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The Halogen Assay – A New Technique for Measuring Airborne Allergen

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Abstract

The Halogen assay is a new technique for measuring airborne allergen. The assay is unique in that it is capable of analyzing allergens and particles together, combining the advantages of morphological approaches and immunoassay. The Halogen assay allows direct observation of the particles that carry the allergen as well as being capable of identifying all the allergen sources an individual is exposed and sensitized to. The assay is sensitive because the extracted allergen is bound to the membrane at a high local concentration within the minute area around each particle and so is easily detected by immunostaining. It is therefore easy to detect few pollen grains.

The Halogen method supersedes other methods commonly used to identify allergens as it is capable of identifying airborne particles that are allergen sources.

Key Words: Allergen; halogen; immunoassay; asthma; mite; cat.

1. Introduction

The term Halogen assay refers to a type of solid phase immunoassay where a visible *halo* of immunostained allergen is formed around the individual particles that are the source of that allergen ***(1)***. To perform this, particles containing allergen such as pollen grains, fungal spores, mite feces, and cat dander are collected and permanently immobilized in contact with a protein-binding membrane. When the particles are wetted, the allergens are extracted from the particles and they bind to the membrane. The bound allergens are then immunostained by passing the reactants through the porous membrane, and the staining appears as halos around the particles (*see* **Fig. 1**).

A long-standing technical goal in aerobiology has been to identify the airborne particles that are allergen sources. The Halogen assay can be used to

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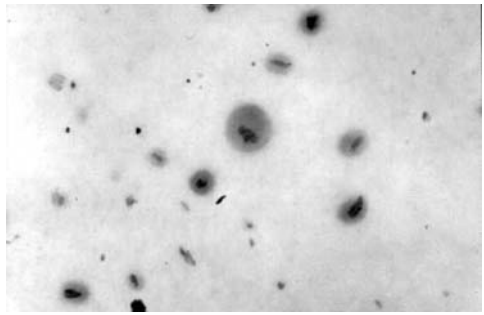

Fig. 1. A small area of the dust collected during active, domestic dust disturbance using a nasal sampler. This has been Halogen immunostained using a monoclonal antibody specific for cat allergen Fel d 1. Particles carrying allergen can be identified by the halo of immunostain around them. These particles differ in size and shape and also in the quantity of allergen per particle. Under these sampling circumstances and in this house, many of the inhaled particles carry cat allergen.

do this. A brief discussion of techniques previously used to detect the allergens associated with particles is provided (*see* **Note 1**).

The main methods commonly used to identify allergens and measure exposure have limitations. Whereas pollen grains and fungal spores can generally be identified based on their morphology, counting them does not provide a reliable measure of exposure to their allergens, as the allergens are also carried by small amorphous particles that cannot be identified ***(2)***. It is also not known how much different allergens are carried by pollen grains or fungal spores at different times. Exposure to the allergens of domestic pets and mites, which are carried by amorphous particles, is generally determined by extracting the allergens from the particles and then measuring them by methods such as ELISA. This approach gives no information about the particles carrying the allergen. Also, the quantities of allergen involved in personal exposure are close to detection limits of most ELISA systems and this limits the types of exposure measurements that can be made.

The Halogen assay combines the advantages of morphological approaches and immunoassays to provide a simple way to analyze allergens and particles together. For example, the use of an allergen-specific antibody allows the direct observation of the particles that carry a specific allergen as well as quantifying that allergen. Alternatively, immunostaining the air samples collected in a subject's environment with their own serum IgE allows the observation of all the allergen sources the subject is both exposed to and allergic to. No other technique can do this.

Halogen assays are also exquisitely sensitive. This sensitivity is achieved because the extracted allergen is bound to the membrane at a high local concentration within the minute area around each particle and so is easily detected by

immunostaining. This means it is as easy to detect one pollen grain, as it is to detect thousands.

Finally, the assays have the potential to measure the quantity of allergen associated with each particle using calculations based on the integrated intensity of each halo measured using computer-based image analysis techniques. These quantities of allergen can then be related to other information about each particle (size, identity, etc.) and can be summed to provide the total amount of allergen in the sample. The technologies for performing these measurements are available in prototype form.

1.1. Three Variants of the Halogen Assay

Halogen immunostaining systems share the common principle of maintaining the collected particles in permanent contact with a matrix capable of nonspecifically binding proteins and other macromolecules. The matrix-bound proteins are then immunostained, allowing the particles that function as the allergen source to be identified. In practice, a protein-binding membrane is used as the matrix, though other options such as protein-binding gels are possible.

Three variants of this, which differ in the way particles are collected and fixed in contact with the protein-binding membrane, are shown in steps 1 and 2 of **Fig. 2**. These are:

1. Collection of particles onto a dry adhesive tape, which is later laminated to a dry protein-binding membrane.
2. Collection of particles onto the dry protein-binding membrane, which is later laminated with a dry adhesive tape.
3. Collection of particles onto a wet protein-binding membrane that has been precoated with an adhesive agarose gel. After collection, this is recoated with more gel to retain the particles.

Variants 1 and 3 are suitable for use with impaction-based collection systems and variant 2 is used with filter-type air samplers (*see* **Section 1.4.**). Binding of allergen to the membrane occurs immediately in the 'wet' system (variant 3) and only after the wetting the dry adhesive/membrane sandwich in variants 1 and 2. The remaining steps 3–5 of the assay process (**Fig. 2**) are common to all variants and are analogous to those used in protein blotting. The components required for performing variants 1–3 are described below.

