

Chapter 40

Production of Monoclonal Antibody by DNA Immunization with Electroporation

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Abstract DNA immunization with in vivo electroporation is an efficient alternative protocol for the production of monoclonal antibodies (mAb). Generation of mAb by DNA immunization is a novel approach to circumvent the following technical hurdles associated with problematic antigens: low abundance and protein instability and use of recombinant proteins that lack posttranslational modifications. This chapter describes the use of a DNA-based immunization protocol for the production of mAb against a house dust mite allergen, designated as Blo t 11, which is a paramyosin homologue found in *Blomia tropicalis* mites. The Blo t 11 cDNA fused at the N terminus to the sequence of a signal peptide was cloned into the pCI mammalian expression vector. The DNA construct was injected intramuscularly with in vivo electroporation into mice, and the specific antibody production in mice was analyzed by enzyme-linked immunosorbent assay (ELISA). Hybridomas were generated by fusing mouse splenocytes with myeloma cells using the ClonaCell™-HY Hybridoma Cloning Kit. Six hybridoma clones secreting Blo t 11 mAb were successfully generated, and these mAb are useful reagents for immunoaffinity purification and immunoassays.

Keywords: monoclonal antibody, DNA immunization, electroporation, hybridoma, dust mite allergen

1. Introduction

Monoclonal antibodies (mAb) are powerful immunological and biochemical tools in basic and applied research. In recent years, mAb have been fully exploited for development of invaluable diagnostic and therapeutic reagents. Conventional hybridoma techniques for the generation of mAb require the use of exogenously produced protein immunogens for immunization in laboratory animals (1). In the case of low abundance proteins or recombinant proteins that lack posttranslational modifications, mAb production may be problematic and challenging. The DNA immunization approach is a simple, cheap, and efficient strategy to circumvent

laborious protein production and purification and, yet, can effectively induce antigen-specific immune response.

Wolff et al. first showed that intramuscular (i.m.) immunization with naked plasmid DNA resulted in direct gene transfer into mouse muscles (2). Subsequently, several reports revealed that DNA immunization is an effective strategy for the delivery of DNA constructs encoding specific immunogens into host cells, inducing both antigen-specific humoral and cellular immune responses (3–6). The method of DNA immunization requires the gene of interest to be cloned in a suitable mammalian expression vector with a strong promoter (e.g., CMV enhancer/promoter region) and a polyadenylation/transcriptional termination sequence. The two commonly used routes of DNA immunization include the intramuscular (i.m.) injection and the use of “gene gun” to propel gold beads with DNA into the epidermis (6).

Intramuscular immunization of naked DNA constructs in mice resulted in the *in situ* expression of the encoded proteins (2), thereby generating antibody responses in the immunized organism (3, 7). In recent years, the use of electroporation in conjunction with intramuscular immunization has attracted considerable attention (8, 9). Muscle electroporation using high-frequency, low-voltage electric pulses has been reported to increase the production and secretion of recombinant erythropoietin in mouse skeletal muscles by more than 100-fold (8). Electroporation was shown to increase the expression of the recombinant protein encoded by the immunized gene and to elevate the immune responses of animals against the expressed protein (9, 10). Electroporation enhances uptake of genes by permeabilization of the muscle fibers, resulting in a very high frequency of transfected bundles as well as higher intensity of expression. The enhancement of immunity apparently follows the increased availability of antigen.

Production of specific antibodies by DNA immunization is initiated by the expression of the protein encoded by the immunized gene (6). The encoded protein is then targeted as an internal immunogen to the host immune system. Antibodies produced by DNA immunization have been reported to recognize both linear and conformational antigens (11). DNA immunization coding for a defined portion of the *Helicobacter pylori* vacuolating cytotoxin in mice resulted in the generation of highly specific mAb recognizing the natural protein (12). Polyclonal antibodies against apobec-1 (13), an editing enzyme that is difficult to purify, and tobacco mosaic virus coat protein (TMV-CP) (14) have been generated by DNA immunization in rabbits. Our laboratory was the first to show that allergen-specific antibody and cellular responses, induced by allergen-gene immunization, were protective against mite allergy (15). Subsequently, mAb for a number of mite allergens have been generated in our laboratory by the DNA immunization approach (16–19). In addition, the DNA immunization approach has been successfully used to generate mAb for native human thyrotropin receptor (20), the phosphoprotein (21), and blood group antigens (22). These studies demonstrate the advantages of DNA immunization, whereby a full spectrum of native epitopes of an antigen can be expressed *in situ* in the host's mammalian system, and thus a better conformation or protein presentation can be achieved.

