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# A methodological approach for production and purification of polyclonal antibody against dog IgG

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## Abstract

Antibodies are a class of biomolecules that has an important role in the immune system and lots of applications in biotechnological methods and in pharmaceutics. Production and purification of antibodies in laboratory animals is one of the first ways to manufacture of these prominent tools. The obtained antibodies from these process could be used in various types of bioassay techniques such as enzyme linked immunosorbent assay (ELISA), radioimmunoassay, etc. Also, antibodies employed in diagnostics applications in humans and other animals in order to detect specific antigens. In this study, we aimed to produce and purify anti-dog IgG via immunizing rabbits with dog IgG in combination with Freund's adjuvant. Polyclonal IgG were purified by ion exchange chromatography and then the purified antibody was labeled with horse radish peroxidase (HPR). Direct ELISA was used to determine the optimum titer and cross-reactivity of HRP conjugated IgG. The purity of various IgG preparations and the optimum dilution of prepared HRP conjugated IgG, respectively, was about 95.00% and 1:8000. This study showed that efficiency ion-exchange chromatography could be an appropriate method for purification of IgG antibodies. This antibody could be a useful tool for future dog immune diagnosis tests. This product characterization shown here sets the foundations for future work on dog IgGs.



## رهیافت روش شناختی برای تولید و خالص سازی پادتن چند دودمانی برعلیه ایمنوگلوبولین G سگ

### چکیده

پادتن ها جزو مولکول های زیستی هستند که نقش مهمی در سیستم ایمنی بدن داشته و کاربرد های بسیاری در روش های زیست فناوری و دارویی دارند. تولید و خالص سازی پادتن در حیوانات آزمایشگاهی از نخستین روش ها برای تولید این ابزارهای برجسته می باشد. پادتن های به دست آمده از این فرایند می تواند در انواع مختلفی از روش های زیست سنجی مانند الایزا و رادیو ایمنواسی استفاده شود. همچنین، پادتن ها کاربردهای تشخیصی در انسان و دیگر حیوانات به منظور تشخیص اختصاصی پادگن ها دارند. هدف ما در این مطالعه تولید و تخلیص پادتن علیه IgG سگ در خرگوش های ایمن شده با IgG سگ همراه با کمکی فروند بود. پادتن چند دودمانی با استفاده از رنگ نگاری تعویض یونی تخلیص شد و پادتن تخلیص شده با آنزیم هورس رادیش پراکسیداز نشاندار گردید. تست الایزای مستقیم به منظور تعیین عیار مطلوب و میزان واکنش متقاطع آمیخته مربوطه انجام شد. خلوص پادتن آمیخته تولیدی در حدود ۹۵/۰۰ درصد و عیار آمیخته ۱:۸۰۰۰ بدست آمد. این مطالعه نشان داد که روش رنگ نگاری تعویض یونی می تواند برای تخلیص پادتن ها بسیار کارآمد باشد. این پادتن تولیدی می تواند یک ابزار مفید برای تست های تشخیصی برای سیستم ایمنی سگ باشد. خصوصیت این محصول نشان داد که می تواند پایه ای برای کارهای بعدی بر روی IgG سگ باشد.

**واژه های کلیدی:** آمیختگی با HRP، ایمنوگلوبولین جی Anti-dog، پادتن چند دودمانی، رنگ نگاری تعویض یونی

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## Introduction

One of the most important parts of the immune system is antibody or immunoglobulin that act as a mediator of the immune system against antigens.[1] There are five kinds of the antibody (Ab) that some of them are on the surface of B cells and the others are free in the blood or lymph acting as the effector of the humoral immune system. Structurally antibodies are glycoproteins with a Y-shaped building includes two identical light chains (24 kD) and two identical heavy chains (55 to 70 kD). Each chain consists of constant (CH and CL) and variable fragment (VL and VH). The two sites of each chain of an antibody (VH and VL) form the antigen detection site in each antibody while C region of each heavy chain namely FC allows biological function. The V region of each antibody is different from the other, however, the C region is common for all antibodies.[1-3]