1.2. Performance Requirements of Adhesive Tapes

There are two major functions of the adhesive tape in Halogen assays. First, it can form the surface on which samples are collected, as in variant 1. Second, if the samples have initially been collected onto the protein-binding membrane, as in variant 2, the tape is subsequently used to maintain the position of the particles on the membrane throughout the assay.

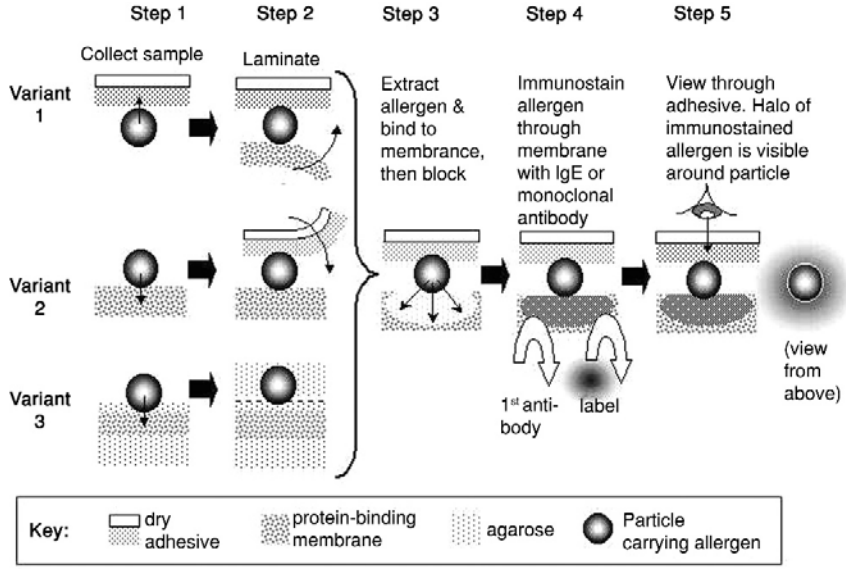

Fig. 2. A simplified schematic view of the five main steps involved in performing three variants of Halogen immunostaining. Interpretation of the symbols is contained in the key.

Of the extensive range of adhesive tapes available, in our experience no commercially available tapes have the required properties combining collection efficiency, optical clarity, noninterference with immunostaining as well as adhesiveness in the aqueous and solvent environments. This led to contracting the development and manufacture of a suitable tape (Inhalix, Woolcock Institute of Medical Research, NSW, Australia).

Low particle bounce is a critical property determining the collection efficiency of adhesive tapes when used for impaction collection. This performance requirement of adhesives could not be predicted from other published characteristics of adhesives. Particles in an impaction sampler may be traveling at well over 1 m/s (a million μm/s) when they hit the tape and they must be captured in the microseconds of contact time before they bounce off. Fibers, which do not make a large contact area with the adhesive surface compared to their contact area with the airstream (generating drag), and large particles, which have a high kinetic energy to surface contact area ratio, tend to be collected least efficiently, whereas small particles are less likely to be impacted. *See **(3)*** for discussion.

Adhesive tapes need to have excellent optical properties, as particles and staining are observed microscopically through them, not on them. When we

compared the collection efficiency of four commercial adhesive tapes against the standard Vaseline-based coating in a Burkard trap, we found that although these tapes had a lower collection efficiency than did Vaseline for particles >10-μm diameter, we also found that the counts of smaller particles were apparently more than twice as high with the tapes than with Vaseline. This was probably because of greater visibility of the small particles on the tape ***(4)***. As mentioned earlier, we subsequently developed more efficient adhesive tapes that collect 88% of *Lolium perenne* pollen at 10 L/min, which are better than the commercial adhesives used in this comparison.

The other properties required of tapes (aqueous adhesiveness, clarity, noninterference in assays) were all determined by experimental evaluation. The only property that was directly predictable from manufacturers' data was toxicity, as tapes used in nasal samplers must contain no hazardous solvents.

Adhesive tapes have been directly used for collection in a variety of impaction-type air samplers including nasal samplers ***(5–7)***, Burkard spore traps ***(4,8)***, and cascade impactors ***(9)***. They could presumably be used in swinging arm impingers, although we have not tested these.

When the tape is used for nonimpaction sampling, such as with 'adhesive lifts' (*see* **Section 1.4.3.**) and 'sprinkle blots' ***(10)*** (and *see* **Section 1.4.4.**) and when used to laminate onto samples already collected onto a porous membrane, performance requirements do not need to include particle collection efficiency.

1.3. Performance Requirements of Protein-Binding Membranes

Many membranes that nonspecifically bind proteins (e.g., nylon, nitrocellulose [NC], mixed cellulose ester [MCE], and polyvinylidene difluoride [PVDF]) with different characteristics are available. Each of these differs in opacity, consistency of microscopic appearance, and protein-binding capacity, which contribute to differences in halo shape, sharpness, intensity, and signal to noise ratios. Good results can be obtained with NC, MCE, and PVDF membranes. In general, membranes with smaller pores, e.g., 0.2–0.45 μm produce more distinct and smaller halos than those with larger pores and in general PVDF and MCE membranes produce halos with a sharper appearance than NC membranes. In addition, some allergens seem to be better detected on PVDF, perhaps because interactions between the allergen and the membrane allow a better presentation of epitopes for binding by particular monoclonal antibodies. MCE membranes are easier to handle than PVDF membranes. The general subject of membrane choice has been widely discussed in the literature on protein blotting, for example see Kirschfink and Terness ***(11)*** who concluded with a similar preference for PVDF membranes. Our most recent experience has tended toward the use of MCE.