compared with administration of recombinant protein expressed and purified *in vitro*. In addition, complex mixtures of antigens can be screened for immunogenicity or protective efficacy using DNA immunization far more rapidly than by purifying the individual protein components.

Here, we describe the generation of mAb for a house dust mite allergen, designated as Blo t 11, which is a paramyosin homologue found in *Blomia tropicalis* mites. The molecular weight of the Blo t 11 protein is ~102 kDa, and the production of intact protein was problematic because of its susceptibility to degradation (23); therefore, it was not feasible to immunize animals with the conventional protein immunization protocol. We have resolved the problem by adopting the DNA immunization approach. The Blo t 11 cDNA fused at the N terminus to the sequence of a signal peptide was cloned into the pCI mammalian expression vector (Promega). The signal peptide sequence used was derived from the cDNA (complementary DNA) encoding for Der p 5 dust mite allergen (15). The DNA construct was

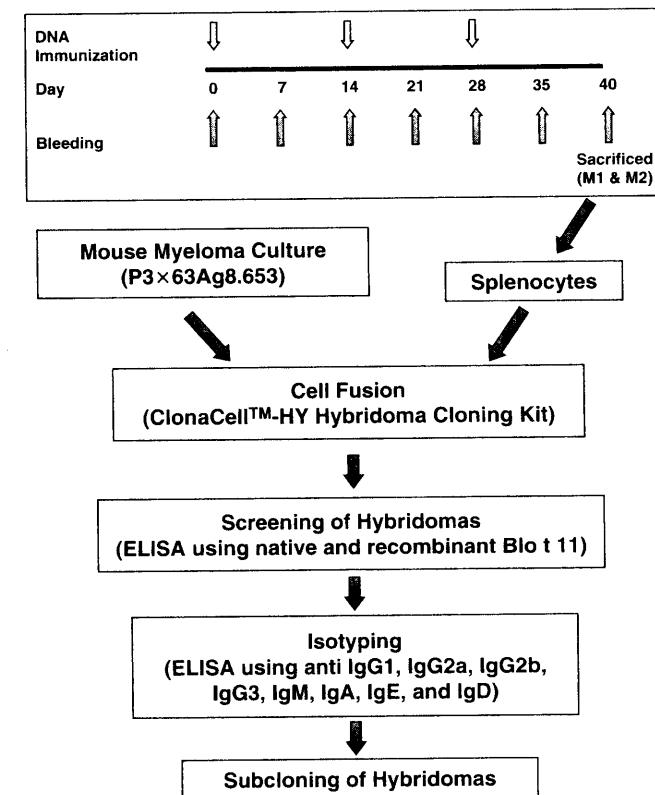


Fig. 40.1 Schematic representation of the DNA immunization regimen in BALB/cJ mice and the stepwise protocols used to generate and screen hybridomas secreting Blo t 11 mAb, followed by production and characterization of the various Blo t 11 mAb

injected intramuscularly with in vivo electroporation into mice and the specific antibody production in mice was analyzed by enzyme-linked immunosorbent assay (ELISA). The generation, screening, and subcloning of hybridomas were performed using the ClonaCell™-HY Hybridoma Cloning Kit (Fig. 40.1).

