

Monoclonal Antibodies

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Abstract

Monoclonal antibodies (mabs) are powerful tools for the quantification, detection, and targeting of specific molecules. Allergen-specific mabs are important for the quantification of major allergens in allergen preparations used for allergen-specific immunotherapy and allergy diagnosis. Indeed, progress in the understanding of the mechanisms of the immunological responses underlying allergic disease would not have been possible without the use of mabs. Quantification assays are also important in the assessment of environmental allergen exposure and monitoring of avoidance procedures.

Mabs against human IgE provide the basis for various test systems for the detection of specific and nonspecific IgE. Mabs raised against IgE or defined cytokines or cytokine receptors have potential as neutralizing reagents *in vivo* for the treatment of allergic diseases.

Allergen-specific mabs are also valuable tools for the localization of allergens within their source material and the characterization of allergens derived from natural sources and by recombinant technologies. Furthermore they are often used for the isolation of allergens from complex extracts by affinity chromatography.

The procedure described in this chapter has been used successfully to produce mabs against numerous allergens from house dust mites, insect venoms, cat, hens egg white, tree-, grass-, and herb pollens, and fungi, with the ultimate aim of obtaining matched antibody pairs to establish two-site binding assays for the quantification of major allergens. The method has also been used successfully to generate mabs against human IgE.

Key Words: Monoclonal antibody; IgE; allergen.

1. Introduction

Monoclonal antibodies (mabs) are powerful tools for the quantification, detection, and targeting of specific molecules. Allergen-specific mabs are important for the quantification of major allergens in allergen preparations used for allergen-specific immunotherapy and allergy diagnosis. They allow the concentrations of major allergens to be defined in these preparations, thus contributing to better

standardized preparations. The quantification assays are also important in the assessment of environmental allergen exposure and monitoring of avoidance procedures.

Allergen-specific mabs are also valuable tools for the localization of allergens within their source material, e.g., pollen grains and mite bodies and the characterization of allergens derived from natural sources and by recombinant technologies. Furthermore they are often used for the isolation of allergens from complex extracts by affinity chromatography.

Mabs against human IgE provide the basis for various test systems for the detection of specific and nonspecific IgE. Humanized mabs raised against IgE or defined cytokines or cytokine receptors have potential as neutralizing reagents *in vivo* for the treatment of allergic diseases.

Progress in the understanding of the mechanisms of the immunological responses underlying allergic disease would not have been possible without the use of mabs. Fluorescence-activated cell analysis, immunohistochemical and cytochemical investigations, and *in situ* hybridization have been undertaken using a diverse range of labeled antibodies in order to identify cell types, specific activation markers, and the sources of various cytokines and chemokines.

Antibodies are a product of B cells. Immunoglobulin-producing B cells from the spleens of mice are not able to survive in culture *in vitro*, as they are only capable of dividing a few times. In order to render them immortal and capable of infinite reproduction, they are fused with cells of a permanent myeloma cell line. The resulting hybridoma cells combine the features of antibody production and perpetual reproduction. The progeny of a single antibody-producing hybridoma cell represent a cell clone which produces antibodies with a single specificity: mabs. The method of mab production was originally described by Köhler and Milstein (1).

In order to ensure that only hybridoma cells but not the nonfused myeloma cells grow after fusion, mutated myeloma cells are used. The mutational defect imparts the inability to synthesize nucleic acids by salvage pathways. The *de novo* pathways of the nucleotide synthesis are inhibited by aminopterin. Only myeloma cells fused with B cells are able to maintain the nucleic acid synthesis by salvage pathways using compounds, e.g., hypoxanthine and thymidine, added to the medium for selection, and it is these cells that are able to survive and multiply in culture. The fusion of the two cell types is facilitated by the addition of polyethylene glycol. In order to obtain antibodies of a defined specificity, the mice are immunized with the desired antigen, and the respective antibody-producing B cells accumulate in the spleens of the mice.