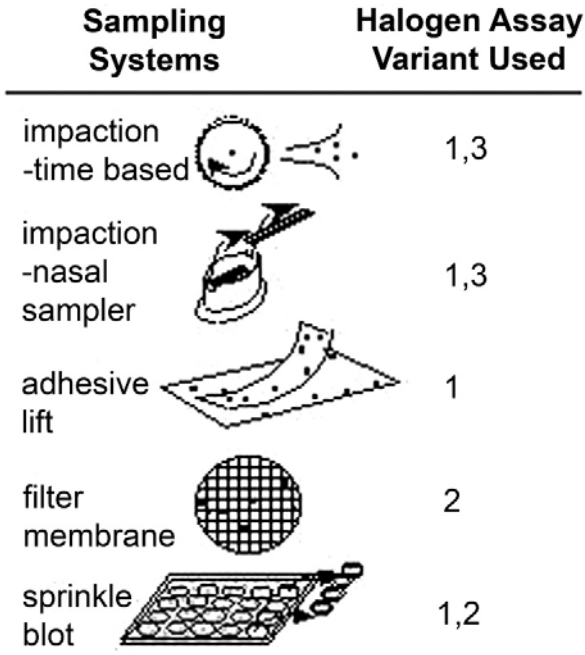

Fig. 3. Four different common methods of sampling allergens, to which one or more of the three variants of Halogen immunostaining systems can be applied, are shown schematically. These include impaction-based systems such as Hirst type spore traps and nasal samplers, collecting particles with an adhesive tape from soft or hard surfaces, and collection by filtering air through porous membranes. In addition, a large area of the laminate can be prepared which contains a known allergen source. This can be divided up for testing or validating assays in multiple assays.

1.4. Sampling of Allergens for Halogen Assay

As shown in **Figs. 2** and **3**, most of the common methods of air and surface sampling of allergens can be modified to allow analysis with Halogen assays. These include impaction systems (Burkard, nasal samplers) and air filtration systems using membranes, plus other applications such as the lifting of particles from collection surfaces as well as sprinkling particles directly onto the adhesive or the membranes. *See* **Note 2** for comments about sampling capacity of membranes and films.

As far as we are aware, Halogen systems can be used to detect all allergens carried by their natural carrier particles. We have measured cat Fel d 1 ***(5)***, mite Der p 1 ***(12,13)*** (as well as Der p 2 and Der f 1, unpublished), Cockroach Bla g1 ***(6)***, dog (unpublished), rat ***(7)***, the grass pollens *L. perenne* and *C. dactylon*

(8,10,14). We have also detected more than eight species of fungi with IgE ***(31)***. Fungi, however, present a challenge, as often only a low-percentage of spores appear to express allergens.

1.4.1. Nasal Air Sampling

The Halogen assay was originally developed for use with nasal air sampling and they are well suited to each other. The technical details of this sampler's performance are available in abstracts of ***(15)*** and ***(16)***. Briefly, nasal air samplers are small impaction collectors that fit just inside the nostril and collect inhaled particles onto a removable adhesive tape (*see* Halogen methods 1 and 3) of approximate dimensions 12 × 3 mm/sampler. The samplers collect most particles of aerodynamic diameter >10 μm and 50% the particles >5-μm diameter. The samplers have negligible air resistance on normal breathing and provide a simple, inexpensive, and silent sampling platform that enables the dose of allergen exposure inhaled by a subject to be determined. The samplers are available from Inhalix, Australia (*see* **Note 3**).

1.4.2. Sampling by Air Filtration with Pumps

For conventional personal air filter sampling, we use portable, low-volume (2 L/min) air pumps, e.g., Airchek 52 (SKC, Eighty Four, PA) attached to IOM sampling heads (Institute of Occupational Medicine, Edinburgh, manufactured by SKC, Blandford, UK). Many other filter holder and air pump assemblies could be used. Again, different types of PVDF, MCE, and NC membranes can be used, provided they will allow sufficient airflow rates and offer the desired performance in immunostaining. We have commonly used 0.45-μm PVDF ***(13)*** and 1-μm pore size PVDF ***(6)***. With more powerful vacuum pumps, airflow rates of up to 10 L/min can be achieved through 25-mm diameter membranes of 1-μm pore size.

1.4.3. Sampling by 'Adhesive Lifts'

Particles carrying allergens may be directly sampled from hard and soft surfaces with adhesive tape. We have measured the level of surface allergen on hard surfaces, such as vinyl floors (Sercombe, unpublished) and on soft surfaces, such as clothing and bedding fabrics. Depending on the circumstances, single or multiple presses can be made from the surface or the adhesive can be wrapped around a small cylinder and rolled across a large surface to sample a large area. In our observation, a single lift will pick up more than 85% of particles from a hard surface. An important consideration is that the tape does not become so saturated with dust that it cannot be later successfully laminated with the protein-binding membrane.