2. Materials

2.1. DNA Construct

1. Mammalian expression vector pCI (Promega) containing Der p 5 signal peptide sequence (Dp5LS) (*see Note 1*).
2. PCR-amplified Blot 11 gene (2,625-bp open reading frame) using forward primer 5' CGTCCGGAATGGCGGCTCGATCAGCA 3' containing *BspE1* restriction enzyme site and reverse primer 5' AGAGAGCTCGAAC TTCTAAGCGGCCGCCG 3' containing *NotI* restriction enzyme site.
3. Pfu polymerase: Store at -20°C .
4. Restriction enzymes *BspE1* and *NotI*: Store at -20°C .
5. T4 DNA ligase: Store at -20°C .
6. Competent *Escherichia coli* DH5 α : Thaw in ice immediately before use. Store in 200 μL aliquots at -80°C .
7. Luria-Bertani agar plates with ampicillin: Dissolve 25 g of Luria-Bertani Miller (Becton Dickinson, Sparks, MD), and 20 g of Bacto Agar™ (Becton Dickinson) in distilled water to a final volume of 1 L. Mix properly using a magnetic stirrer, autoclave for 15 min at 121 psi, cool to $\sim 55^{\circ}\text{C}$, add 1 mL of ampicillin (100 $\mu\text{g}/\text{mL}$), shake, and pour into plates. Store Luria-Bertani agar-ampicillin plates at $4-8^{\circ}\text{C}$.
8. Luria-Bertani broth with ampicillin: Dissolve 25 g of Luria-Bertani in distilled water to a final volume of 1 L. Mix properly using a magnetic stirrer, autoclave for 15 min at 121 psi, cool to $\sim 55^{\circ}\text{C}$, add 1 mL of ampicillin (100 $\mu\text{g}/\text{mL}$), shake, and store at $4-8^{\circ}\text{C}$.
9. Wizard® Plus SV Miniprep DNA Purification System (Promega, Madison, WI): Store at room temperature.
10. QIAGEN Plasmid Giga Kit (Qiagen, Hilden, Germany). Store at room temperature.
11. Agarose gel (1% w/v): Dissolve 1 g of Seakem® LE agarose (BioWhittaker Molecular Applications, Rockland, ME) in 100 mL of 1 $\times$ TAE buffer, microwave mixture, add ethidium bromide (final concentration of 0.5 $\mu\text{g}/\text{mL}$), pour on casting tray, and allow to polymerize.
12. TAE buffer (50 $\times$ stock solution): Dissolve 242 g of Tris base in 800 mL of distilled water. Then 57.1 mL of glacial acetic acid and 100 mL of 0.5 M EDTA are added. Adjust pH to 8.0 and add distilled water to a final volume of 1 L. Store solution at room temperature.

13. ABI Prism 377 Automated DNA Sequencer (PE Applied Biosystems, Foster City, CA).
14. GenElute™ Agarose Spin Column (Sigma-Aldrich, St. Louis, MO).

2.2. DNA Immunization

1. Five 6-week-old female BALB/cJ mice. All mice are housed under conventional conditions and fed with mice food pellets and water ad libitum (*see Note 2*).
2. Electro Square Porator (ECM 830) attached to a two-needle array electrode with cable (model no. 532) (BTX, CA).
3. U-100 insulin syringe with 29-G needle.
4. Phosphate-buffered saline (PBS): Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 , and 0.24 g KH_2PO_4 in distilled water to a final volume of 1 L. Adjust pH to 7.4 with HCl.

2.3. Enzyme-Linked Immunosorbent Assay

1. Antigens: Recombinant and native Blot 11 allergens were used (*see Note 3*). Store in aliquots at -20°C .
2. ELISA plates (Costar Flat bottom high binding plate 3590).
3. ELISA coating buffer: 0.1 M NaHCO_3 , pH 8.3.
4. Washing buffer: 1 $\times$ phosphate-buffered saline, pH 7.4 (sect. 2.2, item 4), containing 0.05% (v/v) Tween 20.
5. ELISA plate washer (Tecan, Austria).
6. Blocking buffer: 1% (w/v) bovine serum albumin in washing buffer.
7. Primary antibody (antisera) is diluted in blocking buffer (*see Note 4*).
8. Secondary antibody: Biotinylated anti-mouse immunoglobulin is diluted 5,000 $\times$ in blocking buffer.
9. Alkaline phosphatase-conjugated ExtrAvidin is diluted 5,000 $\times$ in blocking buffer.
10. Substrate solution: *p*-nitrophenyl phosphate (pNPP) tablets are dissolved and diluted in distilled water (*see Note 5*).
11. ELISA plate reader.