The procedure described here is according to Köhler and Milstein (1) and Goding (2) and has been used successfully to produce mabs against numerous allergens from house dust mites, insect venoms, cat, hens egg white, tree-,

grass-, and herb pollens, and fungi (**3–12**), with the ultimate aim of obtaining matched antibody pairs to establish two-site binding assays for the quantification of major allergens. The method has also been used successfully to generate mabs against human IgE. Purification of the mabs is an important prerequisite for many applications. As most of the mabs belong to the IgG class this is easily done by protein A chromatography. With special exceptions (**12**) two-site binding assays for the quantification of allergens require mabs of different epitope specificity. Thus an epitope analysis using biotinylated mabs and unlabeled mabs is necessary. After selecting mabs of different epitope specificity the next step is to determine whether they are suitably matched for a two-site binding assay. For the quantification of allergens another important step is to provide a suitable standard. After development and validation, the assay is ready for use for the quantification of allergens in the samples.

2. Materials

For each step the general basic laboratory equipment for cell culture is needed: sterile working-bench with laminar flow hood, cell incubator (37°C, 5% CO₂, humidified atmosphere), centrifuge, microscope, autoclave, and for cryopreservation a –80°C freezer and storage container with liquid nitrogen.

2.1. Immunizing Mice

1. Use 6-wk-old female mice of the strain Balb/c (*see Note 1*).
2. Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) (Sigma, Taufkirchen, Germany) (*see Note 2*).
3. Antigen against which mabs are to be generated.
4. Syringes (1–5 mL) and appropriate cannulas (23–25 gauge).

2.2. Preparation of Reagents for Cell Fusion

2.2.1. Myeloma Cell Line

Myeloma cell line P3X63Ag8U.1 (ATCC No. CRL 1597) according to Yelton et al. (**13**).

2.2.2. Media

1. Equipment for sterile filtration of media.
2. Culture medium RPMI 1640 (PAA, Cölbe, Germany).
3. HAT-media supplement (Sigma) (*see Note 3*).
4. HT-media supplement (Sigma) (*see Note 3*).
5. L-Glutamine, 20 mM stock solution (PAA).
6. Penicillin/streptomycin stock solution with 10,000 IU/mL or 10,000 UG/mL (PAA).
7. Na-pyruvate, 100 mM stock solution (PAA).
8. Fetal calf serum (FCS) (supply is variable and depends on prior testing of the respective batch).

9. RPMIØ: RPMI 1640 medium without supplements. Shelf-life: 3 wk at 4°C.
10. RPMI⊕ = RPMI 1640 supplemented with 2 mM glutamine, 1 mM Na-pyruvate, penicillin/streptomycin (100 U/mL), and 5% heat-inactivated FCS. Add the supplements after sterile filtration using a vacuum filtration device. Shelf-life: 3 wk at 4°C.
11. HAT-medium: Same supplements as in RPMI⊕ and additionally HAT-supplement. Reconstitute two vials each with 10 mL sterile purified water and add 12 mL to the basic medium under sterile filtration. Shelf-life: 3 wk at 4°C.
12. Freezing medium: RPMI⊕ with 20% DMSO. Shelf-life: 1 wk at 4°C. Use as a 4°C cold solution.

2.2.3. Preparation of Instruments

1. Scissors and tweezers for the preparation of the spleen from the mouse.
2. Bags for sterilization and a special adhesive tape with sterile indicator.
3. Cell dissociation sieve CD-1 with mesh screen 40 and pestle (Sigma).

2.3. Cell Fusion

See **Section 2.2.** and additionally:

1. Polyethylene glycol (PEG 1500; Roche Diagnostics, Mannheim, Germany).
2. Petri dishes for cell culture, 20-mL volume (Greiner).
3. 96-Well flat bottom culture plates (Nunc, Wiesbaden, Germany).
4. Trypan blue solution (0.4%; Sigma).

2.4. Cultivation of Cells After Cell Fusion

1. 24-Well plates (Greiner).
2. Cryo vials, 2-mL volume (Greiner).
3. HAT-medium.

2.5. Screening for Antibody Production

1. 96-Well round bottom microtiter plates (Greiner).
2. Microplate washer/ELISA washer.
3. Microplate reader/ELISA photometer.
4. Multichannel pipette.
5. Mouse hyperimmune serum (serum taken on the day of the fusion).
6. Antigen to which mabs are desired.
7. Peroxidase (POD)-conjugated goat-anti-mouse IgG (FC-specific) (Dianova/Jackson Immuno Research 115-036-071, Hamburg, Germany) (see **Note 4**).
8. ABTS-substrate [2',2'-azino-bis(3-ethylbenzthiazolin-6-sulphonacid)] tablets (Sigma).
9. Citric acid (Merck, Darmstadt, Germany).
10. Hydrogen peroxide, 30% H₂O₂ (Merck).
11. Sodium azide, NaN₃ (Merck).
12. Carbonate buffer: 1.59 g Na₂CO₃ and 2.93 g NaHCO₃ (Merck), adjust with sterile purified water to 1 L. The pH should be 9.6 ± 0.1. Shelf-life: 4 wk at 4°C.