1.4.4. Sprinkle-Blot Assays for Screening

A homogeneous source of an allergen of known identity and purity, such as commercially available pollen grains or fungal spores from culture, may be directly sprinkled, or in other ways distributed, onto either the adhesive tape or the membrane. This is then laminated with the membrane or adhesive tape, respectively, and small disks cut which will fit ELISA wells. These can be used, for example, to screen monoclonal antibodies for functional activity ***(10,14)*** or to determine other aspects of assay development and optimization.

2. Materials

2.1. Collection and Lamination of Samples for Variants 1 and 2 of Halogen Assay

1. The recommended adhesive tape is supplied by Inhalix, Australia (*see* **Note 3**).
2. For a generally useful PVDF membrane, use 0.45-µm pore polyscreen (NEN Research Products, Boston, MA) or 1-µm pore Millipore BVXA (Millipore Corporation, Bedford, MA). For a generally useful NC membrane use 0.22-µm Nitrobind pure NC (Micron Separations Inc., Westborough, MA) and MCE protein-binding membranes (0.8-µm pore size; Millipore Corp., Bridgewater, MA).

2.2. Collection and Lamination of Samples for Variant 3 of Halogen Assay

1. Agarose ballast: 2% agarose type VI-A (Sigma-Aldrich) in 20% D-sorbitol (Sigma, St Louis) in PBS. PBS is prepared as 8.0 g/L NaCl, 0.2 g/L KCl, 1.44 g/L dibasic sodium phosphate Na_2HPO_4, 0.24 g/L monobasic potassium phosphate (K_2HPO_4). Dissolve in 800 mL MilliQ water, adjust to pH 7.2 with HCl or KOH, adjust volume to 1 L. The above agarose ballast solution is made by adding components together in a conical flask, stirring briefly to mix, and then heating in a microwave until the solution is at boiling point and the agarose has dissolved. Take care the solution does not boil over. Allow to cool to ~50°C for handling.
2. Adhesive coating: 1% agarose type VI-A (Sigma-Aldrich) in 20% D-sorbitol (Sigma), 2% sodium carboxy methyl cellulose (MW 90,000 Da, Sigma-Aldrich) in PBS.
3. Protein-binding membranes as in materials 2.1.2 can be used. New PVDF membranes require prior wetting in 80% ethanol or methanol for approx. 1 min before being transferred to PBS or water. After this, they should not be allowed to dry out.

2.3. Extraction, Blocking, and Immunostaining

1. Different extraction buffers which can be used include:
   a. *Phosphate-buffered saline, pH 7.4 (PBS)* (*see* **Section 2.2.1.**).
   b. *Borate buffer:* Dissolve 12.366 g boric acid in 900 mL MilliQ water. Adjust to pH 8.2 with NaOH and add MilliQ water to a final volume of 1 L.
   c. *Coca's solution:* In 0.5 L MilliQ water dissolve 0.14 g $NaHCO_3$, 0.41 g NaCl, and 0.23 g phenol. Adjust to pH 7.2.
2. Blocking buffer: 5% (w/v) skim milk powder in PBS (*see* **Section 2.2.1.**).

3. Washing buffer: Add 0.5 mL Tween 20 to 1 L PBS.
4. Incubation buffer for first and second antibodies: 2% (w/v) skim milk powder in washing buffer.
5. Immunostaining reagent: 1-step BCIP/NBT (5-bromo-4-chloro-5-indolyl phosphate/nitroblue tetrazolium, Pierce; approximate staining time: 10–30 min). Use neat.
6. Immunostaining reagent: Fast Red TR/naphthol AS-MX (insoluble alkaline phosphatase substrate with 1 mM levamisole, Sigma Chemical Co.); approximate staining time: 30 min–1 h.

2.4. Visualizing Halogen Staining

Either binocular transmission microscope, equipped with 10× eyepieces and 10 and 20× objectives or a 35-mm slide scanner attached to a suitable computer. The slide scanner is useful as an economical way to obtain a digital image of the immunostaining, but is not useful for observation of particles.

2.5. Miscellaneous Materials

1. Precision pipets devices (0–20, 0–200, 0–1000 μL).
2. 24-Well plates (Sarstedt Inc., Newton, NC).
3. Scalpel blades, fine point tweezers, gloves.
4. Pipet attached to a vacuum line and liquid waste trap for removing liquid during washing steps.

3. Methods

3.1. Sample Collection onto Dry Adhesive Tape (Variant 1)

Samples are collected directly onto the adhesive tape as described in **Sections 1.4.1.**, **1.4.3.**, and **1.4.4**. Gloves, either powder free or washed free of powder, should be worn at all times and neither the adhesive nor the corresponding area of the membrane surface involved in particle and allergen presentation should be touched at any time.

The adhesive is removed from the collection device and laminated with the protein-binding membrane by gently ‘rolling’ the adhesive tape, adhesive side down, on top of the protein-binding membrane that has been placed on a clean, hard, smooth surface. The bond between the membrane and the tape is augmented by briefly massaging the back of the adhesive tape with a smooth item, such as a spatula, or a small hard rubber roller used for mounting photos. Take care during handling not to minutely crush areas of the membrane. In our experience, such laminated membranes can generally be stored for prolonged period prior to continuing the assay. The next step is **Section 3.4**.