Having special capacity such as feature selection, flexible structure for labeling, *in vivo* and *in vitro* production capability led to raising the researcher interest to use this molecule in analytical and medical applications.[4] Up to now there are myriad of efforts for enhancing the quality and quantity of production and purification of these biomolecules, however, the increasing need of polyclonal and monoclonal Ab foment the more effort to precise calibrate and enhancing the methods for production of Ab in large scale in commensurate with the amount of industry, health and research sector require. According to the scale, facilities and type of applications various methods can be used in antibody production and purification process.[3,4] According to immune-experiments and diagnostic applications polyclonal antibodies labeled with different labels such as fluorescent dyes, enzyme-labeled antibodies are used in immunoblotting, histo-chemical staining, and enzyme linked immunosorbent assay (ELISA) techniques. They could provide high-resolution inspection for results of immunochemical and histological tests. Horse radish peroxidase (HRP) conjugated IgG against canine immunoglobulins are used in diagnosing some of the dog's diseases by ELISA or western blotting tests, among non-affinity purification techniques.[5] Ion exchange with regard to improvement in rigidity, developing porosities and other characteristics (such as charge density) which have been optimized, is the preferred chromatographic technique for the purification of IgG from plasma.[2-4]

## Materials and Methods

**Preparation of antigen.** Blood samples were collected from three clinically healthy dogs using sterile disposable needles, after clarification by centrifugation at 1000 *g* for 15 min and then diluted 1:1 with phosphate buffer saline (PBS, pH 7.20). In the next step, the equal volumes of diluted serum and saturated ammonium sulfate were mixed by stilly shaking. The centrifugation processed again was employed at 1000 *g* for 20 min and the precipitate was washed twice with 50.00% saturated ammonium sulfate solution. The obtained precipitate was dissolved in PBS followed by overnight dialysis against PBS. The precipitated fraction was dialyzed against 0.05 mM PBS, pH 7.40 and IgG was purified by ion exchange chromatography (DEAE-Sepharose 6B; Pharmacia, Piscataway, USA), which is a simple and economical method. The distinct antibody was eluted from the column through a washing buffer containing 50 mM NaCl (Merck, Darmstadt, Germany), and the fractions were collected at 5 mL in 20 min. Confirmation of the purified fractions was done by SDS-PAGE in reducing condition. Finally, the purified fractions were kept for Immunization of rabbit.[1,6]

**Immunization of rabbit with dog IgG.** From prepared dog IgG in PBS, 300 µg per 300 µL was mixed with equal volumes of Freund's complete adjuvant (Sigma-F5881, Hamburg, Germany) and inoculated intra-muscularly into three 6-month-old New Zealand White rabbits. All procedures were performed according to the guidelines approved by the Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran (Appendix 4-9, 1395).

The rabbits were fed regularly on commercial diets. The second and third inoculations were performed on days 21 and 35 with Freund's incomplete adjuvant, and the fourth inoculation was done on day 45 without any adjuvant. After the final immunization, blood samples were collected from the rabbit and its antibody titer of immunoglobulin was investigated by double diffusion and ELISA test.[7-12]

**Double diffusion test.** Double diffusion test was used for the evaluation of antibody production in rabbit. For double diffusion analysis, 1.20% agar gel was prepared in Barbital buffer, pH 8.60. Then, five wells were made with about one cm distance between each well. Amounts of 10 µL of antigen and serially diluted rabbit sera were poured into central and the peripheral wells, respectively. After the glass plates were incubated for 24 hr at room temperature, Coomassie® Brilliant Blue R-250 (Sigma) was used for the gel staining.[13-15]

**Indirect ELISA.** Indirect ELISA was performed for determining the optimum titer of the produced rabbit polyclonal antibody against dog IgG (anti-dog IgG). Initially, 100 µL of antigen was coated into each well of microtiter plate. The plate was covered with an adhesive plastic and incubated at 4 ˚C overnight. Then, the wells were washed with PBST (PBS buffer with 0.05% Tween-20; Sigma) three times. The wells were blocked with 250 µL of 5.00% skimmed milk. After washing process, 100 µL of each dilution of rabbit sera was added to each well. Then, the wells were washed with PBST three times. Next, 100 µL of HRP conjugated goat anti rabbit antibody was added and incubated for