13. Phosphate-buffered saline/Tween 20 (PBS/TW): 6.845 mM NaCl, 0.135 mM KCl, 0.507 mM Na_2HPO_4 , 0.073 mM KH_2PO_4 , 0.05% Tween 20 (all chemicals from Merck), 0.01% Thimerosal (Sigma). pH 7.4. Shelf-life: 6 wk at RT.
14. PBS/TW/BSA: PBS/TW + 1% BSA (Merck). pH 7.4. Shelf-life: 4 wk at 4°C.
15. Citrate buffer: Dissolve 2.82 g citric acid and 1.5 g Na_2HPO_4 in 250 mL purified water each separately. Adjust the pH of the phosphate solution by adding the citric acid solution until a pH of 4.2 is reached. Bring the volume to 500 mL. Shelf-life: 4 wk at 4°C.
16. Substrate solution: Prepare the solution directly prior to use. Dissolve one tablet of ABTS-substrate in 12 mL citrate buffer and add 12 μL H_2O_2 .
17. Stop solution: 0.002 M NaN_3 .

2.6. Cloning of Selected Hybridomas

1. Trypan blue.
2. Hematocytometer.
3. HAT-medium.
4. Sterile 1-mL Eppendorf cups and 15-mL tubes.
5. 96-Well flat bottom culture plates.
6. 24-Well culture plates.

2.7. Cultivation of Selected Hybridomas

1. HT-medium: Same supplements as in RPMI $\oplus$ and additionally HT-supplement. Reconstitute one vial HT-supplement with 10 mL purified water and add it to the basic medium under sterile filtration. Shelf-life: 4 wk at 4°C.
2. RPMI $\oplus$: See **Section 2.2.2.**, No. 10.

2.8. Two-Site Binding Assay for the Quantification of Allergens

The material is similar to the ELISA equipment described under **Section 2.5**. The additional items needed are

1. POD-conjugated streptavidin (Sigma).
2. Biotin labeling kit (Roche Diagnostics).

3. Methods

For detailed descriptions of any variations of the procedure described here, refer to comprehensive books (**14,15**).

3.1. Immunizing Mice

1. Prime with 10–50 μg purified antigen or 100 μg antigen extract (*see Note 5*). Dissolve the antigen in 100 μL sterile PBS and make an emulsion by adding 100 μL CFA (*see Note 6*). Note that Freund's adjuvants are potentially harmful. Protect the eyes and be careful during the preparation of the emulsion and injection into mice. It is recommended to start the immunization procedure with more than one animal, take five to ten mice.

2. On day 28 after priming perform a boost with the same amount of antigen emulsified in 100 μ L IFA and 100 μ L PBS per intraperitoneal injection (*see Note 7*).
3. After 7–10 d (on day 35–38) take blood from the mice and check for antigen-specific antibodies by ELISA (*see Section 3.5*). Select the best responder for the planned fusion (*see Note 8*).
4. After 42–50 d boost the selected mouse with antigen in the same concentration as above in a total volume of 150 μ L PBS without adjuvant via ip route. Boost the remaining mice as described under **Section 3.1**, using IFA as adjuvant.
5. Take the selected mouse for fusion 3 d later.

3.2. Preparation of Fusion

1. 5–10 d before fusion thaw PU1 myeloma cells and expand in culture to obtain approximately 5×10^7 cells for fusion. Daily splitting of the cells in a 1:4 ratio ensures that they remain in the desired exponential growing phase.
2. Prepare HAT-medium 4 d prior to fusion. Perform a sterile control and a control with PU1 cells. The PU1 cells should not grow in this medium.
3. Boost the selected mouse 3 d prior to fusion (*see Section 3.1*).
4. Prepare sufficient sterile scissors and tweezers. Sterilize the cell dissociation sieve CD-1 with mesh screen 40 and the pestle using the sterilization bags and indicator tape.