2.4. Cell Fusion and Cloning of Hybridomas

1. ClonaCell™-HY Hybridoma Cloning Kit (StemCell Technologies Inc., Vancouver, Canada).
2. Myeloma cells, a sub clone of P3X63Ag8.653 (ATCC, Manassas, VA).
3. 96-well Falcon® tissue culture plates.

4. Biotinylated anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, IgE and IgD.
5. Trypan blue.

2.5. Ascites Production and Purification

1. Dulbecco's modified Eagle's medium (DMEM).
2. 10% fetal Calf Serum.
3. Trypan blue.
4. Pristane (2,6,10,14-tetra-methyl-pentadecane).
5. 8-week-old female BALB/c mice.
6. Protein A- or G-agarose column.

3. Methods

3.1. Preparation of DNA Construct

1. PCR-amplify the 2,625-bp open reading frame of the Blo t 11 gene by using a 25 μ L PCR reaction consisting of 1 μ L of plasmid DNA, (see Note 6) 1 μ L each of 10 μ M forward and reverse primers, 0.5 μ L of 10 mM dNTPs, 2.5 μ L of 10 $\times$ PCR buffer, 0.4 μ L of *Pfu* polymerase enzyme, and 18.6 μ L of deionized distilled water. Amplification is done with 30 cycles: each cycle consists of 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 3 min with a final elongation step of 72 $^{\circ}$ C for 10 min, using the Programmable Thermalcycler.
2. Electrophorese the 25 μ L PCR product in 1% agarose gel for 20 min at 110 V using a BioRad Agarose electrophoresis unit. Run a 1-kb DNA marker to determine the molecular weight of the PCR product. Visualize and document the DNA band using a gel documentation system.
3. Cut the DNA band from the agarose gel examined under a UV transillumination and purify the PCR product using a GenEluteTM Agarose Spin Column.
4. Separately digest the purified Blo t 11 gene PCR product and the pCI-Dp5LS plasmid by using *Bsp*E1 and *Not*I for 1 h at 37 $^{\circ}$ C. Electrophorese and purify the digested samples as in step 3.
5. Ligate the *Bsp*E1- and *Not*I-digested Blo t 11 gene into the *Bsp*E1- and *Not*I-linearized pCI-Dp5LS plasmid for 14–16 h at 16 $^{\circ}$ C.
6. Transform the ligated samples into *E. coli* DH5 α by heat shock method. A transformation reaction mixture of 200 μ L of thawed competent cells with 10 ng of the ligation mixture is incubated on ice for 30 min, followed by heat shock at 42 $^{\circ}$ C for 2 min and 30 s in a water bath, and finally incubated on ice for 10 min.
7. Resuspend the transformation reaction (200 μ L) in 800 μ L of Luria-Bertani broth and incubate at 37 $^{\circ}$ C for 1 h with constant shaking at 250 rpm. Plate cells onto selective Luria-Bertani ampicillin plates and incubate these plates overnight at 37 $^{\circ}$ C (see Note 7).

8. Pick 3–5 colonies and culture them separately in 5 mL Luria-Bertani broth with ampicillin. The broth cultures are incubated overnight at 37 $^{\circ}$ C with constant shaking at 250 rpm. Each of the selected colonies is also plated onto a Luria-Bertani ampicillin plate, which is incubated overnight at 37 $^{\circ}$ C.
9. Pellet bacterial cells from the overnight cultures by centrifugation for the isolation of plasmid DNA using the Wizard[®] Plus SV Miniprep DNA purification system. Presence of insert is analyzed by PCR, RE digest, and DNA sequencing.
10. A clone containing a plasmid with the inserted gene in its proper orientation is cultured in 1 L of Luria-Bertani broth for the large-scale isolation of plasmid DNA. Luria-Bertani broth cultures are set up following similar protocols as in step 8.
11. Plasmids are isolated and purified using QIAGEN Plasmid Giga Kit, and a sample is sequenced using the ABI PrismTM 377 Automated DNA Sequencer.
12. Resuspend the purified plasmid DNA in sterile water, analyze the purified DNA in 1% agarose gel, and quantitate the DNA by spectroscopy. Aliquots are stored at –20 $^{\circ}$ C until further use.
13. Glycerol stocks of the positive transformants consisting of a 1:1 mixture of overnight bacterial culture and sterile 70% glycerol are prepared, mixed properly, and stored at –80 $^{\circ}$ C for future use.