90 min at 25 ˚C. After washing process, 100 µL of 3', 5'-tetramethyl benzidine (TMB; Merck) was loaded for promoting the reaction. Finally, the optical density was determined by ELISA reader.[16]

**Purification of rabbit Abs against anti-dog IgG.** Immunized rabbit serum was collected and precipitated using a 50.00% ammonium sulfate (Merck). After dialysis against PBS and tris-phosphate buffer (pH: 8.10; Merck), concentration of protein was adjusted and then transferred onto an ion-exchange chromatography column packed with diethylamino ethyl (DEAE)-Sepharose® Fast Flow (Pharmacia). The column was washed in two steps using tris-phosphate buffer and tris-phosphate buffer containing 100 mM NaCl (Merck), respectively, for the first and second steps. Assessment of collected protein was done using a UV spectrophotometer (Bio-Rad Laboratories, Redmond, USA) that was adjusted on a 280 nm absorbance. The eluted proteins were collected in 5 mL fractions and analyzed by sodium dodecyl sulfate polyacryl amide gel electrophoresis (SDS-PAGE).

**SDS-PAGE analysis.** The purity of various IgG preparations was checked using SDS-PAGE under reduced and non- reduced conditions. The final concentration of polyacrylamide solution was 12.50%. Samples that boiled with 2.00% SDS for 10 min and were loaded on the electrophoresis gel. After separation, the bands were stained with Coomassie® Brilliant Blue G 250 (Sigma).

**Conjugation of rabbit IgG with HRP.** The periodate method was performed for conjugation with some variations. Two mg of peroxidase (Sigma) was dissolved in 500 µL of distilled water in a dark glass bottle. Then 100 µL sodium periodate (Merck) was added to the solution, and the container was kept at 25 ˚C on a stirrer for 25 min. The blend was dialyzed against a sodium acetate buffer (Merck) (0.10 mM, pH: 4.40) at 4 ˚C overnight followed by the addition of 10 µL of carbonate-bicarbonate buffer (Merck) (0.20 M, pH: 9.50). 4 mg of the purified rabbit anti-dog IgG in 1 mL of carbonate-bicarbonate buffer (Merck) (10 mM, pH: 9.50) was added to the active enzyme, and the bottle was put on the stirrer. Then, 100 µL of fresh sodium borohydrate solution (Merck) was added to the solution and was kept at 4 ˚C for 90 min on the stirrer. The product was then dialyzed overnight against PBS at 4 ˚C with the addition of BioStab antibody stabilizer (Sigma).

**Direct ELISA.** Direct ELISA was used to determine the titer of the HRP conjugated rabbit anti-dog IgG. 100 µL of prepared dog immunoglobulins, which was diluted 1:100 in PBS (10 µg), was added to each well of a 96-well micro-titer plate and incubated at 4 ˚C for 24 hr. The wells were washed with PBS Tween (0.05% Tween 20) three times and blocked with 200 µL of blocking solution (PBS–0.50% Tween 20). After washing step, 100 µL of 1:500, 1:1000, 1:2000, 1:4000, 1:8000 and 1:16000 dilutions of prepared HRP conjugated anti-dog IgG were added to each well. The reaction was developed using 100 µL of 3, 3', 5, 5'-tetra-methylbenzidine (TMB) as a substrate and the absorbance was determined at 450 nm after stopping the reaction using a 5.00% sulfuric acid solution (Sigma).

## Results

**Evaluation of production of anti-dog IgG.** Production of rabbit anti-dog IgG for assessment production of antibody in rabbit and evaluating the effectiveness of immunization, double diffusion, and ELISA tests were employed. The titer of polyclonal anti-dog IgG in double diffusion test was 1:16 (Fig. 1), which appeared as a sharp band between antigen and antibody wells. The titer of anti-dog IgG determined by ELISA was 128000 (Table 1).