3.3. Cell Fusion

1. Prepare 2–3 petri dishes with 37°C warm RPMIØ.
2. Adjust HAT-medium and an aliquot of 500 μ L PEG to 37°C in a water bath.

3.3.1. Preparation of the Spleen

1. Kill the mouse. When desired, take blood from the mouse prior to killing (*see Note 9*).
2. Disinfect the mouse by splashing with 70% ethanol and transfer it to a clean bench.
3. Fix the mouse on its back on a preparation board and open the peritoneum aseptically: Make a little cut in the skin over the gut. Separate the skin from the peritoneal wall with the round end of a pair of tweezers and fix the skin on the preparation board with needles so that the peritoneum is accessible.
4. Open the peritoneum with a vertical cut, taking care not to damage the gut.
5. Put the gut carefully on the right side of the animal. The spleen is now exposed in the upper part of the abdomen (the spleen is smaller than the liver, elongated, and of a dark red color, whereas the liver is of red-brown color and is multilobed).
6. Remove the spleen carefully without damaging the gut and transfer it into a petri dish with prewarmed (37°C) RPMIØ.
7. Put the spleen into the cell sieve and use the pestle to isolate the cells from the tissue by rubbing them carefully through the mesh of the sieve. Rinse the mesh with medium and transfer the cell suspension to a 15-mL tube and adjust the volume to 10–14 mL with RPMIØ (*see Note 10*).
8. Centrifuge at 200g for 10 min.

9. Wash once with 10 mL RPMIØ.
10. Resuspend the spleen cells in 10 mL RPMIØ. Take an aliquot, dilute it 1:10 with Trypan blue solution, and count the cells using a hematocytometer.

3.3.2. Preparation of the Myeloma Cells

1. Check the myeloma cells for their morphology and possible contamination.
2. Pool the cells from several culture dishes in 50-mL tubes and centrifuge at 200g for 10 min.
3. Wash the cells once with RPMIØ and centrifuge again.
4. Resuspend the myeloma cells in 10 mL RPMIØ and count the cells using Trypan blue and a hematocytometer.

3.3.3. Fusion

1. Take a small volume (50 µL) of each cell suspension for sterile and HAT-control in 1 mL HAT-medium.
2. Put the cells in a ratio of 10 spleen cells to 1 myeloma cell together in one 50-mL tube and centrifuge at 200g for 10 min.
3. Decant the supernatant completely, loose the sedimented cells, and add 400 µL prewarmed PEG dropwise under slight agitation over a period of 30 s.
4. After addition of the PEG, agitate the tube slightly for another minute.
5. Dilute the cell-PEG suspension stepwise by slowly adding a defined volume of RPMIØ within a defined time:
 - 1st minute → 1 mL RPMIØ
 - 2nd minute → 1 mL RPMIØ
 - 3rd minute → 8 mL RPMIØ
 - 4th minute → 10 mL RPMIØ
6. Adjust to 50 mL with RPMIØ and centrifuge for 10 min at 200g.
7. Decant the supernatant and suspend the cells carefully in 1–2 mL HAT-medium.
8. Bring the cell suspension to the desired cell concentration (recommended concentration: 1×10^5 cells/well) with HAT-medium, mix carefully in order to obtain a homogenous suspension, and seed into 96-well flat bottom culture plates.
9. Incubate at 37°C, 5% CO₂, and humidified atmosphere.

3.4. Cultivation of Cells After Cell Fusion

1. Check the fusion plates daily for growing cells, any contamination, and the effectiveness of HAT-selection.
2. After 5–7 d evaluate the progress of the growth of the cells and feed with 50–100 µL HAT-medium (*see Note 11*).
3. After 2–4 further days remove the medium completely and add 100–200 µL fresh HAT-medium (*see Note 12*).
4. After 2–4 d check the growing hybridomas by ELISA (*see Section 3.5*).
5. When the cells of a selected hybridoma cover more than half the bottom of the well, transfer them to a 24-well plate and clone (*see Section 3.8*) as soon as possible (*see Note 13*).

6. Harvest the noncloned hybridoma cells from the 24-well culture at 3–5 time-points and freeze in order to save the cells of origin. One fully grown well will give 1 cryo vial (*see Note 14*).