3.2. Sample Collection onto Dry Protein-Binding Membranes (Variant 2)

Particles are directly collected by suction of the air containing the particles onto a protein-binding membrane as described in **Section 1.4.2**. *See* comments

in **Sections 1.4.2.** and **2.1.** about airflow rates through membranes and the types of membranes that can be used.

After sample collection, the membranes are laminated with an adhesive tape to form a permanent sandwich containing the particles, using similar manual techniques described in **Section 3.1.** for laminating the adhesive carrying the particles with a membrane. The next step is **Section 3.4**.

1. Laminate the adhesive with the protein-binding membrane. Trim to size.
2. Place each laminated adhesive/membrane sandwich from a nasal sampler in the well of a labeled 24-well plate, with the membrane side facing upwards.
3. For PVDF only, not NC or MCE, wet in an 80% methanol solution for 60 s until evenly opaque. Do not oversoak or the laminate may separate.
4. For PVDF only, not NC or MCE, remove the methanol solution and rinse 3× with PBS over 5 min. Do not allow the membranes to become dry at any stage.
5. Add 500 μL of elution buffer to all membranes and elute allergens for 1–12 h at room temperature with gentle agitation. Incubation time depends on the allergen, but longer times give a stronger signal.
6. Block with 5% skim milk powder solution for 1 h, then rinse in PBS/Tween (0.05%) for 5 min.

3.3. Sample Collection onto a Wet, Protein-Binding Membrane Precoated with an Aqueous Adhesive Gel (Variant 3)

The preparation and handling of the wet adhesive membranes is technically rather tedious and demanding, although the method can give excellent results. It is included here as it can be used as an alternative to the dry adhesive film, described elsewhere, if these are not available.

NC ***(12)***, MCE, or PVDF ***(13)*** membranes can be used. All these types of membranes should be kept moist once the adhesive gel has been applied and this imposes limits on impaction sampling conditions. The method is suitable for nasal sampling where the flow of humid air during exhalation minimizes drying out. It is only suitable for cascade impaction systems for short sampling times and there is the risk the high-velocity airstream in the final stages of the impaction collector will damage the agarose adhesive layer.

The first step is to coat the back of the membrane with a ~0.5-mm layer of the agarose, in PBS containing sorbitol as a humectant, to function as a water ballast. The calculated volume of agarose is pipeted onto a prewarmed surface in the collecting apparatus (held at about 45°C) and then the damp membrane is laid on top to this. A very thin (20–30 μm) layer of agarose is then applied to the top of the protein-binding membrane. This consists of agarose containing sorbitol plus 2% sodium carboxy methyl cellulose. This is applied using an ultrasonic nebulizer of the type used to deliver asthma medications at home. The jet of nebulized liquid agarose is directed onto the top surface of the membrane,

where it gels. Successive, brief passages allow an even layer of 20–30 μm thickness to be deposited. The thickness is checked with a microscope and some experimentation is required to determine the exact conditions (we use 3–4 passages each of ~2–3 s with the surface to be coated held about 1 cm from the nebulizer's output orifice). After their construction, the adhesive-coated membranes are maintained in humid atmosphere prior to use.

Following sample collection, the membranes are stored in a sealed, humid container at 4°C for 1–24 h to allow the allergens to elute from the particles and to diffuse through the thin agarose layer and bind to the underlying protein-binding membrane. *See* **Section 3.4.1.** for comments on extraction times.

After this incubation, the membranes are again coated using the nebulizer to apply another thin (~50 μm) layer of 0.5% agarose to hold the particles in place during the rigors of immunostaining. It is important during the application of the second agarose coat that the layer is applied slowly so that no liquid agarose is allowed to accumulate. Any agarose flow on the surface at this time can redistribute the particles from the location where allergens have been bound.

The following is carried out to prepare the wet adhesive membrane for both sampling and immunostaining:

1. Wet membrane in 80% methanol solution for 60s until evenly opaque. Remove and rinse 3x with PBS (for PVDF only, not NC or MCE).
2. Coat back of wet PVDF or NC membrane with ~0.5-mm layer of agarose/humectant by pipeting out appropriate volume of agarose onto the base of collection device held at ~45°C and then placing the membrane gently on top of the thin layer of agarose. This needs to be done quickly as the agarose forms a gel in <1 min.
3. Construct the collection surface on the membrane by exposing it to an aerosol of agarose/humectant/adhesive until a thin coat is deposited. Exact conditions are established by prior experience—*see* **Section 3.3**. Store in humid location.
4. Collect sample (in nasal samplers).
5. Allow time for allergens to be extracted from particles (1–24 h).
6. Recoat particles on the collection surface with a layer of agarose using aerosol generator.
7. Block membranes with 5% skim milk powder solution for 1 h and then rinse in PBS/Tween 20 (0.05%) buffer.

3.4. Immunostaining Systems

The general scheme for immunostaining follows that used in protein blotting, *see* ***(11,17,18)***. The scheme is outlined in **Fig. 2** and some variants for probing are shown in **Fig. 4**.