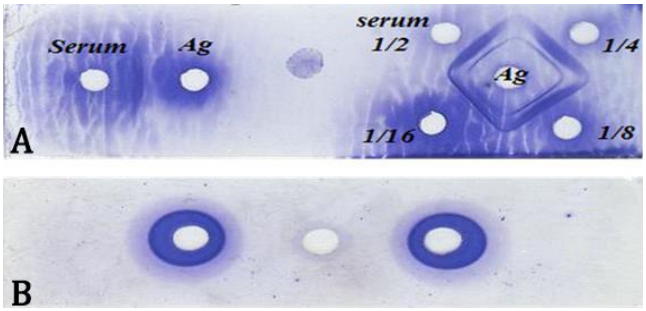


**Fig. 1. A)** Assessment of anti-dog IgG with double diffusion test. Dog IgG and serially diluted of anti-dog IgG were poured into central and peripheral wells, respectively. Precipitation arcs represent production of anti-dog IgG in rabbits. **B)** Evaluation of anti-dog IgG production by single radial immunodiffusion. Circle in this shape showed the antibody production against dog IgG.

**Purification of rabbit anti-dog IgG.** The purification of polyclonal antibody was done from an immunized rabbit ammonium sulfate precipitation followed by DEAE ion-exchange chromatography that resulted in a highly pure fraction. The protein content of this fraction was 12.80 mg which was about 1/4 of primary protein content (Fig. 2).

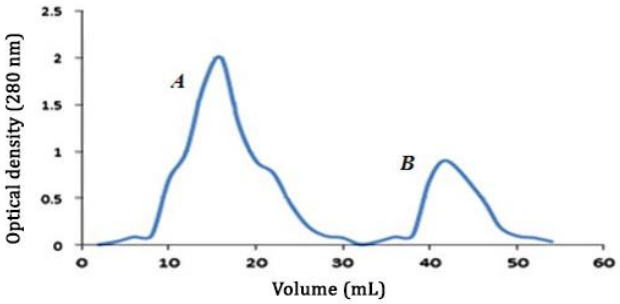


**Fig. 2.** Purification of rabbit polyclonal IgG by ion-exchange chromatography. Peak A: Elution of IgG by tris-phosphate buffer, pH 7.40. Peak B: Elution of other proteins by tris-phosphate buffer containing 100 mM NaCl.

**SDS-PAGE analysis.** The result of SDS-PAGE for determining the purity of rabbit anti-dog IgG is represented in Figure 3, that purified by ion exchange

**Table 1.** Evaluation of produced antibody in the serum of immunized rabbit by ELISA method. The presence of antibody against dog IgG in serum of the rabbit was confirmed.

| Dilution | Negative Control* | 1/4000 | 1/8000 | 1/16000 | 1/32000 | 1/64000 | 1/128000 | 1/256000 |
|---|---|---|---|---|---|---|---|---|
| **Optical density** | 0.11 | 2.84 | 2.66 | 2.18 | 1.88 | 1.55 | 1.13 | 0.89 |

* Negative control with 1/4000 dilution.

**Table 2.** Determine of titer of HRP-conjugated rabbit anti-dog IgG. The optimum dilution of prepared HRP to conjugated IgG was found to be 1:8000.

| Dilution | Negative Control* | 1/1000 | 1/2000 | 1/4000 | 1/8000 | 1/16000 |
|---|---|---|---|---|---|---|
| **Optical density** | 0.13 | 2.48 | 2.03 | 1.76 | 1.18 | 0.89 |

* Negative control with 1/2000 dilution.

chromatography. There are two bands that represent distinct polypeptide band with molecular weight about 50 kDa corresponding to rabbit IgG heavy chains. The diffuse bands between molecular weights of 20 to 30 kDa correspond to rabbit IgG light chains. The SDS-PAGE analysis showed that purification of IgG by Ion-exchange chromatography resulted in a highly pure product.

**Determine of titer of HRP-conjugated rabbit anti-dog IgG.** The purified antibody was conjugated with HRP. Next, a direct ELISA test was used to determine the optimum titer of HRP-conjugated rabbit anti- dog IgG. The optimum dilution of prepared HRP to conjugated IgG was found to be 1:8000 (Table 2).