3.5. Screening for Antibody Production

1. Coat a 96-well microtiter plate with antigen (1–10 $\mu\text{g/mL}$) in 100 μL carbonate buffer per well. Incubate overnight at 4°C (*see Note 15*).
2. Wash five times with PBS/TW using a microtiter plate washer.
3. Transfer 50 μL culture supernatant under sterile conditions from the 96-well culture plate to the test plate and add 50 μL PBS/TW/BSA per well. Use the hyperimmune serum from the donor mouse diluted 1:500 as a positive control and culture medium as negative control. Incubate for 2 h at room temperature (*see Note 16*).
4. Wash five times with PBS/TW.
5. Dilute the POD-conjugated goat-anti-mouse IgG (FC-specific) 1:10,000 with PBS/TW/BSA. Incubate 100 μL /well, 1.5 h at room temperature (*see Note 4*).
6. Wash five times with PBS/TW.
7. Incubate with 100 μL freshly prepared ABTS-substrate per well.
8. Positive wells develop a greenish color. Stop the reaction after 15 min – max. 60 min – by adding 100 μL 0.002 M Na-azide solution.
9. Read the optical density with a microplate reader at a wavelength of 414 nm.

3.6. Cloning of Selected Hybridomas

1. Suspend the cells of a selected hybridoma from a 24 well and count the cells diluted 1:1 with Trypan blue using a hemacytometer.
2. Prepare two sterile tubes with 6 mL HAT-medium, and two Eppendorf cups one with 500 μL and one with 950 μL HAT-medium.
3. Add as many cells as necessary to the 500- μL cup to achieve a final concentration of 12,000 cells/mL by exchanging an equal volume of medium for cell suspension. Mix thoroughly.
4. Add 50 μL of this suspension into the 950- μL cup. Mix thoroughly. The cell suspension of this cup is now 600 cells/mL.
5. Add volumes of this suspension, as indicated below, into 10 mL HAT-medium in order to achieve final concentrations of 2, 1, and 0.5 cells/well and seed 100 μL /well of each suspension into 96-well plates:
333 μL for 2 cells/well
167 μL for 1 cell/well
83 μL for 0.5 cell/well
6. Check for growing clones daily.
7. Feed with 50–100 μL HAT-medium when the bottom of a growing well is approximately one-fourth covered with cells.
8. Perform a screening ELISA 2–4 d after feeding (*see Section 3.5*).
9. Select three clones with the strongest positive reaction. Select the clones from the plates in the order 0.5, 1, 2 cells/well (*see Note 17*).

10. As soon as the cells are spread over more than half of the bottom of the plate, transfer them into wells of a 24-well plate, harvest cell samples at three successive time-points, and freeze in cryo vials.
11. Then transfer the cells into the wells of a 6-well plate and again freeze cells at different time-points (*see Note 18*).

3.7. Cultivation of Selected Positive Hybridoma Cells

1. After the first cloning, convert the cells to HT-medium. Perform this in a stepwise manner so that the HAT-medium is slowly replaced by HT-medium, for example, if the culture has to be divided between two wells adjust the total volume with HT-medium after splitting.
2. 1–2 wk after cultivating the cells in HT-medium, convert to RPMI $\oplus$ medium.

3.8. Establishment of a Two-Site Binding Assay for the Quantification of Allergens

3.8.1. Purification and Biotinylation

1. Expand the selected and cloned hybridoma cells for sufficient mab-containing supernatant (*see Note 19*).
2. Purify the mab from the supernatant (*see Note 20*).
3. Determine the protein content of the purified mab.
4. Aliquot and lyophilize the purified mab.
5. Confirm its activity by a direct ELISA and its purity by SDS-PAGE.
6. Conjugate an aliquot of several mabs with biotin (*see Note 21*).
7. Check the activity of the biotinylated mabs by direct ELISA (*see Note 22*).
8. Store the biotinylated mabs in aliquots at -20°C .

