strips and incubated for 2 hr at 37°C with the supernatants of suitable clones. Again, after 5 washes, the strips were incubated for two hours at 37°C with Rabbit Anti-Mouse IgG conjugate (1/2000 dilution). The strips were washed and detected by DAB (3,3'-Diaminobenzidine) hyperfilm after exposure for 5 min.[14]

Conjugation of monoclonal antibody with gold nanoparticle

To prepare the AuNP conjugate, anti-troponin I antibody(five monoclonal Ab) in PBS (10 L of 1 mg/mL) was added to the mixture of 1 mL AuNP colloid (10 or 20 nm in diameter) and 0.1 mL of borate buffer (0.1 M, pH 8.5). After incubating for 30 min at room temperature, 0.1 mL of 10 mg/mL BSA in PBS was added to the solution to block the AuNP surface. After incubating for 15 min at room temperature, the mixture was centrifuged at 10,000 rpm and 4°C for 20 min. The supernatant was discarded, and 1 mL of 1 mg/mL BSA in PBS was added to the AuNP conjugate to be re-suspended. The centrifugation and suspension process were repeated twice, and the final suspension solution was PBS.

Results

Monoclonal antibodies using ascitic fluid

After priming of the mouse peritoneal with pristane, about 1 million cells related with five mono clone were suspended in 0.5 mL of sterile PBS and injected to each mouse. Approximately ten days later, 5 mL ascitic fluid was collected from each mouse in two times (first time ascitic fluid was harvested from each mouse after ten day and about again ascitic fluid was harvested from their peritoneum for a second time, after 3–5 days (Table 1). Further characterization of this antibody showed that it is an IgG2 isotope with a kappa light chain (Figure 1). The product was precipitated by saturated ammonium sulfate and dialyzed against PBS. Concentration of the dialyzed product in assay with UV at 280 nm was about 35 mg. Purification by Protein-G-Sepharose column affinity chromatography yielded about 5.5 mg of monoclonal antibody.

Monoclonal antibody characterization

The result of purification was confirmed with non-reducing SDS-PAGE. In non-reducing SDS-PAGE, only one 150 KD band was appeared that demonstrator of purified antibody (Figure 2).

The titer of monoclonal antibody in ascitic fluid was assessed by ELISA method. The mean absorbance of non-immune mouse serum, Immune mouse serum, and ascitic fluid was compared in Table 1 at 450 nm. The results showed that its 1/32000 dilution has high absorbance with Human Cardiac troponin I antigen (Table 2).

Result of western blotting for two monoclonal antibodies showed that these antibodies did not demonstrate any cross-reactivity with liver and muscle tissue as negative control and high specificity with cardiac and rat and mice tissue as positive control (Figure 3).

The purified monoclonal antibody was conjugated with gold nanoparticles and used for assay design for diagnostic application. To examine the specific attachment of purified mAb with cardiac troponin I antigen, monoclonal antibodies were attached to gold nanoparticle (Figure 4).

Table 1. Purification of monoclonal antibody from ascites.

No.	Clone name	Ascetic volume (ml)	After ammonium sulfate (mg)	After protein G column (mg)
1	P1D2E3	10 ml	14.5	3.75
2	P2E4D3	5 ml	5.59	2.08
3	P1H1D7	4 ml	8.49	1.86
4	P3A2F4	5 ml	9.86	2.58
5	P1B5D7	5 ml	8.79	2.28

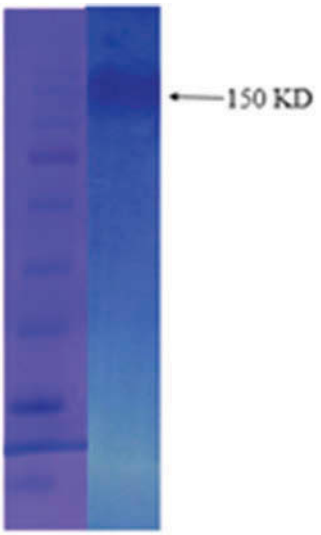

Figure 1. SDS-PAGE analysis of fraction from Protein-G affinity purification. Non-reducing SDS-PAGE of produced monoclonal antibody in non-reducing SDS-PAGE condition, only one band was seen in about 150 kDa.