3.2. Immunization of DNA Constructs

1. Acclimatize five 6-week-old female BALB/cJ for at least a week.
2. Immunize mice intramuscularly with 50 μ g of the pCI-Dp5LS-Blo t 11 plasmid DNA (suspended in 50 μ L of PBS) on the quadriceps muscle of the hind leg by using U-100 insulin syringe with 29-G needle.
3. Immediately after immunization, each mouse is pulsed at the site of the immunization using the Electro Square Porator (ECM 830) attached to a two-needle array electrode with cable (model no. 532) (BTX, Genetronix, CA), using the following parameters: 4 pulses of 82 V with a pulse length of 20 ms and an interval of 200 ms.
4. Boost each mouse twice with electroporation at days 14 and 28 using the same parameters mentioned earlier.
5. Collect blood via infra-orbital every week to monitor the titer of antibody production. Isolate sera from the blood samples and store at –180 $^{\circ}$ C until further use.

3.3. Determination of Antibody Titer Production

1. Suspend 50 μ L of antigens (5 μ g/mL) in ELISA coating buffer and coat the suspension onto 96-well ELISA plates in duplicates by incubation for 14–16 h at 4 $^{\circ}$ C with gentle shaking.

2. Wash ELISA plates thrice with 200 μ L of washing buffer per well using an automated ELISA plate washer.
3. Incubate each well with 200 μ L blocking buffer for 1 h at 22 °C. Use a multi-channel pipette to dispense reagents. Wash plates as in step 2 after the incubation period.
4. Dilute antisera in blocking buffer. Add 50 μ L of different dilutions (see Note 4) of the primary antibody (antisera) extracted from the different immunized mice at different time points to corresponding wells. Incubate the primary antibody for 1 h at room temperature with constant gentle shaking using a horizontal shaker. Wash plates as in step 2 after the incubation period.
5. Dispense 50 μ L of diluted secondary antibody into each well. Incubate for 1 h at room temperature with constant gentle shaking using a horizontal shaker. Wash plates as in step 2 after the incubation period.
6. Incubate each well with 50 μ L of diluted alkaline phosphatase-conjugated ExtrAvidin solution. Incubate for 1 h at room temperature with constant gentle shaking using a horizontal shaker. Wash plates as in step 2 after the incubation period.
7. Develop reaction by incubating each well with 50 μ L of substrate solution. Incubate for 30 min up to 1 h at room temperature in the dark.
8. Read absorbance at 405 nm using an ELISA plate reader. Plot the absorbance and determine the kinetics of antibody titer production of each mouse. An example of antibody response of 2 mice immunized with a DNA construct is shown in Fig. 40.2.

3.4. Cell Fusion and Cloning of Hybridomas

1. A week prior to cell fusion, culture parental myeloma cells to confluence in medium A of the ClonaCell™-HY Hybridoma Cloning Kit. Subculture cells into several flasks reaching the mid-log phase growth on the day of fusion.
2. Determine the viability of the myeloma cells by the trypan blue exclusion assay and count the number using a hemocytometer.
3. Resuspend 2×10^7 viable myeloma cells in 30 mL of medium A of the ClonaCell™-HY Hybridoma Cloning Kit.
4. Euthanize two mice with the highest antibody titer by cervical dislocation and dissect the spleens under sterile conditions. Splenocytes are separated by grinding the spleen tissue using the frosted/coarse end of a glass slide. These cells are resuspended in medium B of the ClonaCell™-HY Hybridoma Cloning Kit.
5. Fuse a total of 1×10^8 viable cells with previously prepared myeloma cells using the supplied PEG solution and medium B in the ClonaCell™-HY Hybridoma Cloning Kit.
6. Incubate the resulting hybridomas overnight at 37 °C in medium C. Then plate them using medium D (methylcellulose-based medium) and incubate them for 14 days at 37 °C until isolated colonies appear.