3.8.2. Epitope Analysis

1. Coat a 96-well microtiter plate with antigen as described under **Section 3.5**.
2. Wash five times with PBS/TW.
3. The biotinylated mab should be used in a dilution referring to approximately 80% binding in the direct ELISA. Make a dilution which is twice concentrated to that and incubate it with an equal volume of unconjugated mab in 10 twofold serial dilutions, starting with 80 $\mu\text{g/mL}$ in tubes. Preincubate for 30 min in the tubes and then transfer the mixture to the microtiter plate (100 $\mu\text{L/well}$) and incubate for 2½ h at room temperature.
4. Wash five times with PBS/TW.
5. Incubate with POD-conjugated streptavidin diluted 1:1000 with PBS/TW/BSA for 30 min at room temperature.
6. Wash five times with PBS/TW.
7. The substrate incubation is as described under **Section 3.5**.
8. Plot the optical density against the concentration of the unlabeled mab.
9. Mabs which inhibit the binding of the biotinylated mab to the allergen coated onto the microtiter well yield a sigmoid curve and recognize a similar epitope on the

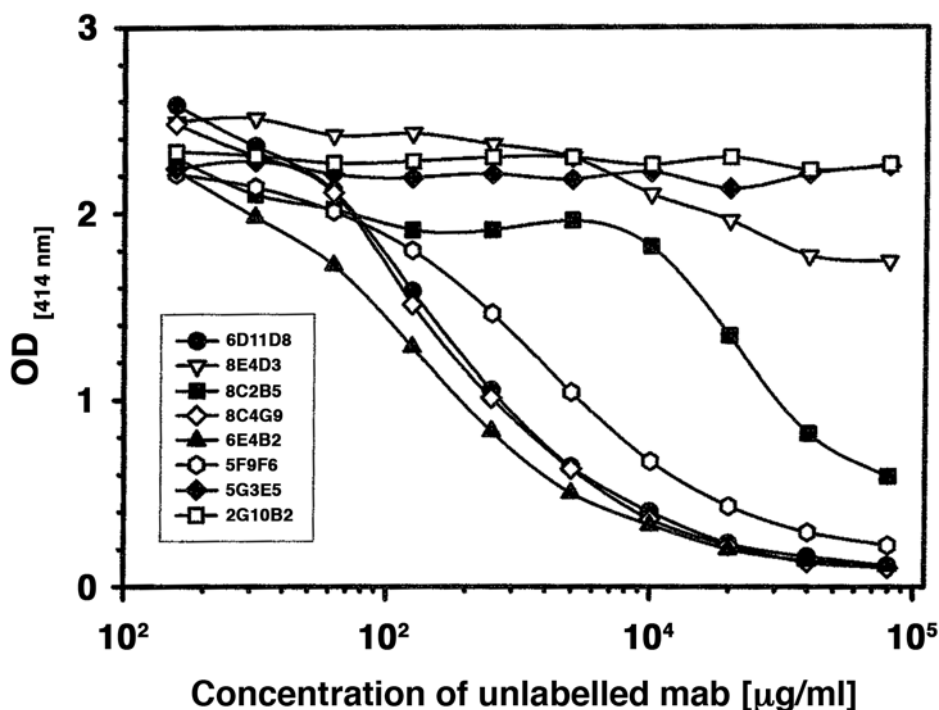


Fig. 1. Epitope analysis of monoclonal antibodies against the major hazel pollen allergen Cor a 1 with biotin-labeled 8C4G9. The mab 5G3E5, 2G10B2 and partly 8E4D3 do not inhibit the binding of biotin labelled mab 8C4G9 to solid phase bound Cor a 1 and are thus candidates for a two-site binding assay in combination with mab 8C4G9.

allergen as the biotinylated mab. These mabs are generally not suited for a two-site binding assay (*see Note 23*).

10. Mabs which do not inhibit the binding of the biotinylated mab recognize a different epitope and are candidates for matched antibody pairs in an ELISA (*see Fig. 1*).

3.8.3. Two-Site Binding Assay

1. Coat an unlabeled mab to a 96-well microtiter plate in a concentration of 1–10 $\mu\text{g/mL}$ in carbonate buffer. Incubate overnight at 4°C.
2. Wash five times with PBS/TW.
3. Block free binding sites with 200 μL PBS/TW/BSA for 15 min. Then discard the blocking solution.
4. Apply purified allergen as a standard and the samples in which the allergen should be quantified in two- to threefold serial dilutions. Often the starting solution of the allergen standard is 1 μg protein/mL whereas the samples should be used in higher protein concentrations (*see Note 24*).