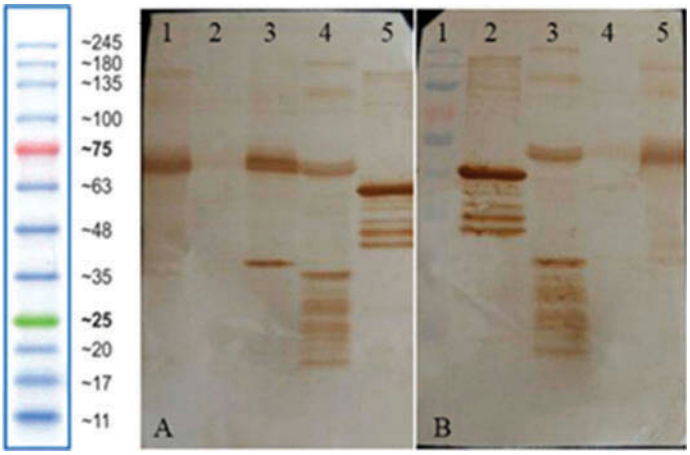

Figure 2. Western blotting of monoclonal antibodies: A(P1D2E3) clone; line 1: mice cardiac tissue; line 2:rat liver tissue (negative control); line 3: human cardiac tissue; line 4: rat cardiac tissue; line 5: recombinant troponin I.B (P2E4D3) clone; line 1: protein marker, line 2: recombinant troponin I,line3:rat cardiac tissue,line4:rat muscle tissue(negative control); line 5: human cardiac tissue.

Discussion

Monoclonal antibodies are now critical biological devices for biomedical research. These molecules are also very important for designing of diagnostic kits. Nowadays, various applications have been introduced for monoclonal

Table 2. Titration of purified monoclonal antibodies from ascites with ELISA.

Clones	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
P1D2E3	3.1	2.6	2.1	1.6	1.2	0.85	0.70
P2E4D3	3.3	2.8	2.4	2	1.6	1	0.8
P1H1D7	3.2	2.5	2.2	1.6	1.4	1	0.6
P3A2F4	3.2	2.6	2.2	1.7	1.2	0.8	0.4
P1B5D7	3.3	2.8	2.2	1.7	1.2	0.7	0.5

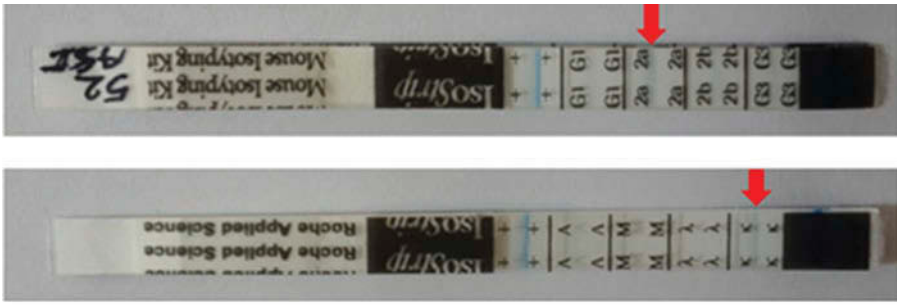

Figure 3. Characterization of antibody (P3A2F4) with Isostrip kit.

Figure 4. Gold conjugation of three monoclonal antibodies. Five monoclonal Ab conjugated with nanogold as detection Ab and as capture Ab concentration of troponin I is 1 ng/mL.

antibodies.[15] Koehler and Milstein developed the basic methods of producing monoclonal antibodies in hybridoma cells of the mouse in 1975 which has changed slightly to date.[16]

One of the best methods for mass production of monoclonal antibodies uses ascitic fluid. The production of monoclonal antibody in the ascitic fluid is commercially useful for mass production. In this study, five clones of monoclonal antibody against human cardiac troponin I was selected for mass production of monoclonal antibody.