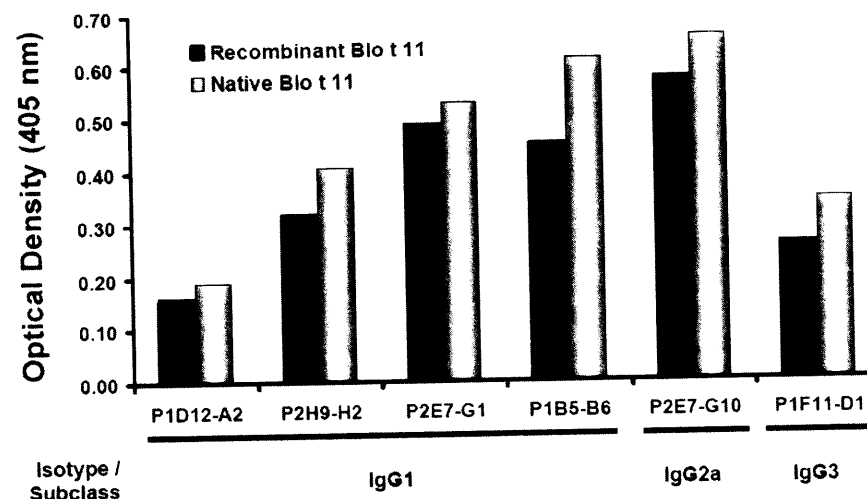


Fig. 40.2 Kinetics of native Blo t 11 specific antibody productions of the two high responder mice (M1 and M2) immunized intramuscularly with pCI-Dp5LS-Blo t 11 construct via electroporation according to methods described in sect. 3.2. Immunization regimen used is shown in Fig. 40.1. Mice were bled weekly for antibody analysis by ELISA, as described in sect. 2.3. Mice were euthanized on day 40, and suspension cells prepared from the spleens were used for hybridoma production according to protocols summarized in Fig. 40.1

7. Pick isolated colonies using a pipette tip and culture hybridomas in 96-well Falcon® tissue culture plates.
8. Collect hybridoma clone supernatants and assay for recombinant and native Blo t-11-specific antibody by ELISA, as described earlier.
9. Subculture antigen-specific antibody-producing clones and expand into 6-well Falcon® culture plates using DMEM medium supplemented with 10% fetal calf serum.
10. Isotype each positive clone by ELISA (as described earlier) using biotinylated anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, IgE and IgD.

3.5. Ascites Production and Purification

1. One week prior to inoculation of hybridoma cells, inject intraperitoneally 1 mL pristane into each mouse by using a 22-G needle.
2. Culture high antibody titer producing hybridoma clones in DMEM medium supplemented with 10% fetal calf serum and expand for inoculation into mice for ascites production.
3. Inject 5×10^6 viable cells in 1 mL PBS intraperitoneally into each mouse by using a 22-G needle.