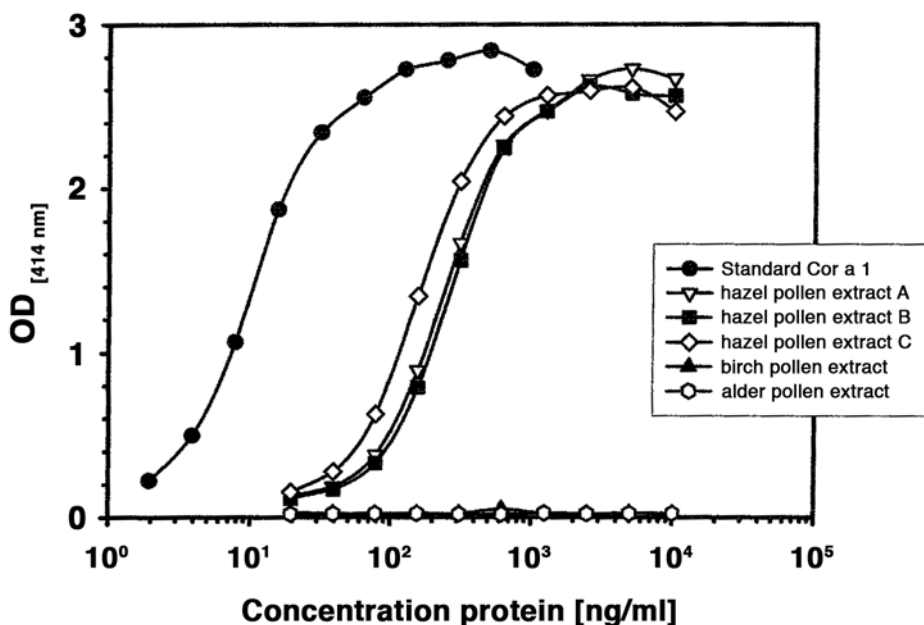


Fig. 2. Two-site binding assay for the major hazel pollen allergen Cor a 1 with mab combination 8C4G9-2G10B2:B using affinity purified Cor a 1 as a standard. Cor a 1 was detected in different batches of hazel pollen extract but not in birch or alder pollen extracts.

5. Incubate for 2 h at room temperature.
6. Wash five times with PBS/TW.
7. Apply the biotinylated mab in constant dilution. Incubate for 1 h at room temperature.
8. Wash five times with PBS/TW.
9. Apply POD-streptavidin diluted 1:1000 in PBS/TW/BSA.
10. Wash five times with PBS/TW.
11. Perform the substrate incubation as described under **Section 3.5**.
12. Calculate the amount of allergen in the samples from the linear part of the standard curve considering the dilution factor.

An example for a two-site binding assay is given in **Fig. 2**.

4. Notes

1. Other strains of mice may be useful for the production of mabs against particular antigens (16). For example, we eventually obtained suitable mabs for the house dust mite allergens Der p 1 and partly for Der p 2 when using A/J mice instead of Balb/c mice.
2. Other adjuvants like aluminum hydroxide or new formulations like TiterMax (Vaxcel, Norcross, Georgia) may be used.