At first, ascitic fluids were collected from the peritoneal cavity and the titer of monoclonal antibody was assessed by indirect ELISA method. The results showed that 1/32000 dilution has high absorbance with human cardiac troponin I antigen. The suitable method for antibody purification depends on the intended applications of the antibodies, isotyping of antibodies, and the available resources.[17]

The subclass of monoclonal antibody was IgG2 and its light chain was "kappa" type. The affinity chromatography method seems to be an easy, inexpensive,

one-step, and affordable method in comparison with the other chromatography methods such as ion-exchange chromatography. Accordingly, this method was used for purification of produced mAb.

Evaluation of antibody purification was done with SDS-PAGE. Therefore, purity was evaluated by SDS-PAGE in non-reducing condition. Suitability of the purified antibody was confirmed by the results of SDS PAGE analysis.

The purified monoclonal antibody was conjugated with gold nanoparticles. Specific binding of purified mAb with human cardiac troponin I antigen was monitored using gold nanoparticle techniques. The results have clearly showed that our antibodies strongly react with human cardiac troponin I antigen (Figure 4). Five monoclonal antibodies conjugated with gold nanoparticles were used for designing and evaluation of their affinity and suitability to be employed as capture and detection antibodies. To achieve this goal, each of the five antibodies was used as capture and detection antibody. Our results showed that one of the antibody pairs has the best properties for a diagnostic human cardiac troponin I test design.

Nowadays, two methods for mass production of the antibody are available: the mouse ascites method and the tissue-culture method which could be performed in vivo and in vitro.[18]

Cell-culture method requires experience, specific media, and can be expensive and time-consuming. In vitro methods of mAb production generally requires FBS, which is a concern from the animal-welfare perspective and some hybridomas do not grow well in culture or are lost in culture

Generally, in vivo production method requires, at first, pristane or Freund's incomplete adjuvant injection to suppress the immune systems and then the multiplied hybridoma cells form antibody-rich ascitic fluid in the peritoneal cavity. The ascites technique has some advantages, specially, high levels of antibody production up to 20 mg/mL. In addition, this technique is not expensive and does not need cell culture methods for antibody production.[7] Briefly, ascitic fluid production enriched with mAb in mice is a rapid and economic method.

One of the most important factors in the ascitic fluid production is the amount of the injected pristane and the interval of priming with hybridoma cells. In addition, side effects of tumor growth can be more severe due to incorrect i.p. injection of hybridoma cells as a result of insemination of hybridoma cells in abdominal organs, such as urinary bladder or intestines. The number of the injected cells to the peritoneum of mouse is of great significance, due to its importance in production of ascitic fluid.[19]

After the priming of monoclonal antibody cell, animals should be watch out daily at least for the first week to monitor the amount of abdominal swelling and signs of illness.[19] In the Peterson et al. study, no significant evidence of distress was obtained in the animals evaluating the effect of pristine injection and ascites production.[20]

In all these studies, the ascites methods were preferred for production of monoclonal antibody due to its economical and efficient nature for production of mAbs in high concentrations.

Then monoclonal antibodies were purified by affinity chromatography on protein G Sepharose.[21] Based on documented evidence, analysis of mAb produced in tissue culture reveals that a desired antibody function is diminished or lost. Furthermore, tissue culture might be maintained for long periods, and some mAbs were denatured during concentration or purification processes. However, for many reasons in vivo methods have been underestimated. The most important reasons are as follow: significant pain and distress in mice; high-quality in vitro production systems such as fermenter systems; and contamination of produced mAbs with infectious agents, such as viruses and other microorganisms.[22]