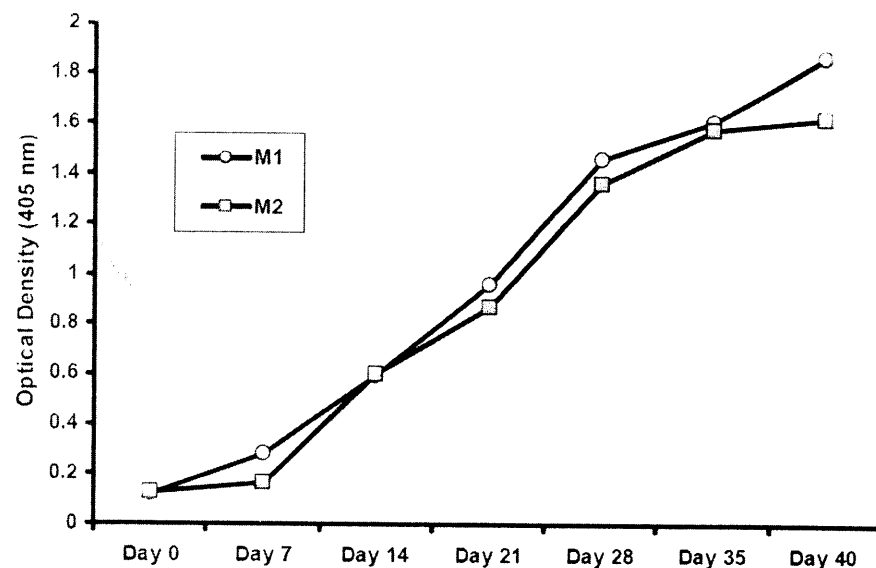


Fig. 40.3 The IgG reactivity of six representative mAb (C1 – C6) to native and recombinant Blo t 11 proteins. The antibody titers were determined by ELISA and presented as optical densities measured at a wavelength of 405 nm. The indicated subclasses of these mAb were determined by ELISA, as described in sect. 3.5

- About 1–2 weeks after cell inoculation, collect ascites fluid from each mouse by gravity flow into 15-mL conical centrifuge tubes using an 18-G needle inserted 1–2 cm into the abdominal cavity (*see Note 8*).
- Assay ascites fluid for antibody titer and specificity by ELISA, as described earlier, using recombinant and native antigens (*see Note 9*). A panel showing the IgG reactivity of 6 different mAb to Blo t 11 allergen is shown in Fig. 40.3.
- Purify the mAb from the pooled ascites fluid by affinity chromatography using protein A or G agarose column (*see Note 10*).

Acknowledgments This study was supported by grants from the Agency for Science, Technology, and Research (A*STAR), Singapore (R-178-000-010-303, R-178-000-101-305), and Academic Research Fund, National University of Singapore, Singapore (R-178-000-010-112).

4. Notes

- The induction of protein-specific antibodies can be enhanced by increasing the levels of protein expression in vivo following DNA immunization. Inclusion of a signal peptide sequence in a DNA construct will help to enhance the in vivo expression of the secretory protein. Our previous studies showed that up to a 10-fold increase in the titers of antibody production in immunized mice were observed with constructs containing the signal peptide sequence

derived from Der p 5 mite allergen cDNA (24). Der p 5 derived signal peptide sequence can be replaced with signal peptide sequences from other genes. We had shown that the use of pSecTag2 vectors (Invitrogen Life Technologies), which are expression vectors designed for high-level protein expression in mammalian hosts, could significantly increase the magnitude of antibody responses in DNA-immunized mice. Proteins expressed from pSecTag2 are fused at the N terminus with Ig κ -chain signal peptide sequence for protein secretion.

- All animals should be treated humanely and all animal experiments were performed according to Institutional Guidelines for Animal Care and Handling of National University of Singapore.
- Recombinant Blo t 11 allergen was expressed in *E. coli* as a GST-fusion protein (25) while the native Blo t 11 was immunoaffinity purified from a crude *B. tropicalis* extract (23).
- We recommend determining the best dilution at which the least noise to background ratio can be observed by diluting the antisera at 10 $\times$, 100 $\times$, 1,000 $\times$, and 5,000 $\times$.
- p*-nitrophenyl phosphate solution is light-sensitive. Turn off lights or close windows when pipetting the solution. Use the recommended volume of distilled water for dilution of each tablet according to the manufacturer's instructions.
- The full-length Blo t 11 gene was cloned by screening a *B. tropicalis* (Bt) cDNA library by plaque lift method, 5' rapid amplification of cDNA ends (RACE), and by long-distance PCR (25). The Bt cDNA library was constructed according to standard protocol by inserting cDNA into *Eco*RI and *Xho*I site of Uni-ZAP[®] expression vector (Stratagene, La Jolla, CA).
- It is a common practice to plate 200 and 800 μ L of the transformation reaction into two separate Luria-Bertani plates, as the efficiency of the ligation and transformation procedures vary from one experiment to another. The goal is to produce well-isolated bacterial colonies after the overnight incubation.
- Mice were euthanized after two collections of ascites fluid.
- The mAb assays could also be performed by Western blot analysis and dot blot analysis.
- It is also a good idea to assay the purified mAb for specificity using different immunoassays with both recombinant and native antigens.

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