3. HAT/HT-medium supplements = hypoxanthine, aminopterin, thymidine. H and T are supplements for nucleotide synthesis salvage pathways; A is an inhibitor of the *de novo* nucleotide pathway for nucleotide synthesis.
4. This antibody enables the selection of hybridomas of the IgG isotype right from the beginning and avoids selection of IgM hybridomas, which are unstable and more difficult to purify.
5. The amount of antigen depends on the availability of purified antigen. For most purified allergens good results are generally obtained with immunization doses of 20 μg protein/mouse. When only allergen extracts are available, a dose of 100 μg protein/mouse should be used.
6. Prepare the emulsion by using two syringes connected by a valve and push the mixture between the two syringes until it becomes difficult to move. Transfer the emulsion in one of the syringes and connect an appropriate cannula (23–25 gauge). Remove any air from the syringe before injecting it intraperitoneally. Alternatively, when there is only a small volume of antigen solution it is possible to aspirate the two components with a syringe (with luer locks) and then push the mixture out into a small cup. Repeat this procedure several times until it becomes difficult. Then proceed as described above.
7. Other immunization intervals may be chosen, for example, a short immunization protocol with priming at day 1 and first boost at day 14, second boost at day 25, and fusion 3 d later. Sometimes the final boost is done by iv injections or by daily injections without adjuvants 3 d prior to fusion; for more possibilities, *see Refs. (14–17)*.
8. Dilute the mouse hyperimmune serum 1:100 and then make threefold serial dilutions over 8–10 steps. Good responders show a plateau of their titer with serum dilutions of 1:3,000–1:10,000.
9. This may be done, for example, by puncture of the retro-orbital plexus. For detailed descriptions on bleeding mice, *see Refs. (14,15)*.
10. Alternatively, the spleen cells may be released by fragmenting the spleen mechanically using scissors and repeated aspiration through a cannula followed by sedimentation (let the tube sit for 2 min) of remaining larger tissue pieces. Experience has shown that the method of using a cell sieve as described under **Section 3.3.1** is faster and more elegant.
11. When the cells grow slowly, then it is recommended that the smaller volume of 50 μL is added. Stronger growing cells should receive 100 μL medium. Under certain conditions it might be necessary to transfer strongly growing cells to larger culture wells (24-well plate) prior to screening. Experience shows that in most cases strongly growing cells at this time-point of the fusion do not produce the desired antibodies.
12. This step is necessary because unfused B lymphocytes may still produce antibodies in the first hours after fusion before they die. Because these antibodies are not derived from hybridoma cells this may lead to false results in screening ELISA and thus useless work. Remove the medium using a pasteur pipet connected to a vacuum device and aspirate the supernatant by holding the pipet at an angle to the wall of the well. If this is done carefully the cells will remain in the well.

13. It is recommended to perform the cloning as soon as possible because hybridomas which produce no antibodies or antibodies of a different specificity may be present in the origin well and may overgrow the desired hybridoma cells.
14. Freezing procedure: Suspend the desired cells in the well and transfer them into a centrifuge tube. Spin them down 10 min at 200g. Discard the supernatant and suspend the cells in 0.5 mL cold (4°C) RPMI $\oplus$ and then add 0.5 mL of freezing medium, mix, close the vial, and transfer it immediately into a styropor box and the box into a -80°C freezer. This procedure is especially recommended when cells of different hybridomas should be handled and frozen at the same time because a prolonged exposure to DMSO (final concentration 10%) should be avoided because of cell damage at higher temperatures.
15. The optimal concentration of antigen has to be determined individually. In general, 1 μ g/mL of purified allergen is appropriate for plate coating and 10 μ g protein/mL if only allergen extracts are available.
16. Choose a pipeting scheme that allows the positive and negative controls to be included in duplicate wells on each test plate. It is good practice to seed only the center 60 wells of a 96-well culture plate, leaving the peripheral wells empty. It is then possible to test 1½ fusion plates on one ELISA test plate with the indicated controls.
17. In order to achieve the highest probability of monoclonal cells it is best to select the hybridomas from the plate with a seeding concentration of 0.5 cells/well, but when growing clones are visible only in the plates with 1 or 2 cells/well, then these clones should be taken.
18. In order to obtain stable clones, a second cloning procedure is recommended.
19. For a first estimation of the suitability of the selected mab we usually take 500 mL supernatant of hybridoma culture. Harvesting of the supernatant is performed when more than 90% of the cells are dead.
20. Good results for mabs of the subclasses IgG1, IgG2a, and IgG2b are obtained using 50% saturated ammonium sulfate precipitation in the first step for enrichment and concentration of the antibody and protein A chromatography in the second step for purification.
21. For example, with the biotin labeling kit from Roche Diagnostics.
22. Most of the antiallergen antibodies keep their activity after biotin labeling, but it has to be considered that sometimes the labeling occurs in the antigen binding region of the antibody resulting in a loss of activity. It is also possible that the biotin-labeled antibody retains its reactivity to soluble allergen but loses it when the allergen is coated to microtiter plates. We experienced this with biotin-labeled mabs for Der p 1 which perform very well in two-site binding assays but fail to show reactivity to Der p 1 coated to microtiter plates.
23. For allergens which occur in the form of homodimers it is not necessary to obtain a pair of antibodies with different specificities because the same antibody may serve as both capture mab and detector. A two-site binding assay for Alt a 1 from *Alternaria alternata* has been established on this basis (12).
24. The final concentrations of standard and samples have to be assessed individually.

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