3.4.1. Elution of Allergens from Particles and then Blocking

In variant 3 (**Fig. 2**), which uses a wet collection surface, the elution of allergens from particles begins as soon as they are collected. In systems with dry

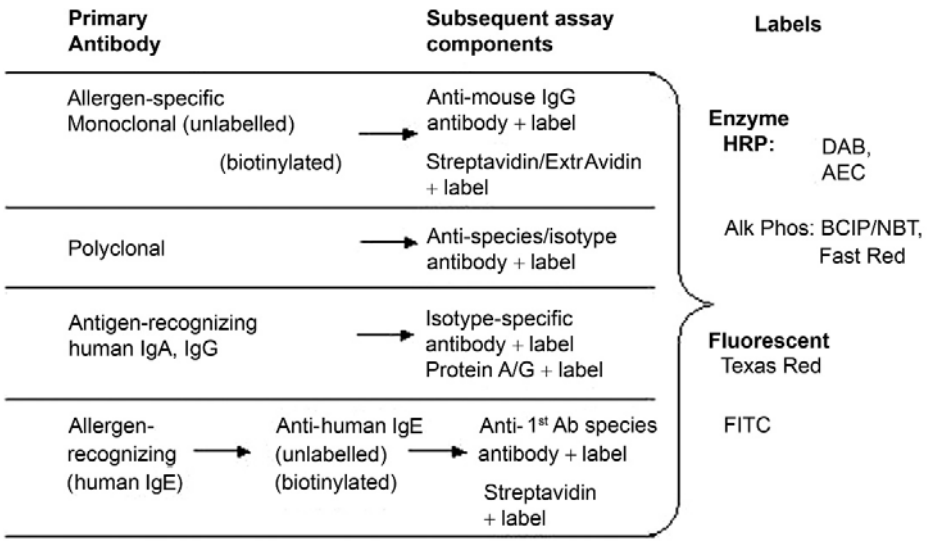

Fig. 4. An overview of some different combinations of primary antibodies and labeling systems that can be used for probing in Halogen assays.

collection surfaces (variants 1 and 2), extraction occurs only after lamination has been performed and the sandwich has been wetted. This event can be delayed until a convenient time.

The initial procedure for wetting the membrane/adhesive laminate depends on whether NC/MCE or PVDF membranes are used (*see* **Section 3.2.**).

Various extraction buffers can be used, *see* **Section 2.3.1**. These should not contain any detergents or proteins which can either block the membrane or interfere with the adhesive/membrane sandwich.

Extraction times vary from 1 to 24 h. Whereas for sources such as pollen this extraction mainly occurs in less than an hour, with mite, cat, and dog allergens, elution substantially occurs in the first hour, although longer incubations up to 12 h generally produce more intense halos. For fungi the extraction is slower and more variable and is best performed for up to 24 h. For membranes carrying fungi, storing the unlaminated membrane at 100% RH and 25°C for 24 h can lead to hyphal growth and enhanced allergen expression. This is followed by lamination, and this variation of the protocol shows improved immunostaining of both fungal conidia and hyphae ***(31)***.

After extraction, the protein-binding membrane is blocked to prevent binding of antibodies to vacant protein-binding sites on the membrane. This is generally performed with 5% skim milk powder in PBS for an hour, although many blocking agents would be potentially suitable. The final choice may require

experimentation. Based on the experience in the literature of blotting proteins from SDS gels, the choice of blocking agent can effect the subsequent detection of some allergens. In our experience with Halogen assays, the blocking agent may have a small effect on halo intensity.

The use of a sample for Halogen assay and for other types of assay is discussed (*see* **Note 4**).

3.4.2. Antibody Probing with a Monoclonal Antibody

Allergens are detected using a primary antibody of known specificity for that allergen. In our experience, monoclonal antibodies are preferred. Monospecific polyclonal antibodies have also been used ***(8)***, but there is the risk that the animals they are raised in will have natural antibodies against a range of common environmental antigens which will also be detected by the serum. This requires either that these unwanted antibodies are adsorbed out or affinity purification of specific antibodies is performed. Techniques for this can be found in specialist text books of immunological methods. Small amounts of specific purified antibodies can also be prepared directly from protein blots ***(20)***.

In Halogen assays, as the allergens are extracted from their native sources (unlike allergens blotted from SDS gels), the allergens are in their native conformation and should be detectable with monoclonal antibodies directed against conformational epitopes. However, there can be subtle differences in the shape of the allergen's epitopes following binding to different solid phases. Antibodies that were selected in an ELISA may not be optimal for Halogen applications and vice versa. In general, antibodies should be screened for performance in the application they will be used in.

Primary antibodies are generally used at a concentration of 1–2 µg/mL for incubation periods ranging from 1 h up to overnight. These times depend on the concentration and avidity of the antibody and the need to maximize the signal and minimize background and are best optimized by experiment. Incubation buffer is 2% skim milk in PBS–Tween.

After incubation with primary antibody the laminated membrane is washed three times for a total of 30 min, with rocking, to remove any unbound residual primary antibody. The bound primary antibody is detected with a secondary antibody, for example with antimouse antibody conjugated to alkaline phosphatase (Sigma) followed by washing and detection with BCIP/NBT (Pierce) substrate. Alternatively biotinylated primary antibody can be used and detected with ExtrAvidin or streptavidin–enzyme (peroxidase or alkaline phosphatase). In our experience the former system has lower backgrounds. Further comments on washing and incubation times are given (*see* **Note 5**).