In vivo and in vitro methods for production of monoclonal antibodies have their own pros and cons. In ascites production some disadvantages include daily observation of mice, contamination of produced antibody with the undesired proteins (which makes the purification necessary) and significant pain and distress in mouse. The disadvantages of in vitro production includes: use of FBS in monoclonal product, that limits utilization of some monoclonal antibodies; mAb concentration tends to be low in the supernatant; generally more expensive than the ascites method for small-scale or medium-scale production of mAb. Nevertheless, the mouse ascites method usually produces very high mAb concentrations that often do not require further concentration procedures specially avoid the effects of contaminants in in vitro batch-culture fluid; avoids the need to teach the antibody producer tissue-culture methods.[23]

According to the above discussion it seems that ascites production of monoclonal antibody is very versatile, inexpensive, and economically useful.

Acknowledgments

The authors wish to thank Tarbiat Modares University, Qazvin Biomedical Technology Incubator of Qazvin University of Medical Sciences (QUMS), and Iran National Science Foundation (INSF) for supporting this research.

References

1. (a) Pandey, S. Hybridoma Technology for Production of Monoclonal Antibodies. *Hybridoma* **2010**, *1* (2), 017; (b) Zhang, C. Hybridoma Technology for the Generation of Monoclonal Antibodies. *Antibody Meth. Protocols* **2012**, 117–135.
2. Katrukha, I. Human Cardiac Troponin Complex. Structure and Functions. *Biochemistry* (Moscow) **2013**, *78*(13), 1447–1465.
3. (a) Babuin, L.; Jaffe, A.S. Troponin: The Biomarker of Choice for the Detection of Cardiac Injury. *Can. Med. Assoc. J.* **2005**, *173*(10), 1191–1202; (b) Reichlin, T.; Hochholzer, W.; Bassetti, S.; Steuer, S.; Stelzig, C.; Hartwiger, S.; Biedert, S.; Schaub,