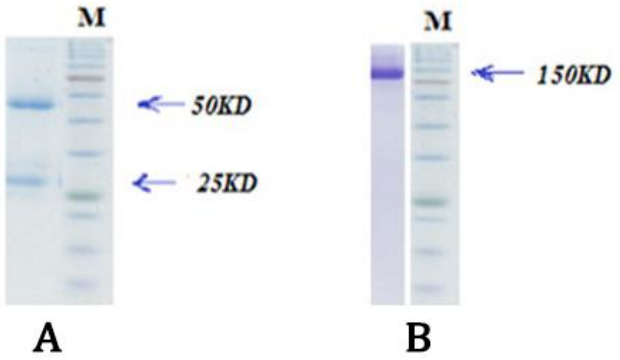


**Fig. 3.** SDS-PAGE analysis of purified rabbit polyclonal IgG by ion-exchange chromatography. **A)** In reduced form, two bands were seen in 50 and 25 kDa MW positions; **B)** In non-reduced condition only one band was seen in about 150 kDa MW position.

## Discussion

Despite great efforts and investment in the past few decades, the researchers still are looking for alternative methods and improving available methods to enhance quality and quantity of antibodies to achieve reliable antibodies for therapeutic and detecting methods.[17-19] In this study, the ELISA test was applied in order to screen polyclonal antibody titer with a straightforward assessment of antigen-antibody complex that results in a high-resolution outcome. The titer of a product of this study was 128000 that represent a high-quality result and also good optimization of a work process. On the other hand, the amount of obtained IgGs that was collected from each rabbit showed an acceptable efficiency of this work methods to obviate at least the part of our need for research and educational programs. However, the attentions are paid to monoclonal antibodies more than polyclonal, but in some case polyclonal antibodies acts better than monoclonal antibodies. For example, when an antigen with role of target in a study have various epitopes polyclonal antibody can connect to the more connective sites resulting better sensitivity, and also in some application like electron microscopy polyclonal antibody acts better.[19] The process that needs to pass to immunoglobulins purification has special challenges that need to be obviated. There are several items that need to be optimized for preparation and separation and recoveries of antibodies like buffer type and pH, a length of the gradient, flow rate of the mobile phase, ionic strength and nature of counter ion, and characteristic of the proteins. It needs to calibrate and optimize all of conditions and parameters to obtain high-quality proteins like antibodies. Although some alternative techniques for the purification of antibodies such as affinity and immunoaffinity chromatography have its advantages but ion-exchange chromatography is considered more satisfactory in terms of economy.[19-22] In this study after purification step, protein with the approximate purity of 95.00% was obtained and showed the acceptable performance for the process followed in this study. SDS-PAGE analysis showed that a bond contained the protein with approximately 50 kDa MW that was rabbit IgG heavy chains. The light chain of rabbit IgG was appeared as a diffused band of 20 to 30 kDa molecular weights. It was likely that diffused band of the light chain could be related to the different level of deglycosylation of protein during manipulation process.[23]

ELISA method was done and the results showed that the harvested polyclonal antibody recognized dog IgG. Also, anti-IgG polyclonal antibody (PAb) interacted with dog IgG with a very high specificity and affinity. Polyclonal anti-dog IgG is very important, significant and as a key reagent for its recognition. The PAbs with high affinity are useful tools in biomedical and biochemical research. In addition, it is applicable in immunoassay tests for detection and quantitation of dog IgG subclass levels in immunoassay tests such as: ELISA, western blot, immunofluorescence, immunohistochemistry, immuno-electrophoresis, and immunodiffusion tests.

This product was suitable for conjugation with enzymes, radiolabels, fluorochromes or other markers, and for attachment to solid supports for use as an immunoadsorbant. Also, this product was applicable in immunoelectrophoresis, immunodiffusion, immunohistochemistry and flow cytometry tests. Furthermore, antibody against dog IgG was suitable for use as a primary reagent in enzyme immunoassays, western blot and cell or tissue immunostaining.

There is a need for quick, rapid, reliable serological assays to diagnose dog diseases, including autoimmune disorders, inflammatory diseases, etc.[5,23] Nevertheless, use of polyclonal and monoclonal antibodies against immuno-globulins are the most appropriate option, very few specific monoclonal and polyclonal antibodies to canine immunoglobulins have been reported to date. Accordingly, this body of study lays the basis and foundation for evaluating dog immunoglobulin for therapeutic antibody development and builds upon the fundamental knowledge of dog immunology and a deep understanding of the dog immune system.[24,25]

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## Conflicts of Interests

The authors declare that they have no conflict of interest with regard to this manuscrip.

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