1. Incubate with allergen-specific monoclonal antibody (generally at 1:500) in 2% skim milk in PBS/0.05% Tween 20 for 1 h at room temperature on the plate shaker.

A container is chosen to provide the minimum but sufficient area for the membrane to remain flat; for small items 24-well cell culture plates are convenient. The volume used should be the minimum needed to adequately cover the laminated membrane, for example 400 μL/well in a 24-well plate, and care should be taken that the adhesive side of the laminated membrane is kept facing down.
2. Wash 3 × 10 min in PBS/Tween.
3. Add 1:500 antimouse alkaline phosphatase conjugate. Incubate for 1 h at room temperature with agitation on the plate shaker.
4. Wash 3 × 10 min in PBS/Tween.
5. Develop membranes with BCIP/NBT substrate. The reaction is stopped after membranes have been stained light-medium purple (approximately 10–30 min) by rinsing 3× in deionized water.
6. Stained samples can be stored in deionized water at 4°C for up to 3 d before analysis.

3.4.3. Antibody Probing with IgE

IgE from allergic subjects can be used to specifically detect what are, by definition, allergens on the membrane. Usually the primary incubation is performed with RAST class 3 or 4 serum, overnight, at 4°C. However, depending on the antibody titer of the serum and the allergen involved, the serum can sometimes be diluted up to fivefold with no loss of halo quality or number and a reduction of background staining. In one study we found a direct correlation between the numbers of halos detected and the titer of the sera, with serum dilutions going down to 128-fold for a 35% reduction in the numbers of halos. After incubation with serum and then washing, the bound IgE is detected using suitable second antibody, such as biotinylated antihuman IgE (for example KPL, Gaithersburg, MA), at a dilution of 1 in 500. This is followed by incubation with streptavidin–alkaline phosphatase conjugate (1 in 1000) and BCIP/NBT development.

1. Add neat human sera from allergic patient (preferably RAST class 3 or 4) to the laminated membrane. Dilutions of serum may later be used after confirming their activity by trial. *See* **Note 1** in **Section 3.4.2.** on advice about containers and volumes to use.
2. Incubate overnight on an orbital shaker at room temperature.
3. Wash 3 × 10 min in PBS/Tween.
4. Incubate with biotin-labeled goat antihuman biotin IgE diluted 1:500 in 5% skim milk, PBS–Tween for 1.5–2 h at room temperature.
5. Wash 3 × 10 min in PBS/Tween.
6. Incubate with ExtrAvidin alkaline phosphatase conjugate diluted 1:1000 in 2% skim milk, PBS–Tween for 1.5–2 h at room temperature.
7. Wash 3 × 10 min in PBS/Tween.
8. Develop membranes with either BCIP/NBT for 10–30 min or Fast Red TR/naphthol AS-MX for 30–60 min at room temperature.
9. Stop the staining reactions by rinsing in distilled water.

10. Stained samples can be stored in deionized water at 4°C for up to 3 d before analysis.

3.5. Enzyme and Fluorescent Immunostaining Systems

Halogen staining uses similar immunostaining techniques that have been used for protein blotting. When the enzyme label horse radish peroxidase is used, the insoluble, colored enzyme substrates are used, for example, a precipitating TMB (3,3′,5,5′-tetramethyl benzidine; Pierce) and when the enzyme label alkaline phosphatase is used the substrates used are BCIP/NBT or Fast Red.

In general, the intensity of background color is developed to a darker level in Halogen than in protein-blotting assays. For example, BCIP/NBT is allowed to develop until the membrane background is stained to a medium blue to the eye, although when viewed through the microscope using illumination directed from the underside, this background appears quite light. Epi-illumination can also be used if available, which allows the particles to be more closely observed.

We have found enzyme-labeling systems to be more sensitive than fluorescent labels such as Texas Red and FITC and would be easier to quantify. We have recently published a highly sensitive method using dual immunofluorescence and confocal microscopy ***(32)***.

Immunogold staining was found not to be sensitive, presumably because of the hindrance of migration of the gold–second antibody complexes by the membrane. Chemiluminescence has not been evaluated, as it would require a sophisticated system of signal capture.

PVDF membranes can be cleared by briefly incubating in ethylene glycol/glycerol solution (9:1) ***(21)***. This is useful particularly when heavy staining has been used to maximize sensitivity. NC membranes can be cleared when dry using microscope immersion oil (e.g., Olympus nd 1.516).

Further comments about multiprobe labeling can be found (*see* **Note 6**).

3.6. Staining of all Proteins or Particles

For some applications it maybe of additional interest to be able to stain all the proteins or other types of macromolecules extracted from particles and bound to the membrane. For example, it is surprising to see the amounts of protein extracted from fungal spores even though there may be little immunostaining of allergens. Proteins can be biotinylated directly on the membrane (prior to blocking) using kits such as NHS-Biotin Total Protein Detection kit (BioRad) and then probed with a streptavidin–enzyme system. It should also be possible to do this with India ink on NC after Tween blocking or with other protein-staining systems prior to blocking, although we have not tested this. Additionally it may be of interest to stain all the translucent dust particles to aid their counting. This can be done with 0.25% Safranin O in 10% ethanol (DeLucca, unpublished).

3.7. Interpretation of Halogen Assays

The simplest way to interpret Halogen assays is to directly visualize the membranes using a conventional laboratory compound light microscope. If a video camera is attached, this has the advantage that images can be preserved, captured, and communicated.