N.; Buerge, C.; Potocki, M. Early Diagnosis of Myocardial Infarction with Sensitive Cardiac Troponin Assays. *New Engl. J. Med.* **2009**, *361*(9), 858–867.
4. O'Gara, P.T.; Kushner, F.G.; Ascheim, D.D.; Casey, D.E.; Chung, M.K.; De Lemos, J.A.; Ettinger, S.M.; Fang, J.C.; Fesmire, F.M.; Franklin, B.A. 2013 ACCF/AHA Guideline for the Management of ST-elevation Myocardial Infarction: A Report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J. Am. College Cardiol.* **2013**, *61*(4), e78–e140.
5. Tiwari, R.P.; Jain, A.; Khan, Z.; Kohli, V.; Bharmal, R.; Kartikeyan, S.; Bisen, P.S. Cardiac Troponins I and T: Molecular Markers for Early Diagnosis, Prognosis, and Accurate Triaging of Patients with Acute Myocardial Infarction. *Molec. Diagn. Therapy* **2012**, *16*(6), 371–381.
6. Lee, G.; Liu, S. Monoclonal Antibodies Against Human Cardiac Troponin I for Immunoassays II. *Monoclonal Antibod. Immunodiagn. Immunother.* **2015**, *34*(3), 169–173.
7. Brodeur, B.R.; Tsang, P.S. High Yield Monoclonal Antibody Production in Ascites. *J. Immunolog. Meth.* **1986**, *86*(2), 239–241.
8 (a) McArdle, J. Alternatives to Ascites Production of Monoclonal Antibodies. *Animal Welfare Inform. Center Newslett.* (USA) **1997**; (b) Falkenberg, F.; Weichert, H.; Krane, M.; Bartels, I.; Palme, M.; Nagels, H.-O.; Fiebig, H. In vitro Production of Monoclonal Antibodies in High Concentration in a New and Easy to Handle Modular Minifermenter. *J. Immunolog. Meth.* **1995**, *179*(1), 13–29; (c) Antibodies, N.R.C.C.o. M.o.P.M. In Vitro Production of Monoclonal Antibody, **1999**.
9. Baradaran, B.; Majidi, J.; Hassan, Z.; Abdolalizadeh, J. Large Scale Production and Characterization of Anti-human IgG Monoclonal Antibody in Peritoneum of Balb/c Mice. *Am. J. Biochem. Biotechnol.* **2006**, *1*(4), 190–193.
10. Hadavi, R.; Zarnani, A.H.; Ahmadvand, N.; Mahmoudi, A.R.; Bayat, A.A.; Mahmoudian, J.; Sadeghi, M.-R.; Soltanghoraee, H.; Akhondi, M.M.; Tarahomi, M. Production of Monoclonal Antibody against Human Nestin. *Avicenna J. Med. Biotechnol.* **2010**, *2*(2), 69–77.
11. Costagliola, S.; Franssen, J.; Bonomi, M.; Urizar, E.; Willnich, M.; Bergmann, A.; Vassart, G. Generation of a Mouse Monoclonal TSH Receptor Antibody with Stimulating Activity. *Biochem. Biophys. Res. Commun.* **2002**, *299*(5), 891–896.
12. Grodzki, A.C.; Berenstein, E. Antibody Purification: Affinity Chromatography–Protein A and Protein G Sepharose. *Immunocytochem. Meth. Protocols* **2010**, 33–41.
13. Craig, T.A.; Sommer, S.L.; Beito, T.G.; Kumar, R. Production and Characterization of Monoclonal Antibodies to Human Sclerostin. *Hybridoma* **2009**, *28*(5), 377–381.
14. Nejad-Moghaddam, A.; Abolhassani, M. Production and Characterization of Monoclonal Antibodies Recognizing a Common 57-kDa Antigen of Leishmania Species. *Iran. Biomed. J.* **2009**, *13*(4), 245.
15. Tabll, A.; Abbas, A.T.; El-Kafrawy, S.; Wahid, A. Monoclonal Antibodies: Principles and Applications of Immmunodiagnosis and Immunotherapy for Hepatitis C virus. *World J. Hepatol.* 2015, *7*(22), 2369.
16. Ecker, D.M.; Jones, S.D.; Levine, H.L. In *The Therapeutic Monoclonal Antibody Market, MAbs*. Taylor & Francis, 2015; pp. 9–14.
17. Ezzatifar, F.; Majidi, J.; Baradaran, B.; Maleki, L.A.; Abdolalizadeh, J.; Yousefi, M. Large Scale Generation and Characterization of Anti-Human IgA Monoclonal Antibody in Ascitic Fluid of Balb/c Mice. *Adv. Pharmaceut. Bull.* **2015**, *5*(1), 97.
18. Peterson, N.C.; Peavey, J.E. Comparison of in vitro Monoclonal Antibody Production Methods with an in vivo Ascites Production Technique. *J. Am. Assoc. Lab. Animal Sci.*, **1998**, *37*(5), 61–66.

19. Kozlowski, S.; Swann, P. Current and Future Issues in the Manufacturing and Development of Monoclonal Antibodies. *Adv. Drug Deliv. Rev*. **2006**, *58*(5), 707–722.
20. Peterson, N.C. Behavioral, Clinical, and Physiologic Analysis of Mice used for Ascites Monoclonal Antibody Production. *Compar. Med*. **2000**, *50*(5), 516–526.
21. Darcy, E.; Leonard, P.; Fitzgerald, J.; Danaher, M.; O'Kennedy, R. Purification of Antibodies using Affinity Chromatography. *Protein Chromatogr. Meth. Protocols* **2011**, 369–382.
22. National Research Council Committee on Methods of Producing Monoclonal, A. The National Academies Collection: Reports funded by National Institutes of Health. In Monoclonal Antibody Production, National Academies Press (US), National Academy of Sciences.: Washington (DC), 1999.
23. Etheridge, M.E. Alternatives to Monoclonal Antibody Production (Proceedings). Avialable at http://altweb.jhsph.edu/pubs/proceedings/mab/etheridge.html.