An image can provide a more dramatic interpretation than the quantity of allergen expressed as weight. Any patient shown a picture can fully grasp the story "you did not think you were exposed to cat allergen, but you can see in the staining of this air sample from your house, that many of the airborne particles carry cat allergen".

The next level of interpretation is to count the number of particles carrying allergen. This provides a useful proxy for quantity that is dependent on the distribution of allergen content/particle being similar between samples. This is likely to be the case when large numbers of particles are collected under similar circumstances, but is less likely when the total number of particles is small or some samples contain a disproportionate number of large particles carrying more allergen. These impose limitations on the interpretation of counts of numbers of particles with halos.

The most accurate interpretation of quantity is provided by image analysis of stained membranes to determine the integrated optical density of each halo and then interpret this into absolute quantities of allergens. This is performed by reference to a set of standards of known allergen concentration which have been microdotted onto the membrane and immunoprobed simultaneously. Prototype hardware and software for performing this is available in our laboratory, but overall, the results were inconsistent and the method is technically demanding.

The use of an image analysis-based system provides additional information about the size and shape of particles carrying allergen and about the quantity of allergen per particle. Experience with 'thunderstorm asthma' shows that inhaling many small particles has quite different clinical outcomes than inhaling a few large particles even if the total amount of allergen is similar ***(22)***. The important distinction of particle size, which determines the site of deposition of the particles in the respiratory system, would be missed by an assay that relied on allergen quantification only.

The original aim of the project that led to our development of the nasal air sampler and Halogen assay was to provide an integrated system complete with automated pollen and fungal identification. Although considerable progress has been made ***(23–25)*** including a stand-alone CD-ROM Database (Inhalix, Sydney) of allergenic pollen grains and fungal spores ***(26)***, such a fully integrated system remains a future project.

4. Notes

1. Halogen immunostaining differs from other published methods of immunostaining particles and their allergens. It differs from precipitation systems ***(27)*** that involve specific antibodies of a predetermined specificity to immobilize only the specified antigens around the particle; from press-blotting systems ***(28,29)*** where the association of particle and allergens is not permanently maintained and immunostaining cannot be associated on a one-to-one basis with individual particulate sources; from fixation techniques where the allergen is either fixed inside the particle ***(22)*** and from fixation techniques where allergens are fixed to nonprotein-binding membranes ***(30,31)***.
2. The sampling capacity is limited by the particle loading of the adhesive or the membrane surface used for sample collection. It would probably be unwise for the samples to exceed 5% of the total surface area, as further loading may reduce sampling efficiency, interfere with subsequent lamination and/or with the resolution of halos. With adhesive tapes, we are not aware that they directly lose their adhesiveness from exposure to air alone. Time *per se* is not an issue and we have intermittently sampled onto tapes in Burkard samplers and onto membranes (using a timer on the pump) for sampling periods of over a week.
3. Inhalix, was a spin-off from the Institute of Respiratory Medicine, now called the Woolcock Institute of Medical Research. Although Inhalix was wound up in 2002, most materials are still available for research purposes by contacting Dr Euan Tovey, Rm 461, Blackburn Building, DO6, University of Sydney, NSW 2006, Australia or fax: 61-2-9351 7451.
4. More than one type of assay can be performed on samples collected both in methods 1 and 2. Assuming there is sufficient density of sample on a tape or membrane to enable smaller sections to be representative, the adhesive tapes can be cut into sections and allergen extracted off a section by immersion in buffer that is then subject to liquid phase immunoassay. The remaining section can be analyzed by Halogen assay. Conversely, if samples are collected on PVDF membranes, prior to lamination of the membrane, allergens can be eluted from part of the membrane in an elution buffer such as 1% BSA, PBS with no Tween. PVDF does not 'wet' under these conditions, whereas NC would bind the allergens. In this way, samples collected on filters or membranes can be divided and one section can be analyzed by Halogen assay and the remaining section analyzed in parallel by amplified ELISA.
5. Because all reactants both enter and leave through only one side of the protein-binding membrane in Methods 1 and 2 (the other side being sealed with the adhesive), reagent incubation and washing times are longer in comparison with those used for protein-blotting methods. Larger pore membranes and gentle shaking of reactions aid the more rapid diffusion of reagents.
6. We have attempted to develop multiprobe immunolabeling systems with only partial success. It is feasible to probe two different antigen sources with two different antibodies on the same blot. This is performed by using an unlabeled primary antibody followed by an antimouse antibody labeled with for example alkaline phosphatase, then followed with a biotin-labeled primary antibody of different specificity and

with HRP–streptavidin. This produces a blot with different colored halos for each allergen. However, the colabeling of the same allergen with two differently labeled primary antibodies (for example, an allergen-specific monoclonal and human IgE) so far has been less satisfactory using either different fluorescent probes (e.g., FITC and Texas Red) or a fluorescent probe and an insoluble substrate. It would seem the presence of one antibody system interferes with the expression of the other's signal either by quenching or by steric hindrance. Four recent papers ***(32–35)*** describe methods for dual staining of fungi with human IgE and a monoclonal. These techniques are operational but require on-going development. We would be very pleased to receive correspondence on techniques suitable for dual staining of the same allergen.

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